

**THE DEVELOPMENT AND APPLICATIONS OF  
POLYCLONAL AND MONOCLONAL ANTIBODIES FOR  
THE DETECTION OF ILLICIT DRUGS IN SALIVA  
SAMPLES**

**A thesis submitted for the degree of  
Doctorate of Philosophy**

**by**

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## Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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## **Publications**

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## Abstract

Anti-tetrahydrocannabinol (THC), anti-cocaine and anti-morphine polyclonal antibodies were produced. These antibodies were successfully applied to an ELISA format for the detection of THC, cocaine, and morphine in saliva samples.

Monoclonal antibodies against amphetamine and its derivatives were produced using two different conjugates, amphetamine-bovine serum albumin and methamphetamine-bovine serum albumin. Two successful clones were produced, and the antibodies were applied to an ELISA format for the detection of amphetamine, methamphetamine, and the other common amphetamine derivatives, such as methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA). The ELISA was developed using saliva as the matrix. During the screening stage of the production of these antibodies, particular attention was given to their cross reactivity profiles. Among the molecules tested for cross-reactivity, were legally available medications such as ephedrine, as other commercially available antibodies show cross reactivity. The resulting monoclonal antibodies detected amphetamine and other designer derivatives, and showed negligible cross reactivity with the legal structurally related molecules. The antibodies were applied to a biosensor (BIAcore) assay for the detection of amphetamine and methamphetamine in saliva samples. The affinity constants for the antibodies were determined by ELISA and BIAcore methods. The values obtained were found to be similar by both methods.

A novel automated prototype device, developed by our collaborators, Envitec, was optimised and the anti-THC polyclonal antibody was applied to it for the screening of saliva samples for the presence of THC. This was a rapid, qualitative test, and it could be performed in less than 20 minutes. The basis of the assay was competition between horseradish peroxidase-labeled THC and THC present in the saliva samples, for binding to the anti-THC polyclonal antibodies that coated the reaction wells of the device.

## Abbreviations

APC	antigen presenting cell
BDB	benzodioxole-5-butanamine
BEC	benzoylecgonine
BIA	biomolecular interaction analysis
BSA	bovine serum albumin
BtG	bovine thyroglobulin
CDR	complementarity determining regions of antibody
CE	capillary electrophoresis
CV	coefficient of variation
DNA	deoxyribonucleic acid
EDC	N-ethyl-N'-(dimethylaminopropyl) carbodiimide
EDTA	ethylenediaminetetra acetic acid
EME	ecgonine methyl ester
ELISA	enzyme-linked immunosorbent assay
EMIT	enzyme-multiplied immunoassay technique
Fab	binding region of antibody above the hinge region
Fc	constant region of antibody molecule
FCS	foetal calf serum
FPIA	fluorescence polarisation immunoassay
Fv	variable binding fragment of antibody
GC/MS	gas chromatography/mass spectroscopy
HAT	hypoxanthine aminopterin thymidine
HPLC	high performance liquid chromatography
HT	hypoxanthine thymidine
HBS	Hepes buffered saline
IgG	immunoglobulin class G
IgA	immunoglobulin class A
IgD	immunoglobulin class D
IgE	immunoglobulin class E
IgM	immunoglobulin class M
$K_A$	equilibrium association affinity constant
$k_a$	association rate constant

$K_D$	equilibrium dissociation affinity constant
$k_d$	dissociation rate constant
MAb	monoclonal antibody
MDA	3,4-methylenedioxyamphetamine
MDMA	3,4-methylenedioxymethamphetamine
MBDB	3,4-methylenedioxyphenyl-2-butanamine
MDEA	3,4-methylenedioxy-N-ethylamphetamine
MW	molecular weight
NEAA	non-essential amino acids
NIDA	National Institute of Drugs of Abuse
NHS	N-hydroxysuccinimide
OVA	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PEG	polyethylene glycol
pH	log of the hydrogen ion concentration
SAMHSA	Substance Abuse and Mental Health Service Administration
scFv	single chain Fv antibody derivative
SE	standard error
SDS	sodium dodecyl sulphate
SMT	Standard, Measurements and Testing Framework
S/P	saliva/plasma ratio
SPR	surface plasmon resonance
THC	tetrahydrocannabinol
THC-COOH	tetrahydrocannabinol carboxylic acid
THY	thyroglobulin
UV	ultraviolet
$V_H$	variable region of heavy chain
$V_L$	variable region of light chain
WHO	World Health Organisation

## Units

°C	degrees Celcius
cm	centimetres
g	grams
KDa	kilodaltons
Kg	kilogram
l	litre
µg	microgram
µl	microlitre
µM	micromolar
M	molar
mg	milligram
min	minute
ml	millilitre
mm	millimetres
nm	nanometre
nM	nanomolar
mol	molar
rpm	revolutions per minute
RU	response units
sec, s	seconds
v/v	volume per unit volume
w/v	weight per unit volume



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# *Chapter 1*

## *Introduction*

## ***1.1 Drugs of abuse in saliva - Background***

The use of saliva as an alternative matrix for the detection of drug abuse is currently being investigated by analytical laboratories and international agencies. The obvious advantages of using saliva as a matrix is that the collection is non-invasive and does not intrude on the subjects privacy, is relatively easy, and can be performed under close supervision. From an analytical point of view, saliva is a relatively uncomplex matrix and does not contain some interfering substances that are found in plasma. A simple freeze-thaw cycle followed by centrifugation is the usual method of preparation of saliva before laboratory analysis (W. Lambert, Personal Communication). Another key advantage of using saliva as a matrix is that the presence of certain drugs and metabolites can be a better indication of recent drug use and current levels of intoxication as compared to the more traditional matrices of blood and urine (Cone, 1993). There are some publications that examine the correlation of drug and metabolite concentrations and the correlation with level of intoxication, these are discussed later. It would appear that additional studies would need to be performed before definitive guidelines could be established for all drugs of abuse.

The main disadvantage of using saliva is that only low quantities of drugs and metabolites are found in comparison to blood and urine. This means that the analytical assays developed must have high sensitivity, with low limits of detection. Another major disadvantage is the level of contamination that can occur with drugs that are inhaled or smoked. This can lead to buccal and nasal contamination. Also, passive smoking may lead to contamination. One way to avoid this problem is to raise the cut off levels of detection of substances such as THC. In this way, subjects who are contaminated by passive smoking do not test positive. The presence of buccal contamination can be considered an advantage if the purpose of the test is to detect evidence of drug abuse at any time point after smoking.

### ***1.1.1 Saliva as a matrix***

The concentration of free drug found in saliva is dependent on the different chemical and physiological properties of the drugs and saliva. The lipid membrane of the thin layer of epithelial cells that separates the saliva from the systemic circulation, is responsible for determining the molecules that cross the layer into the saliva. The drugs

can potentially be transported by active transport, diffusion through pores in the membrane, or by passive diffusion across a concentration gradient. It is well documented that the presence of drugs in saliva are achieved largely through passive diffusion across the lipid layer of the epithelial (acinus) cells (Haeckel & Hanecke, 1996). Drugs are commonly assigned an S/P ratio (saliva/plasma) to represent the concentration ratio found between the saliva and plasma. Ethanol is a very good example of a molecule with an S/P ratio of close to 1. This is achieved through its low molecular mass and high lipophilicity, allowing diffusion into the saliva. It is also un-ionised in blood and not protein bound. These properties are the factors responsible for influencing the diffusion of molecules into saliva. The molecular mass and lipid solubility of the drug are important properties controlling passage through the membrane. The degree of ionisation of a drug is of importance, as described by the partition phenomena, and this in turn is influenced by the pH of the saliva (Mucklow *et al.*, 1978). When a molecule crosses into the saliva, the degree of accumulation is dependent on the pH of each side of the membrane. Once the molecule reaches equilibrium the concentration is influenced by the pH of the saliva as described by the classic Henderson-Hasselbach equation and the equation for mass balance.

$$\text{pH} = \text{pKa} + \log \frac{[\text{A}^-]}{[\text{HA}]} \quad \text{Henderson-Hasselbach Equation}$$

$$[\text{A}] = [\text{A}^-] + [\text{HA}] \quad \text{Mass Balance Equation}$$

*Where:*

*A = total concentration of drug in both ionised and non-ionised forms*

This gives the total amount of drug in either form described by the following:

$$\frac{[\text{A}]}{[\text{HA}]} = 1 + 10^{(\text{pH} - \text{pKa})}$$

The Saliva/Plasma (S/P) ratio can then be determined by:

$$\text{S/P} = \frac{[\text{A}_{\text{saliva}}] [\text{HA}_{\text{plasma}}]}{[\text{A}_{\text{plasma}}] [\text{HA}_{\text{saliva}}]}$$

$$\text{S/P} = \frac{1 + 10^{(\text{pH}_{\text{saliva}} - \text{pKa})}}{1 + 10^{(\text{pH}_{\text{plasma}} - \text{pKa})}}$$

At this point consideration must be given to drugs binding to proteins in the plasma and saliva. Since we have assumed that the concentration of HA must be the same in

plasma and saliva as it was this form that was transported, the equation becomes the following:

$$S/P \text{ (acidic drug)} = \left\{ 1 + 10^{(\text{pH}_{\text{saliva}} - \text{pK}_a)} / 1 + 10^{(\text{pH}_{\text{plasma}} - \text{pK}_a)} \right\} \cdot [F_p]/[F_s]$$

Where:

$[F_p]$  = free drug in plasma

$[F_s]$  = free drug in saliva

For basic drugs a similar equation can be deduced as the un-ionised form of the drug is transported across the membrane.

$$S/P \text{ (basic drug)} = \left\{ 1 + 10^{-(\text{pH}_{\text{saliva}} - \text{pK}_a)} / 1 + 10^{-(\text{pH}_{\text{plasma}} - \text{pK}_a)} \right\} \cdot [F_p]/[F_s]$$

(Rasmussen, 1964; Haeckel & Hanecke, 1996)

The usefulness of saliva for detection of drugs has expanded far beyond the scope of drugs of abuse, for example, for therapeutic drug monitoring (Horning *et al.* 1997). For management of chronic treatment of patients, it is necessary to determine the concentration of free drug in plasma, as this is the pharmacologically active form. This again serves as a considerable advantage, as there is minimal protein binding of drugs in saliva. The noninvasive nature of collection is very convenient, in the case of children. Horning *et al.* (1977) investigated the S/P ratios of anti-epilepsy agents by measuring concentrations by GC-MS and immunoassay. Saliva is also used for measurement of hormone levels, to monitor fertility cycles, menopausal fluctuations, and other diurnal fluctuations (Hofman, 2001).

### ***1.1.2 pH of saliva and influence on drug concentrations***

The normal pH of blood is 7.4 while the pH of saliva is around 6.4, but this can vary from 5.6 to 7 (Haeckel & Hanecke, 1996; Kidwell *et al.*, 1998). For neutral, weakly basic and weakly acidic molecules, the change in saliva pH has little effect on the S/P ratio. For acidic, ionised drugs, the S/P ratio increases with an increase in pH. Conversely, for basic drugs the S/P ratio decreases with an increase in pH. The relatively small variability of the pH of saliva is also another advantage of using saliva as a matrix for the screening of drugs of abuse. The pH of saliva is determined by the secretions that originate in the plasma, through the parotid, sublingual and submandibular and other minor glands. The volume of saliva that is produced can vary, and this is another point of consideration. Low volumes are typically seen in the case of drug abusers and methadone users where they are dehydrated and so the collection of a sufficient amount of saliva maybe a problem (Drobitch & Svensson, 1992). The solution to this would be to stimulate saliva secretion through chewing on gum, or citric acid coated sweets.

However, stimulation of saliva leads to an increase in bicarbonate secretion resulting in an increase in pH. For non-ionic or weakly acidic and weakly basic drugs the change in pH will have little effect on the S/P ratio. Many drugs, however, are affected by these changes in salivary pH. This, therefore, must be taken into account when the samples are being collected and a consistent mechanism of collection should be followed. Many of the saliva tests for drugs of abuse are qualitative and so this argument for using stimulated versus unstimulated saliva becomes less important. Indeed, the very definition of stimulated saliva can be vague as it could be suggested that even spitting for the purposes of collection of a sample is stimulated. A variety of different saliva collection devices are commercially available. Most of them utilise an absorbant pad which is placed in the mouth and after enough saliva has been absorbed it is removed and the saliva extracted through a filtration device or by mechanically forcing the fluid from the pad. The important factor relating to salivary pH is that it is more difficult to change through external means and so the concentrations of drugs present cannot be interfered with, for example by ingestion of certain acid or bases. This is not the case with urine, and experienced drug abusers can modify the pH through ingestion of different substances.

## ***1.2 Other alternative biological matrices***

The most commonly used biological fluid used for screening of drugs of abuse is urine. Blood, when available, is most commonly used for post-mortem samples for forensic analysis. The analysis of urine and blood for drugs of abuse is well documented, (Braithwaite, 1995). The ease of collection of the other fluids and the information that can be obtained from these samples has initiated the scientific and commercial sectors to investigate their worth. The cost saving implications of using oral testing as compared to urine testing have been presented by the commercial sector in relation to work place testing, (Kunsmann, 2000). Substance abuse or dependence is seen in 33 – 50% of chronic mentally ill patients indicating that drugs of abuse testing is also hugely important in this area (Shearer *et al.*, 1998).

In addition to saliva, sweat and hair also provide options for matrix selection for analysis. Sweat was investigated recently for the detection of cocaine (Spiehlner, 1996; Preston *et al.*, 1999), and opiates (Fogerson, 1997; Huestis, 2000), methadone, (Skopp *et al.*, 1996), and amphetamine and MBDB, (Fay *et al.*, 1996; Kintz, 1997). Skopp and Pötsch (1999) have reviewed aspects of saliva and sweat in the realm of roadside testing, including the concentration and form of drugs found in sweat, and the transport mechanisms from the blood to the skin. The potential pathways include perspiration and sebum, intracellular diffusion along the cell membrane complex and transcellular diffusion and transport by the keratinocytes. The conclusions made by the reviewers suggest that the main drug form found on the skin surface is the parent form. The time interval between the consumption of drugs and the appearance of the drug on the skin surface is drug specific and there seems to be a considerable time delay of several hours. This would imply that the detection of drugs on the skin surface in sweat may not be suitable as a means of detecting current intoxication.

Hair has been investigated as an alternative matrix with particular application in forensic science. The analysis of hair for drugs of abuse is useful for long term examination, and this in itself is advantageous compared to the other matrices for forensic cases. It is believed that there are two pathways for the passage of drugs into hair; incorporation from the blood into the shaft and adsorption from sweat and other environmental factors. The analysis of hair is a convoluted procedure with extensive preparatory steps for sample extraction. Even the actual process of removing the complete hair is intricate as it is important to get the root end as this has the highest

concentration of drugs. Another factor that has to be considered is the effect of bleaching, perming and other cosmetic treatments on the drug in the hair (Skopp *et al.*, 1997; Yegles, 2000). Nakahara, (1999), has extensively reviewed the basic aspects and analytical studies of hair for drugs of abuse.

The nature of many forensic cases demands the ingenuity of scientists to develop analytical methods for detecting drugs of abuse in many different matrices. Diverse matrices for the detection of drugs of abuse range for nails (Engelhart, 1998), to meconium, (Halstead, 1997). Wolff *et al.* (1999) have reviewed practical considerations and clinical usefulness of the different biological fluids. Table 1.1A and 1.1B outlines the specific issues for each matrix.



**Table 1A:** Characteristics of using blood, urine and saliva as matrices for the detection of drugs of abuse.

	<b>Blood</b>	<b>Urine</b>	<b>Saliva</b>
<b>Collection Procedure</b>	<ul style="list-style-type: none"> <li>• Invasive</li> <li>• Requires healthcare professional</li> <li>• Exposes professional to health risk</li> </ul>	<ul style="list-style-type: none"> <li>• Invasion of privacy</li> <li>• Requires additional staff to monitor procedure</li> </ul>	<ul style="list-style-type: none"> <li>• Non-invasive</li> </ul>
<b>Sample Preparation</b>	<ul style="list-style-type: none"> <li>• Yes, extensive</li> </ul>	<ul style="list-style-type: none"> <li>• Yes</li> </ul>	<ul style="list-style-type: none"> <li>• Minimal</li> </ul>
<b>Period &amp; Level of Detection</b>	<ul style="list-style-type: none"> <li>• Dependent on metabolism of drug – indicates recent use</li> <li>• Low concentrations</li> </ul>	<ul style="list-style-type: none"> <li>• Cannot be correlated to plasma levels</li> <li>• Indicates prior use over last few days and subsequent meatabolism</li> <li>• Higher concentrations build up</li> </ul>	<ul style="list-style-type: none"> <li>• Dependent on metabolism of drug – indicates recent use</li> <li>• Can be correlated to plasma levels</li> <li>• Low concentrations</li> </ul>
<b>Correlation to current intoxication</b>	<ul style="list-style-type: none"> <li>• Correlation can be made</li> </ul>	<ul style="list-style-type: none"> <li>• Correlation cannot be made</li> </ul>	<ul style="list-style-type: none"> <li>• Correlation can be made</li> </ul>
<b>Possible Problems</b>	<ul style="list-style-type: none"> <li>• Small sample volume</li> </ul>	<ul style="list-style-type: none"> <li>• pH can be altered and concentrations of drugs/metabolites affected</li> <li>• Dilute concentration</li> </ul>	<ul style="list-style-type: none"> <li>• Small sample volume</li> <li>• Buccal &amp; oral contamination</li> </ul>

**Table 1B:** Characteristics of using sweat, hair, and breath as matrices for the detection of drugs of abuse.

	<b>Sweat</b>	<b>Hair</b>	<b>Breath</b>
<b>Collection Procedure</b>	<ul style="list-style-type: none"> <li>• Non-invasive</li> </ul>	<ul style="list-style-type: none"> <li>• Non-invasive</li> </ul>	<ul style="list-style-type: none"> <li>• Non-invasive</li> </ul>
<b>Sample Preparation</b>	<ul style="list-style-type: none"> <li>• Minimal</li> </ul>	<ul style="list-style-type: none"> <li>• Yes - extensive</li> </ul>	<ul style="list-style-type: none"> <li>• None</li> </ul>
<b>Period &amp; Level of Detection</b>	<ul style="list-style-type: none"> <li>• Dependent on metabolism of drug – indicates recent drug use</li> <li>• Low concentrations</li> </ul>	<ul style="list-style-type: none"> <li>• Can not be correlated to plasma levels</li> <li>• Indicates prior use over previous weeks</li> <li>• Higher Concentrations</li> </ul>	<ul style="list-style-type: none"> <li>• Correlate to plasma levels</li> </ul>
<b>Correlation to current intoxication</b>	<ul style="list-style-type: none"> <li>• Correlation can be made to an extent</li> </ul>	<ul style="list-style-type: none"> <li>• Correlation can not be made</li> </ul>	<ul style="list-style-type: none"> <li>• Correlation can be made</li> </ul>
<b>Possible Problems</b>	<ul style="list-style-type: none"> <li>• Small sample volume</li> </ul>	<ul style="list-style-type: none"> <li>• Interference by external chemicals such as hair dyes and contaminants</li> </ul>	<ul style="list-style-type: none"> <li>• Only certain analytes suitable</li> </ul>

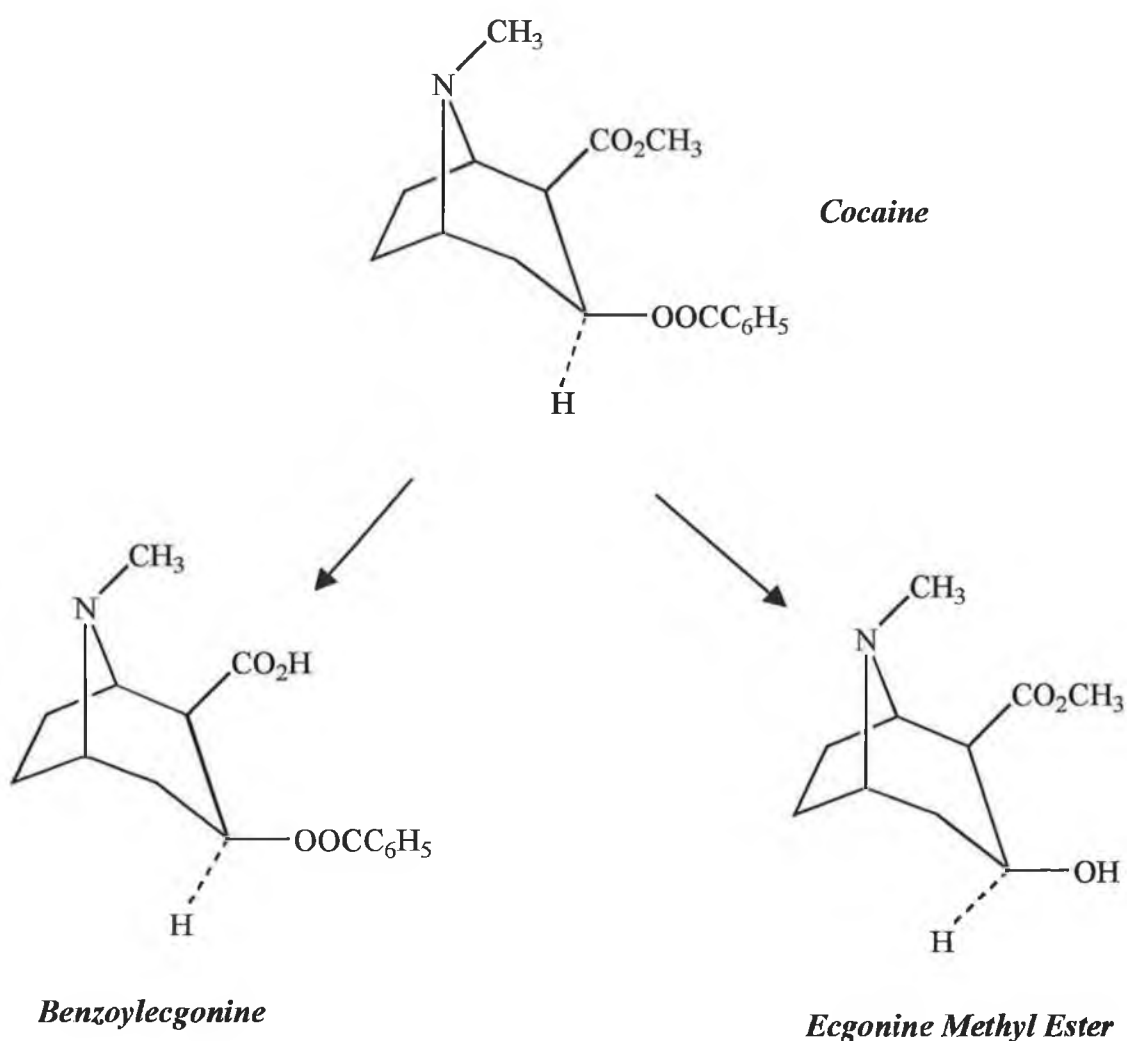
### *1.3 Legal status of alternative biological matrices*

Cone (2001), discusses the legal, workplace and treatment drug testing using alternate biological matrices in different countries. Illicit drug dealing and abuse continue to increase around the world, with implications for drug testing in the workplace, in treatment programs, in roadside testing for driving under the influence and in other forensic investigations. The analytical advances made using matrices such as saliva, and hair have not been appropriately addressed in the laws of a majority of countries. Guidelines are under development and review by various bodies across the US, Europe and Asia. The important points to consider in such guidelines are the collection and storage procedures, what drug form or metabolite is analysed, and the cut-off values that differentiate between positive and negative samples. The guidelines must be an evolving standard that are updated to take into account the advancements that are being made in technology and analytical techniques. Currently, there are no established guidelines for the detection of drugs of abuse in saliva samples, with regard to the form of the drug or concentrations.

#### 1.4 Drugs of Abuse: metabolism, form and concentrations found in saliva

The first step in the development of an assay for the detection of illicit drugs in saliva is to establish the concentration of these drugs and their metabolites in the saliva of drug users. The following section outlines findings from publications regarding the form of the drug found in saliva after intake and comparison to the form and concentration found in plasma and urine for each drug of interest.

##### 1.4.1 Cocaine



**Figure 1.1:** The metabolism of cocaine to benzoylecgonine and ecgonine methyl ester.

Cocaine is a fast acting drug with accompanying immediate mood changes after ingestion. The main metabolites are benzoylecgonine (BEC) and ecgonine methyl ester (EME), as shown in Figure 1.1, formed by the enzymatic activity of plasma cholinesterase, (Inaba *et al.*, 1978). The main routes of administration of cocaine are intranasal, intravenous and smoking of crack cocaine, which is now the predominant route used. The most common method used to determine the concentration of cocaine and the metabolites in biological fluids is gas chromatography/mass spectrometry (Cone, 1995). Many studies have been conducted to obtain information regarding the elimination half-time of cocaine and the main metabolite, BEC. Cone *et al.* (1988) investigated the correlation between cocaine-induced behaviour and mood effects and cocaine concentrations in plasma and saliva as measured by GC/MS. They observed a significant correlation between cocaine-induced pharmacological effects and saliva and plasma concentrations, with an elimination half-time of 34.9 minutes in plasma and 34.7 minutes in saliva. The subjects experienced the “rush” feeling quickly after administration, followed by the “high” feeling, which lasted approximately 30 minutes. Other behavioural and physiological effects returned to baseline within one hour after administration. This correlation between the time period for the pharmacological effects and the elimination half-life is encouraging for the development of an assay to detect recent use or current intoxication with cocaine. They detected cocaine in the saliva following intravenous administration. This demonstrated that the cocaine in the saliva came from the blood. The concentration of the main drug found in saliva may be higher due to contamination of the oral and nasal cavities by the smoking and intranasal routes of administration.

Kato *et al.* (1993) investigated the cocaine and metabolite concentrations in saliva and their results were consistent with the elimination half-time of approximately 35 minutes found by Cone *et al.* (1988). A longer elimination half time of 7.5 hours was calculated for BEC by Cone & Weddington (1989). In this study the subjects, were chronic cocaine users, and the elimination half-time of cocaine in saliva ranged from 21.6 to 110.4 hours. Cocaine could be detected in saliva for up to ten days post administration. The presence of cocaine in saliva after such a long period of use suggests that chronic use and high exposure leads to a build up of the lipophilic cocaine in tissue, and this is slowly released back into the circulation and excreted. Jufer *et al.* (2000) investigated the elimination of cocaine and metabolites following repeated oral administration to

chronic cocaine users. Two phases of elimination of cocaine and metabolites were observed. An initial elimination phase that gave results similar to previous investigations was observed with mean cocaine elimination half-times in plasma, saliva and urine were 1.5, 1.2, and 4.1 hours, respectively. The urinary elimination half-time for the second phase was 19 hours. This suggests the accumulation of cocaine in the body after chronic use resulting in a longer elimination phase.

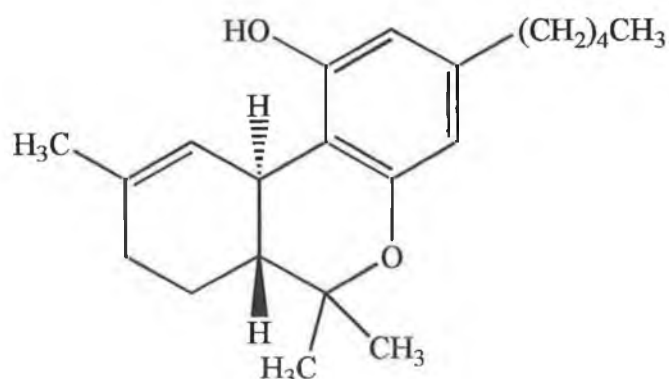
Cone *et al.* (1993) found that after cocaine administration by three different routes, intravenous, smoking and intranasal, cocaine was the major compound found in saliva. BEC and EME were present in minor amounts and the concentrations of these peaked later, corresponding to longer elimination half-times.

Schramm *et al.* (1993) looked at the levels of cocaine and benzoylecgonine in saliva, serum and urine. They found cocaine concentrations were 4.9 times higher in saliva than in serum, and benzoylecgonine levels were 2.5 times higher in serum. Thompson *et al.* (1987), found significant correlation between the plasma and saliva levels in one patient who received doses of cocaine. The S/P ratio varied from 2.96 to 0.5 over time. Cone *et al.* (1994), found S/P ratios of about 3.0 for cocaine, and, as found previously, the levels of benzoylecgonine in saliva were lower than in plasma.

Cone *et al.* (1997) investigated the concentrations of cocaine, BEC and EME in saliva by GC/MS and immunoassay. Subjects were administered different doses of cocaine by the intravenous, smoked and intranasal routes of administration. They found that BEC and EME concentrations were consistently lower in saliva compared to the cocaine concentration. The concentrations were only comparable with cocaine when the cocaine levels had fallen to below 100ng/ml. Cocaine appeared in saliva 0.08 hours after administration. The peak concentrations for the various doses and routes of administration were 258-1303 ng/ml. However, the levels decreased rapidly and reached the limit of detection by 12 hours after administration. BEC and EME levels could be detected 0.08 hour and 1 hour, respectively, after cocaine administration. The peak concentrations were detected 0.17 to 4 hours after administration and were consistently lower than cocaine concentrations (less than 100 ng/ml).

In conclusion, these results indicate that BEC could be used as the analyte in an immunoassay to detect recent cocaine use because of its longer detection period compared to cocaine. For the purposes of development of the immunoassay, the detection of cocaine is sufficient given the similarity in structure and likelihood that an antibody raised against BEC would also recognise cocaine and vice versa.

### 1.4.2 Tetrahydrocannabinol



**Figure 1.2:** Structure of tetrahydrocannabinol (THC), the active constituent of marijuana (cannabis).

The pharmacological active constituent of cannabis is delta-9-tetrahydrocannabinol (THC), as shown in Figure 1.2. The main route of administration of cannabis, (also known as marijuana), is by smoking however it may also be taken orally. THC is metabolised to an active metabolite, 11-hydroxy-delta-9-THC, which is then metabolised to 11-nor-9-carboxy-delta-9-THC, (THC-COOH). This carboxy metabolite, as the glucuronide conjugate, is the most common metabolite detected in the screening of urine (Cone, 1993).

Lemberger *et al.* (1970) investigated the metabolism of delta-9-THC in humans after IV administration. They found THC had an elimination half-life, ( $t_{1/2}$ ), of 56 hours in plasma. The time period of behavioural effects of cannabis in humans is well documented, the peak effects occur 15 minutes after administration. These effects are diminished between 30 minutes and 1 hour post intake and are generally absent after 3 hours. When the time period for the behavioural effects of cannabis is compared with the half-life of THC in plasma it would seem reasonable to assume that the detection of THC in plasma could be an indicator of current or recent use, within a couple of days, of cannabis.

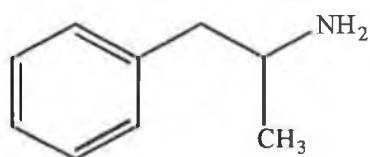
For the purposes of this project it was important to review data regarding the correlation of the plasma levels of THC to the saliva levels. Gross *et al.* (1985) found that the salivary THC concentration did indicate positive results in the blood but the two concentrations did not correlate well, a much higher concentration being found in saliva. Based on the dissociation constants for the compounds and the Henderson-



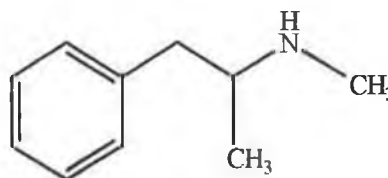
Hasselbach equation the calculated saliva/plasma ratio should be around 0.1. One reason for the high concentrations of THC and the metabolite 11-hydroxy-delta-9-THC in saliva is that due to the smoking route of administration the nasal and oral cavities become contaminated with the THC. It is now generally accepted that the presence of THC in saliva is as a result of contamination and has not been passed from the plasma. Menkes *et al.* (1991) found that subjective intoxication and elevated heart rate were significantly correlated with the salivary THC concentration. Maseda *et al.* (1986), found that THC could be detected in saliva by capillary GC, with a lower limit of detection of 1ng/ml, for at least four hours after smoking.

Schramm *et al.* (1992) presented preliminary results of HPLC analysis on a urine and saliva sample collected simultaneously from a subject who had smoked cannabis. THC-COOH was the main metabolite found in the urine sample. The saliva sample contained THC-COOH, THC, cannabidiol, and 11-hydroxy-delta-9-THC. However, information concerning the time frame for when the samples were collected after administration of the drug were missing from this study as was information regarding the subject's prior history of cannabis use. If the subject was a chronic user of marijuana this would have implications regarding the build up of metabolites in the urine and saliva. Kintz *et al.* (2000), investigated the presence of cannabis in saliva and sweat from drivers involved in road traffic accidents. Of the 22 subjects who tested positive for THC-COOH in urine, 14 and 16 of these tested positive for THC in saliva and sweat, respectively. Urine is the ideal matrix from an analytical viewpoint for the testing of cannabis use, as the cannabinoids can be detected for longer in urine (Cone 1993). However, from the viewpoint of testing for recent use and current intoxication, saliva is the most appropriate method. It would seem more appropriate to screen for THC in saliva, as opposed to THC-COOH in urine, as it is considered the only form of the drug found in saliva, and is as a result of contamination and not passed from the plasma.

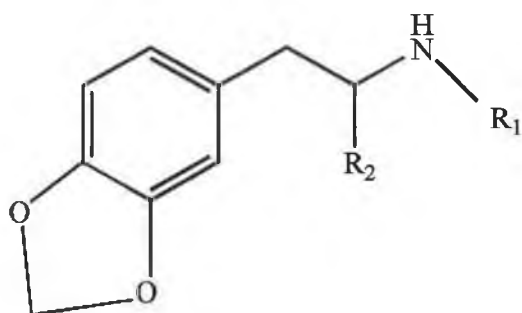
### 1.4.3 Amphetamines



*Amphetamine*



*Methamphetamine*

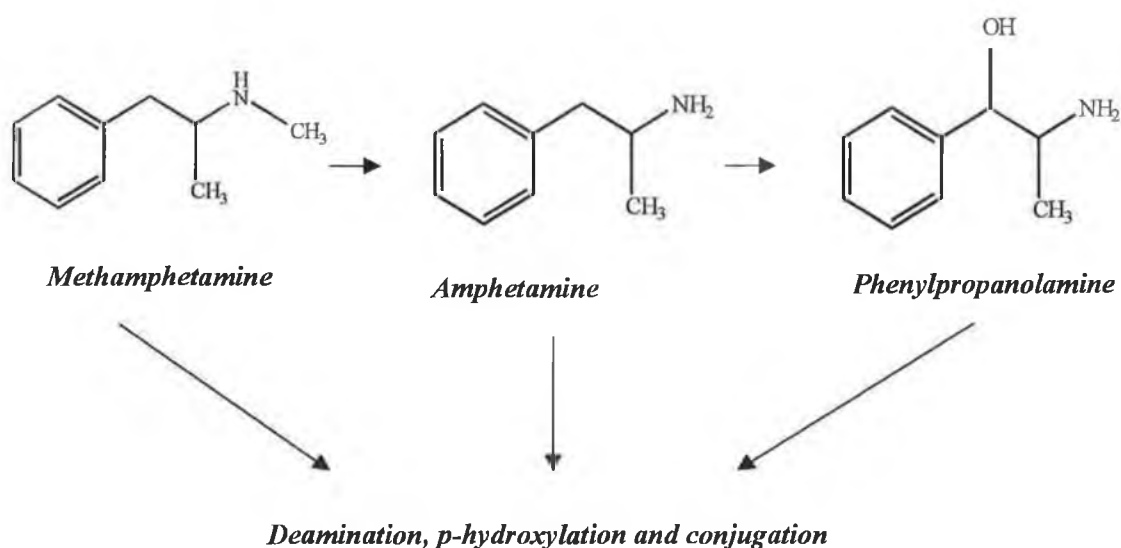


<i>MDMA</i>	$R_1 = \text{CH}_3$	$R_2 = \text{CH}_3$
<i>MDA</i>	$R_1 = \text{H}$	$R_2 = \text{CH}_3$
<i>MDE</i>	$R_1 = \text{C}_2\text{H}_5$	$R_2 = \text{CH}_3$
<i>MBDB</i>	$R_1 = \text{CH}_3$	$R_2 = \text{C}_2\text{H}_5$

**Figure 1.3:** Structures of amphetamine, methamphetamine and the ‘designer’ amphetamine derivatives.

In the 1970's, amphetamines were commonly used for the treatment of obesity because of their anorexic effects. The use of amphetamines for this indication was dramatically decreased because of the associated abuse. The psychological effects of amphetamines include mood elevation, increased blood pressure, increased energy and alertness and decreased appetite. Amphetamines are available in the d- and the l- isomeric forms and also in the racemic mixture form. (Cone, 1993). The common routes of administration of amphetamines are oral and intravenous. Methamphetamine, also called by its street name ‘ice’, and other substituted derivatives are commonly administered by oral, intravenous or smoking routes. The most commonly abused substituted derivative of amphetamines are 3,4-methylenedioxymethamphetamine, (MDMA) also called by its street name, “ecstasy”, 3,4-methylenedioxyamphetamine, (MDA), and 3,4-methylenedioxyethylamphetamine, (MDEA). These drugs are abused to a huge extent particularly at "rave" parties where the pharmacological effects of increased energy and alertness are exploited. Other amphetamine derivatives such as ephedrine, pseudoephedrine and phenylpropanolamine are used in medicinal allergy and cold formulations (Cone, 1993). This is an important consideration when developing a

detection system for amphetamine abuse. The cross reactivity of the immunoassay with these common cold medications must be investigated, as false positive results could have significant legal and financial implications. An example of such a case currently in the news involves the British Olympic medalist skier who tested positive for methamphetamine. He claimed that it was a result of using the ‘over the counter’ Vicks inhaler, available in the US, that contains l-methamphetamine.



**Figure 1.4:** Metabolism of methamphetamine and amphetamine. (Braithwaite et al., 1995)

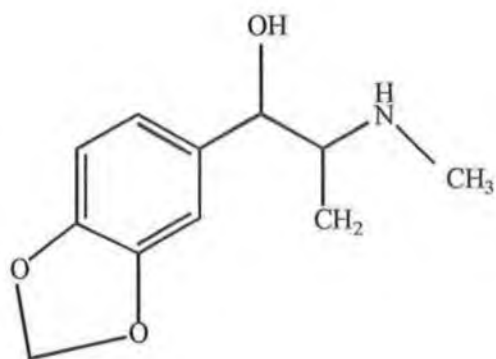
Beckett & Rowland (1965), examined the clinical effects and excretion of the d- and the l- isomers of amphetamine. d-amphetamine was found to be the more potent isomer with regard to clinical effects, such as, central nervous stimulation and dryness of the mouth. These clinical effects were not seen when the l-isomer was administered. They found that the excretion of amphetamines is highly dependent on the pH of the urine. Under normal urinary pH conditions, up to 40% of amphetamine is eliminated unchanged in the urine. The amphetamine can be detected in the urine 20 minutes after the dose is administered (Braithwaite, 1995). Amphetamine is metabolized by the hydroxylation of the phenolic ring to p-hydroxyamphetamine and by deamination to phenol acetone which is oxidised to benzoic acid (Figure 1.4). Methamphetamine is

metabolized to amphetamine. When the urine is acidic, renal excretion is the major pathway of elimination of amphetamines from the body. Wan *et al.* (1978) found that the elimination of the amphetamines was dependent on the urinary pH. The salivary pH in the subjects they tested remained constant even under conditions used to induce alkaline and acidic urinary conditions. These findings indicate that saliva would be a more suitable matrix for detection of amphetamine use as the levels of amphetamine measured in urine are too highly dependant on the urinary pH to give reliable results. Experienced drug abusers can manipulate this property by taking large quantities of sodium bicarbonate concurrently with the dose of amphetamines. This results in basic urinary conditions and so the amphetamines are metabolised in the plasma rather than being excreted, in the original form, via the renal route. This prolongs the clinical effects of the drug and also reduces the excretion of amphetamines into the urine, hence reducing the likelihood of detection in urine (Braithwaite 1995).

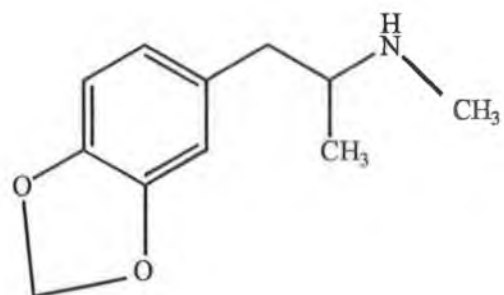
The relationship between the plasma and saliva concentrations of a drug can be predicted on the acidity/basicity of the drug as discussed above. Amphetamines have a S/P ratio greater than 1.0, due to their pKa greater than 5.5, (Haeckel and Hänecke, 1996). This theoretical greater concentration of amphetamines in saliva compared to plasma is another advantage of using saliva for detection. Wan *et al.* (1978), investigated the kinetics and salivary excretion of the d- and l- isomers. They found that the major difference between the isomers is in the elimination half-time. The d-isomer has a more rapid half-time than the l-isomer. The d-isomer is rapidly metabolised under basic urine conditions when metabolism rather than renal excretion is the major route of elimination. The authors proposed that the stereoselectivity of the deamination process, which is more complicated for the d-isomer, is the basis of the stereoselective metabolism. The difference in the half-time between the isomers is decreased when renal excretion is the major route of elimination and this would occur under acidic urine pH conditions.

Kintz & Samyn (1999) reviewed the determination of designer amphetamines in saliva, sweat and hair. In saliva, it was found that the parent drug is detected in higher concentration compared to its metabolites. An investigation into the excretion of MBDB and BDB in saliva and sweat also supported these results by finding that MBDB was present in higher concentrations than its metabolite, BDB, (Kintz, 1997). The only other publication examining the concentration and form of MDMA found after administration is by Navarro *et al.* (2001A). They measured the concentration of

MDMA, and its metabolites, MDA and 4-hydroxy-3-methoxymetamphetamine, (HMMA), in saliva and plasma after administration of 100mg of MDMA. MDMA was the major form found, at concentrations between 1728 and 6510 ng/ml in the saliva, with the peak concentration occurring at 1.5 hours after administration. The saliva/plasma ratio was 32.3 – 1.2. The reports from a different study of users, showed that the subjective psychological feelings mirrored the profile of the salivary concentrations, with subjective feelings reaching a peak at 1.5 hours. Another interesting parameter measured in the study was salivary pH. The pH decreased by 0.6 units at 1.5 hours after administration, probably related to the accumulation of the drug in the saliva. There is a lack of publications regarding the concentrations of amphetamines found in saliva. The concentrations reported, in the limited number of publications are in the nanogram/ml to microgram/ml range. However, it could be presumed, given the previous discussions, that it is an appropriate matrix for its detection.



*Ephedrine*

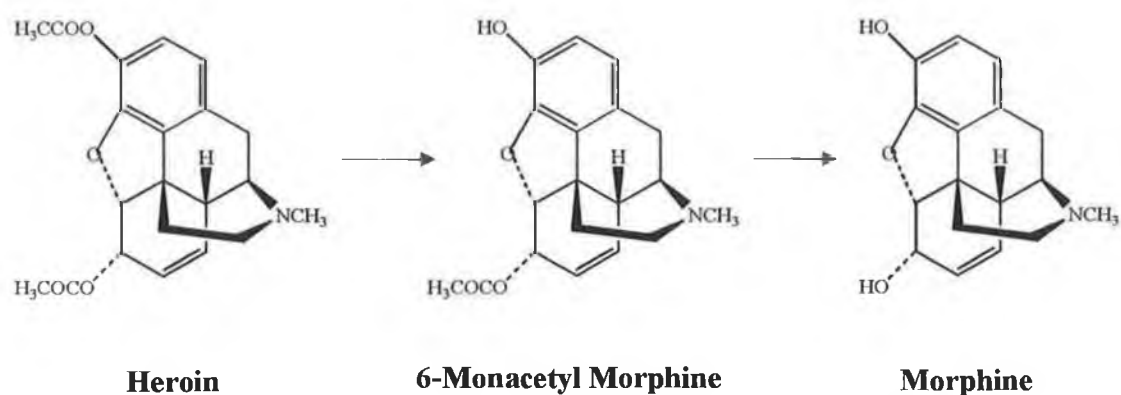


*Methamphetamine*

**Figure 1.5:** Structure of ephedrine, found in 'over the counter' flu and cold remedies and structure of methamphetamine. (Braithwaite *et al.*, 1995)

#### 1.4.4 Opioids

Opioids are used for the relief of severe pain in the clinical setting. The effects include analgesic, drowsiness, mental clouding, decreased gastrointestinal motility, nausea, and vomiting and respiratory depression. Heroin is the probably the best known of the opiates due to its addictive characteristic. Heroin abuse remains a serious problem in many cities. Heroin abuse and addiction arises from the psychological effects.



*Mainly - conjugation with glucuronic acid mainly at 3-position*  
*Small amounts*      - conjugation with glucuronic acid at 6-position  
                                 - demethylation to normorphine  
                                 - methylation to codeine

**Figure 1.5:** The metabolism of heroin and morphine.

Heroin is the diacetyl derivative of morphine, it is more lipid soluble and gets into the central nervous system faster than morphine. Directly after heroin administration, the user describes a "rush" feeling. Heroin is rapidly metabolised to 6-acetyl morphine, which is the active metabolite and responsible for the clinical effects. The nature of the reported initial rush is not clear as it may be the anticipation experienced by the abuser rather than the actual clinical effect. (Palfai and Jankiewicz, 1997).

Opioids are rapidly absorbed from the gastrointestinal tract but do undergo significant first pass elimination on passage through the liver. Morphine is metabolised mainly by conjugation with glucuronic acid at the 3- or 6- position. Codeine is a less potent

analgesic than morphine or heroin. It is metabolised in the liver to morphine by 3-O-demethylation and to norcodeine by N-demethylation. (Braithwaite *et al.*, 1995). Boerner *et al.* (1975) summarised the experimental results regarding the metabolism of heroin and morphine. Very low concentrations of heroin and 6-acetyl morphine, 0.13% and 1.3%, respectively, were recovered in the urine of heroin users. The major metabolite recovered is morphine-3-glucuronide. Trace amounts of free morphine, morphine-6-glucuronide, morphine-3-etheral sulphate, free normorphine, normorphine conjugates, free norcodeine and codeine were recovered.

Heroin abusers generally administer the drug by the intravenous, subcutaneous, intramuscular, intranasal, and smoking routes, and also by heating the powder and inhaling the vapours. Jenkins *et al.* (1995) measured the heroin, 6-acetyl morphine and morphine concentrations in blood and saliva samples from subjects after they had been administered heroin by the smoking and intravenous routes. The samples were collected over a 24 hour period and analysed by gas chromatography/mass spectrometry. The limit of detection of the assay was 1 ng/ml. The results showed significantly higher concentrations of heroin, morphine and 6-acetyl morphine in salivary samples following administration by the smoking route. This is obviously due to contamination of the nasal cavities with the drugs by the smoking route. The concentration of morphine in the blood declined more slowly than heroin or 6-acetyl morphine and it was detected for up to 2 hours after smoking and up to 12 hours after intravenous administration. Following heroin administration via the smoking route, the peak concentration of morphine in saliva ranged from 6 to 142 ng/ml. Morphine salivary concentrations were less than 16 ng/ml after administration by the intravenous route.

Wang *et al.* (1994) analysed saliva from subjects who had received 12 mg of heroin by the intranasal route of administration. The analysis was performed by solid phase extraction and gas chromatography/mass spectrometry. They found that heroin and 6-acetyl morphine concentrations reached their peak 10 minutes after administration, being 307.8 ng/ml and 58.7 ng/ml, respectively, and then decreased over a period of one hour. Morphine concentrations peaked at one hour after administration, (25.4 ng/ml), and declined to 1 ng/ml, the limit of detection of the assay, by 3 hours. Cone (1990), found that morphine shows increasing concentrations in saliva, plasma and urine in that order. The presence of opiates, (dihydrocodeine, dihydromorphine, codeine, morphine, 6-monoacetylmorphine) in saliva and urine, was investigated by



GC-MS in subjects participating in a drug withdrawal program. The correlation of the results of the saliva samples with urine results was over 90%, concluding that saliva may be adequate for the detection of the opiates, (Speckl *et al.*, 1999).

The rapid metabolism of heroin to 6-acetyl morphine and its subsequent metabolism to morphine with a longer elimination half-life would lead to the conclusion that it is appropriate to focus on morphine as the analyte in an immunoassay to detect recent heroin or morphine use. The main concern in relation to analysing saliva samples for recent heroin or morphine use is of the quantitative nature given the results presented by investigators (Cone, 1993) in which low concentrations were detected. However, other studies have shown that the concentration of morphine found after a short time of administration can be as high as 20µg/ml, (Chapter 6; Leute *et al.*, 1972). Thus, the concentrations of morphine and indeed heroin and 6-acetyl morphine detected in saliva samples of opioid users needs to be considered when determining the cut off concentration and limit of sensitivity in an immunoassay.

### 1.5 Levels of detection of assays and cut off levels

Currently, there are no established cut off levels for drugs of abuse in saliva samples. For the purposes of the Standards, Measurements and Testing (SMT) project, discussions were held between the following; School of Biotechnology, Dublin City University; Department of Toxicology, University of Ghent, Belgium; Envitec GmbH, Germany, and Nunc, Denmark; to establish suitable cut off levels for THC, cocaine, morphine and amphetamines in saliva samples. The current international guidelines recognised by the Substance Abuse and Mental Health Service Administration (SAMHSA), National Institute of Drugs of Abuse (NIDA), and World Health Organisation (WHO), for cut off levels in urine and plasma were considered and extrapolations made to the salivary concentration. The cut off level for THC was raised as compared to the plasma level due to high degree of contamination and also the possibility of positive samples by passive smoking and ingestion of cannabis containing (hemp) products .

The following were established as guidelines for our assay development, (Table 1.2).

**Table 1.2:** *Cut off concentrations of drugs for the SMT project.*

	Saliva Cut-Off Level (ng/ml)	Urine Cut-Off Level (ng/ml)	Plasma Cut-Off Level (ng/ml)
<b>THC</b>	200	50 (THC-COOH)	2
<b>Cocaine</b>	50	300 (BEC)	50
<b>Morphine</b>	20	300	20
<b>Amphetamines</b>	50	1000	50

## ***1.6 Methods of Detection of Drugs of Abuse***

The methods for analysis of drugs of abuse include thin-layer chromatography (TLC), gas chromatography coupled with mass spectroscopy (GC-MS), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). GC-MS is the gold standard test for the confirmation of the presence of drugs of abuse in biological samples (Braithwaite, 1995). This test would only be performed on samples that had previously been screened using a more rapid method for example immunoassay, TLC. The extensive preparation of the samples required in order to run these chromatographic tests is well documented (Braithwaite, 1995). Modified solvent extractions and solid phase extraction techniques are used and commercial kits are available for these purposes.

The current chromatographic methods used for drugs of abuse are concisely reported on by Braithwaite *et al.* (1995). The focus of the following section will be the immunological methods, biosensors and commercial tests available.

### ***1.6.1 Immunoassays***

Immunoassays are widely used for the detection of drugs of abuse. They can be developed to be highly specific, sensitive, relatively rapid and usually require little or no preparative clean up steps for the test matrix. The following are types of immunoassays that are used for detection of drugs of abuse: enzyme-linked immunosorbent assay (ELISA), enzyme-multiplied immunoassay techniques (EMIT), fluorescence polarisation immunoassays (FPIA) and up-converting phosphor technology (Braithwaite, 1995; Niedbala, 2001). Immunoassays can be divided into two types, heterogenous and homogenous. In heterogenous assays, the antigen antibody mixtures are separated from the free antigen or antibody by a solid support, such as an immobilised conjugate. In homogenous assays, there is no such separation. There are many more sub-divisions and types of immunoassays. The basis of all enzyme immunoassays is the binding of the antibody to the antigen of interest. This binding is detected using an enzyme, with the enzyme acting on a substrate producing a coloured product which is subsequently measured. Two broad classifications of heterogenous immunoassay are competitive, and non-competitive, e.g. sandwich ELISA.

### *1.6.1.1 Competitive Immunoassay*

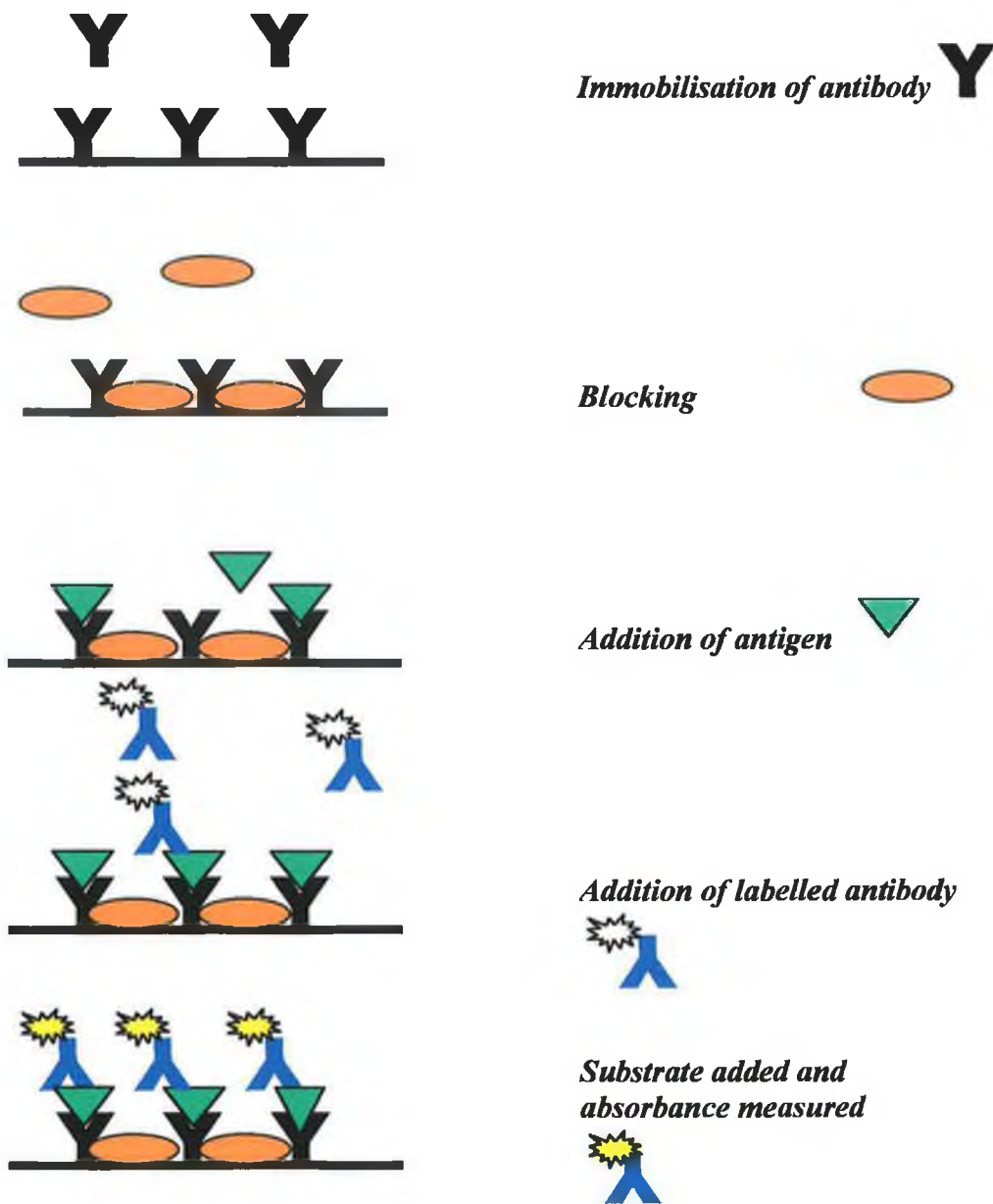
In a competitive immunoassay, one species is immobilised onto the ELISA plate, a mixture of a second and third species are added. Competition is created through two of the species binding to the antibody. An example of a competitive ELISA is shown in Figure 1.8. Antigen is immobilised, and a mixture of antibody and free antigen are added. The amount of free antibody available to bind to the immobilised antigen is inversely proportional to the amount of free antigen in the solution. The subsequent substrate colour change is inversely proportional to the antigen in the solution. An example of a variation of the competitive assay is the inhibition assay. This is the immobilisation of antigen, followed by the addition of a sample of antigen free in solution, followed by addition of antibody. During the incubation period, competition occurs between the immobilised antigen and the antigen free in solution for binding by the antibody. This step is then followed by incubation with anti-species antibody that is enzyme-labelled. The resulting change in substrate colour in the final step is inversely proportional to the amount of free antigen in the test solution. The difference between the inhibition assay and the competitive assay is subtle. In the inhibition assay, the antigen and antibody are not equilibrated before each are added to the antigen-coated wells.

### *1.6.1.2 Non-Competitive Immunoassay*

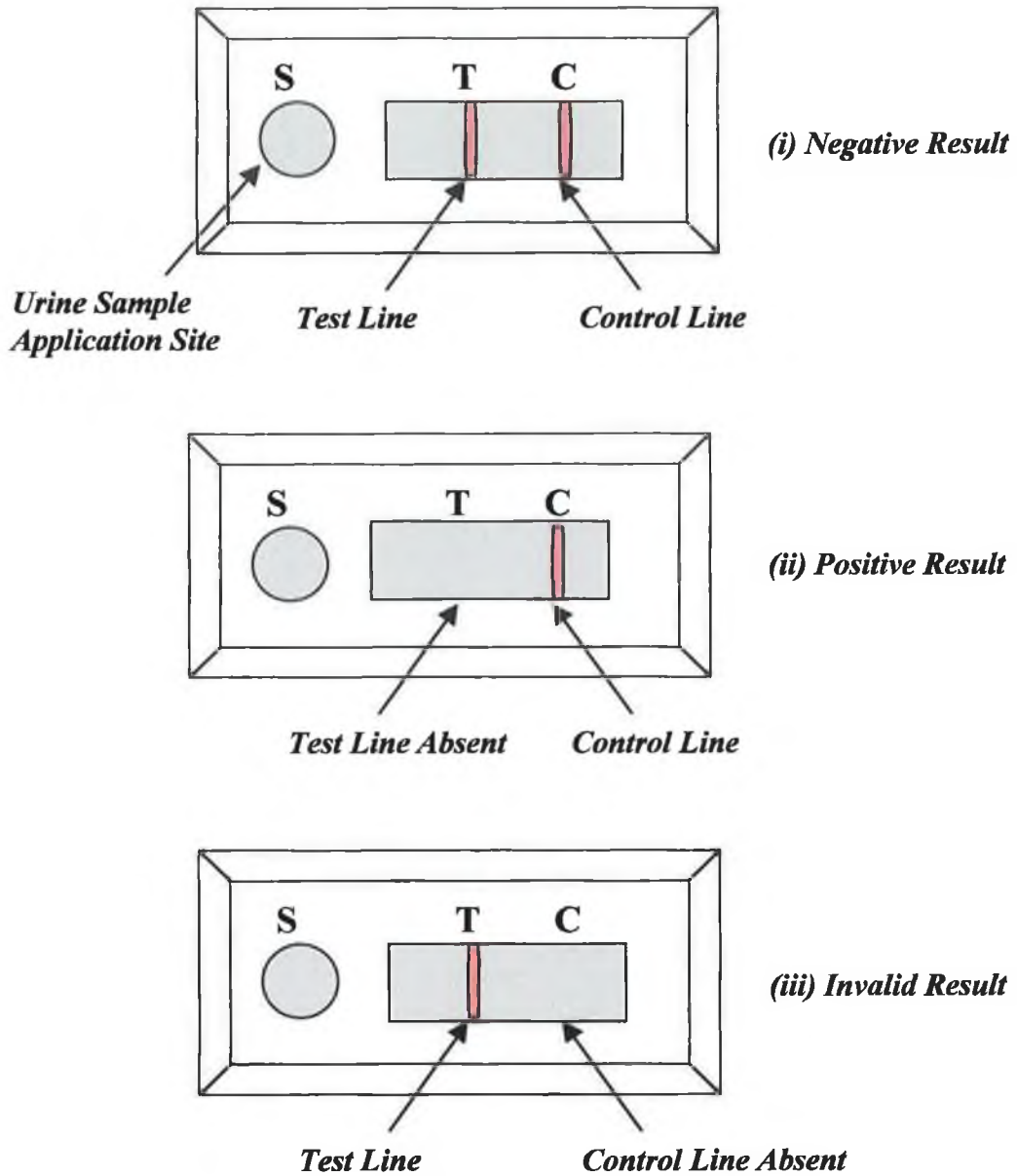
In a sandwich ELISA, (Figure 1.7), two different antibodies, reactive with different epitopes of the antigen are required. One antibody is immobilised to the solid phase, and the antigen is then added. This is followed by the addition of another antibody that is specific for a different epitope of the antigen.

One of the most common rapid assays available currently are the dip-stick or test strip immunoassays. These involve antibodies being coated on surfaces such as nitrocellulose strips. Test strip assays usually employ a sandwich or competitive immunoassay format and lateral flow of the applied sample facilitates accumulation at a region pre-coated with antigen, (Figure 1.9). An example of a format used is the One-Step Rapid Opiates Test, (Craig Medical, USA), for detection of opiates in urine. The urine sample is applied to the chromatographic strip and reacts with labeled antibody-dye conjugate. They laterally flow along the strip and any unbound antibody-dye conjugate binds to immobilised antigen conjugate in the test zone of the strip. This produces a specific

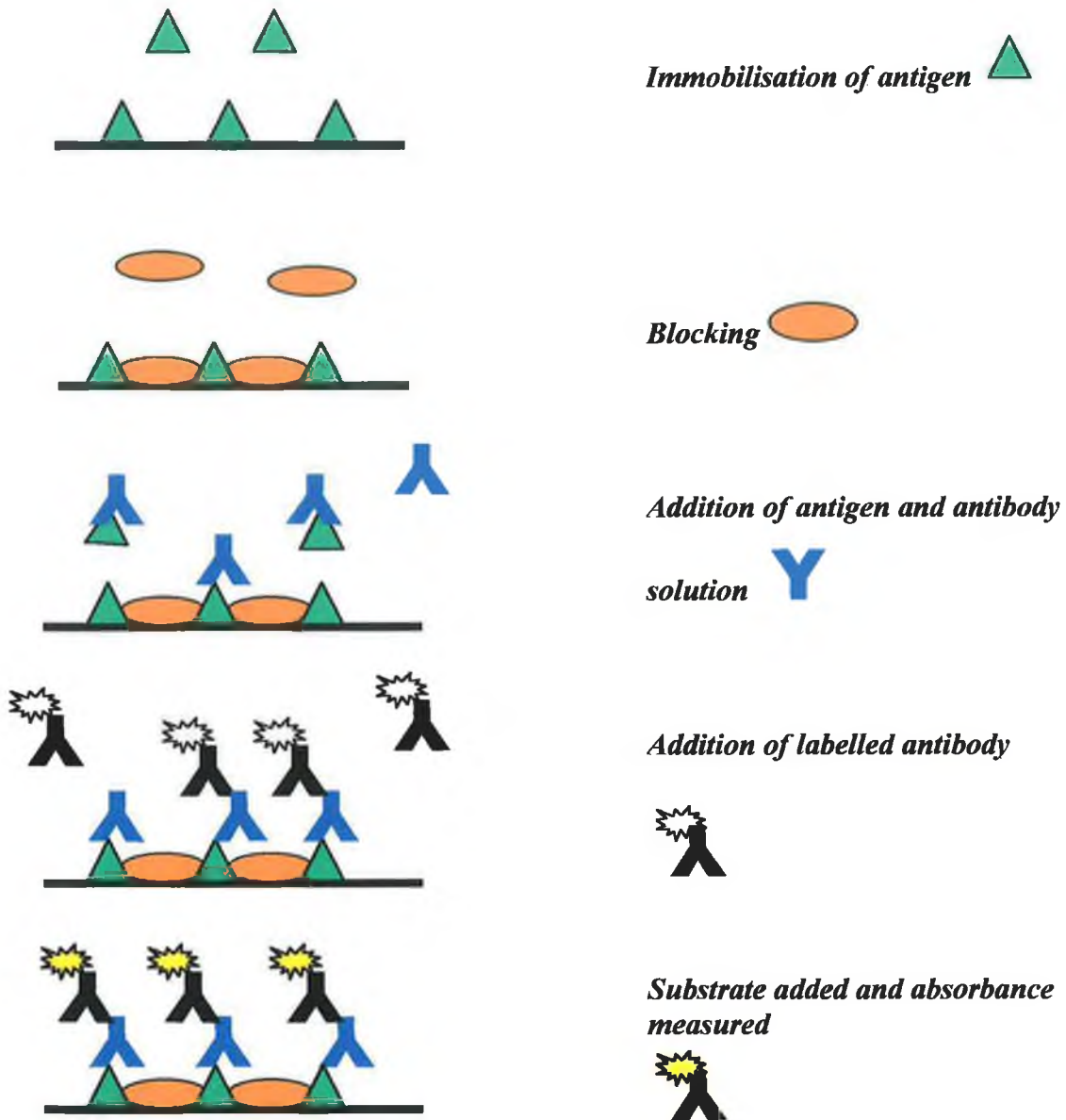
colour line in the result window of the strip, which indicates a negative result. On the other hand, if the urine contains opiates, at a concentration above the cut-off level, the antibody-dye conjugate binds to the free drug in the urine and forms an antigen-antibody-dye complex. This complex competes with the immobilised antigen conjugate in the test zone, preventing the development of a coloured line. A positive control is built in by incorporating a non-specific sandwich dye conjugate reaction.



**Figure 1.7:** Schematic diagram of a non-competitive sandwich ELISA. Two antibodies of different antigenic specificity are used, one of which is labelled with an enzyme. The unlabelled antibody is used to coat the wells. Antigen in solution binds to this antibody. The enzyme-labelled antibody is then added and will bind to the antigen. Substrate is added and the absorbance measured. The intensity of the response is directly proportional to the concentration of antigen that was in the test solution.



**Figure 1.9:** Diagram of example of lateral flow 'dip stick'-type immunoassay for the detection of drugs in a urine sample. The development of the two lines, a test line and a control line, indicates a negative test for the targeted drug (i). The development of the control line and absence of the test line indicates a positive result (ii). The absence of a control line in the window indicates an invalid result regardless of the test line result (iii).



**Figure 1.8:** Schematic diagram of a competitive ELISA. Antigen is immobilised on the wells. A mixture of the sample containing antigen, and a constant amount of antibody are added. Competition occurs between the immobilised antigen and the free antigen for binding to the antibody. Labelled secondary antibody is added, that recognises the bound antibody. Substrate is added and the absorbance measured. The intensity of the response is inversely proportional to the concentration of antigen that was in the test solution.



### ***1.6.2 Enzyme-Multiplied Immunoassay Technique***

EMIT is an homogenous competitive assay. The antigen is labelled with an enzyme and mixed with the sample antigen free in solution and antibodies. Competitive binding takes place, and the binding of the enzyme-labelled antigen, sterically hinders the active site of the enzyme thereby preventing enzyme activity. When unlabelled free antigen is added, it competes with the labelled antigen for binding to the antibody. The greater the level of antigen added, the greater the level of unbound enzyme-labelled antigen resulting in greater enzyme activity. Behring Diagnostics Inc supply a number of EMIT kits for the detection of cannabinoids, opiates, cocaine and amphetamine.

### ***1.6.3 Fluorescence Polarisation Immunoassay***

FPIA is a homogenous competitive assay in which a known amount of antigen or drug analog is labelled with fluorescein and mixed with sample antigen and antibody in free solution. The labelled and sample antigen compete for binding to the antibodies and detection is by means of a vertically polarised detector. The detection is based on the difference in the rotation speeds of the free and bound fluorescein-antigen. The free fluorescein-antigen rotates at higher speeds and results in emission of light in a different plane to the incident light, so it will not be detected. The bound fluorescein is not free to rotate and so the emitted light is almost in the same plane as the incident light and so it is picked up. A major advantage of the FPIA is that it is homogenous and there is no need for the immobilisation step.

### ***1.6.4 Detection of analytes by immunoassay using up-converting phosphor technology.***

Up-converting phosphor technology is based on lanthanide-containing, ceramic particles that can absorb infrared light and emit visible light. The important distinction between fluorescence and phosphorescent is that biological matrices do not up-convert and so there is no background sample autofluorescence. Niedbala *et al.* (2001A) have developed lateral flow immunoassay strips for the detection of drugs of abuse using this up-converting phosphor technology, (UPT). The assay strips are designed like a lateral

flow test that uses colloidal gold or latex particles. The up-converting phosphor particles, about 400nm in diameter, are covalently conjugated to the antibodies using EDC/NHS chemistry. The basis of the test is that in the competitive format used, the UPT-antibody-drug complex will not bind to the test line, immobilised drug-protein conjugate, in the presence of drug in the sample. If the drug is not present in the sample, the UPT-antibody binds to the immobilised drug-protein on the test line giving a signal. This response at the test line is inversely proportional to the amount of drug in the sample.

### **1.6.5 Agglutination**

Agglutination assays are common, and easy to perform. The basis of the assay is the specific mixture of antibody and antigen and visible aggregation of particles. They are homogenous, as they do not require the separation of free and antibody-bound fractions of the analyte. A variation of the agglutination assays include haemagglutination and haemagglutination-inhibition. In the case of haemagglutination the antigen-antibody interaction is mediated using red blood cells pre-coated with the antigen of interest. The addition of test sample containing antibodies results in a visual agglutination, (Fitzpatrick *et al*, 2000). Latex agglutination is similar to haemagglutination assay, in this case the antigen or antibody is coated to latex beads. An example of one popular commercially available agglutination test for drugs of abuse in urine, is the Ontrak® kits, by Roche Diagnostics.

### **1.6.6 Biosensors**

One description of a biosensor is as follows: a sensing device that incorporates a biological entity as a fundamental part of the sensing process (Diamond, 1998). Biosensors have been applied to the field of detection of drugs of abuse, e.g., Ogert *et al*. (1992), developed a continuous flow immunosensor for the detection of cocaine based on a fluorescence assay. The immunosensor is based on the displacement of the fluorophore-labeled cocaine from immobilised antibody. It consists of a sepharose microcolumn with immobilised anti-cocaine antibodies. A buffer flows into the column and exits to an on-line fluorimeter. The immunosensor depends on the immobilised antibodies for specific recognition of cocaine and its closely related metabolites. The

displacement of fluorophore-labelled benzoylecgonine from the immobilised antibodies by samples containing cocaine produces the fluorescent signal. The limit of detection of cocaine was 5ng/ml.

Devine *et al.* (1995) developed a fibre optic biosensor for the detection of cocaine. Anti-benzoylecgonine monoclonal antibodies were immobilised onto quartz fibres and a flow fluorometer was used to detect changes in the fluorescence. A BEC-fluorescein conjugate was produced, and it bound to the immobilised antibody. The cocaine in the test sample competed for binding to the antibody in a concentration-dependant manner, and so reduced the initial rate or steady state fluorescence. The regenerable nature of this assay and that described by Ogert *et al.* (1992), is key to its success. The detection limit for cocaine in this assay was 5ng/ml and for benzoylecgonine was 30ng/ml. Yu *et al.* (1996) presented results from a similar flow immunosensor for the detection of benzoylecgonine in urine samples, giving a 97% correlation with results from samples analysed by GC-MS.

Another variation on the theme of fluoroimmunoassay-based biosensors is described by O'Connell *et al.*, (1999). They evaluated a fluoroimmunoassay using microbeads instead of quartz fibers as a solid support, and the commercial system KinExA™ (Kinetic Exclusion assay) for the bead handling flow fluorometer system. A quantity of beads are coated with antibody and are introduced into a capillary flow cell and retained on a screen. Free benzoylecgonine in the test solution competes with fluorescein-benzoylecgonine conjugate for binding to the screen, and so the bead-bound fluorescence is reduced in the competitive format. The system monitors the binding of the fluorescent conjugate to the fiber in real-time and so it is also designed to measure association and dissociation rate constants of antigen-antibody complexes, similar to surface plasmon resonance technology.

Analyte 2000 (Research International, Woodinville, WA, USA) is a fibre optic biosensor that has been applied to the analysis of cocaine and its metabolites in human urine using a competitive fluorescence immunoassay, (Nath *et al.*, 1999). In this case, the binding of anti-benzoylecgonine monoclonal antibody to casein-benzoylecgonine antigen-coated optical fibres was inhibited by the presence of cocaine. The bound antibody, which is inversely correlated to the cocaine concentration in the sample, is measured by the fluorescence produced by the subsequent binding of cyanine dye-tagged anti-mouse antibody. The use of evanescent excitation of fluorescence in fibre

optic biosensors ensures that only fluorophore bound to the surface of the fibre optic is detected, and so the sensor does not detect any sample constituents or unbound fluorophores. The minimum level of detection in this assay was 0.75ng/ml. The Analyte 2000 is composed of four single-fibre optics and so can perform the analysis of four drugs in the one sample. Currently, the main disadvantages with the systems is the lack of automation and the labour intensive preparation of the fibres. These problems however, are likely to be addressed and improved upon as further applications are developed. For all of the types of immunoassays described it must be reiterated, that their performance in terms of sensitivity and specificity are fundamentally associated with the quality of the antibodies that are used.

As mentioned previously, the detection of drugs by chromatographic methods requires an extraction step, as opposed to immunoassays that usually do not require a sample pretreatment step. There has been considerable research into amperometric and piezoelectric immunosensors (Cassidy, 1998), however, they do not seem to have found a place yet in the routine analytical laboratory.

Surface plasmon resonance-based biosensors are very successful analytical tools and are being used increasingly in research and analytical labs. They are discussed in Chapter 5, in detail.

A novel, non-immunological-based biosensor using frog melanophores to detect opioids has been developed by Karlsson *et al.* (2002). This sensor harnessed the ability of lower vertebrates such as fish and frogs to change colour. In response to specific stimuli, chromophores change colour by redistributing their pigment granules within the cell. Melanophores which are a particular type of chromophore that contains brown melanin pigment granules, were transfected with human opioid receptor 3 and cultured and used for opiate detection. In the presence of opioids, the pigment granules aggregated in a dose-dependant response in the melanophores. This technique of transfection of melanophores with different receptors may create an alternative biosensor for other substances. An example of another novel non-immunological-based assay is the bioluminescent assay for heroin and morphine that uses heroin esterase and morphine dehydrogenase linked to bacterial luciferase (Holt, 1996).

### 1.7 Commercial Tests

The ROSITA project, funded by the European Commission, produced comprehensive papers on roadside drug testing in Europe. Work packages on different aspects of the project include:

- Drugs and medicines that are suspected to have a detrimental impact on road user performance, (Maes *et al.*, 1999, [www.rosita.org](http://www.rosita.org)).
- Inventory of state-of-the-art roadside drug testing equipment, (Samyn *et al.*, 1999).
- Operational, user and legal requirements across EU member states for roadside drug testing equipment, (Moller *et al.*, 1999, [www.rosita.org](http://www.rosita.org)).
- Evaluation of different roadside drug tests, (Verstraete & Puddu, 2000, [www.rosita.org](http://www.rosita.org)).
- General conclusions and recommendations, (Verstraete & Puddu, 2000).

These informative documents can be accessed on the Rosita website at [www.rosita.org](http://www.rosita.org).

There are numerous commercial kits available for the screening of urine for drugs of abuse. However, because of the different form and concentrations of the drugs found in saliva, they are not ideally suited to saliva screening, and are not marketed for such an application. Three devices are commercially available for the purposes of roadside drug testing in saliva samples. These are Drugwipe (Securetec GmbH, Germany), Oral Screen (Avitar Technologies Inc, USA), and Rapiscan, (Cozart Biosciences Ltd., UK). The advantages and disadvantages of each are detailed in the above reports. Drugwipe is used for the detection of drugs on surfaces. Different tastes are available for the detection of opiates, cocaine, cannabis and amphetamines. The test is a lateral flow membrane immunoassay, that consists of a wiping section and the chromatographic reaction cartridge. The substances captured on the wiping device are applied to the strip. The addition of water allows the progression of the immunoassay, (Securetec, Germany). Drugwipe, as reported by the Rosita project, is available for the detection of recent use of cocaine, amphetamine, and designer amphetamines. They found that it was unreliable for detection of cannabis and some false negative results with regard to the detection of opiates. The Drugwipe for opiates is supposed to give a positive test with 5ng/ml of heroin, morphine or codeine. Kintz (1998), found an unacceptable number of false negative results in a study in which volunteers were administered codeine and subsequently tested using Drugwipe and saliva samples were also analysed

by GC-MS. Part of the problem contributing to the false negatives may have been the inadequate sample collection or too low a cut-off mark for the analyte. An advantage of this test is that it does not involve saliva collection, instead, the tongue is wiped with a device. This test has also been marketed for drug detection in sweat samples. Navarro *et al.* (2001B) investigated the concentration of MDMA, in saliva with Drugwipe in a controlled study where recreational users were administered MDMA in a double-blind, cross-over, placebo clinical trial. Saliva samples were collected in addition to the 'on-site' procedure and these samples were retested in the lab using Drugwipe and also tested using GC-MS. At 1.5 and 4 hours after administration, all eight subjects gave a positive result. The cut-off level determined by comparison to the GC-MS results was 650ng/ml.

OralScreen is available for the detection of opiates, cocaine and cannabis, ([www.avitarinc.com](http://www.avitarinc.com)). The ORALscreen System contains an oral fluid collection device, and a test device containing a lateral flow membrane immunoassay. The main disadvantage documented by the ROSITA project with this device, was the difficulties with the saliva collection procedure. Many of the testers were in direct contact with the saliva during the procedure. Barrett *et al.* (2001) of Avitar conducted a study comparing the ORALscreen test for THC, cocaine, opiates and methamphetamine in oral fluid with a laboratory-based urine screen that involved EMIT testing for THC, cocaine metabolite, and opiates and by CEDIA® (Microgenics, USA) testing for amphetamine and methamphetamine. CEDIA testing is a commercial immunoassay that can be run on automated clinical chemical analyzers. The urine samples that tested positive by these screening tests were then analysed by GC-MS confirmation assays. The results show very good correlation for detection of cocaine and opiates for 2.5 and 3 days after use, respectively. THC was detected by the ORALscreen on the day of use and one day following use. There was good correlation between the urine and oral fluid testing for methamphetamine. However the number of days following use was not determined.

Rapiscan, produced by Cozart Bioscience, is probably the most advanced roadside testing device as it incorporates a digital read-out of the results. It has been tested in a major trial by UK authorities. The test consists of three components, a test swab, for saliva collection, a disposable cartridge, and an instrument which displays the results. The sample is placed in the cartridge in the hand held device and the immunochemical

reactions take place, giving results after five minutes, ([www.cozart.co.uk](http://www.cozart.co.uk)). This test detects cannabinoids, ecstasy, cocaine, opiates and benzodiazepines in a saliva sample (Cozart Biosciences, 1999). The Rosita researchers described the device as 'problematic' during the course of their field test. The problems included insufficient saliva collected, the test taking too long, and being too cumbersome. An advantage, however, was the availability of the remainder of saliva that could be used for laboratory confirmation. A recent paper by Jehanli *et al.* (2001), of Cozart Biosciences, examined the results of a trials that involved testing saliva samples from 43 volunteers after consumption of codeine and cannabis. The results from the Rapiscan device was compared with results obtained from immunoassays and GC/MS. They found that positive opiates results were obtained for up to nine hours after ingestion of the codeine, however, the cut-level for the cannabis test was too high at 10ng/ml THC, to detect marijuana use for more than a few hours after ingestion.

In the USA, Intercept developed by OraSure Technologies, Inc, (Bethlehem, PA) is a test kit in which the saliva is collected and then shipped to the designated lab for analysis in an immunoassay format, ([www.Orasure.com](http://www.Orasure.com)). Niedbala *et al.* (2001B & C) have presented the results of the saliva analysis and corresponding urine analysis. Using immunoassay cut-off values of 10ng/ml and 2000ng/ml for positive opiate presence in saliva and urine, respectively, it was found that the correlation was 93.6%. In the corresponding test for marijuana, they found that the time course of the concentration of THC in oral fluid is similar to that described for THC in plasma. This is remarkable as the presence of THC in saliva is believed to be as a consequence of impregnation, rather than diffusion of the THC from the plasma. Concentration of THC in the oral fluid declined in a multi-phasic nature that suggests the presence of fast and slow releases of sequestered THC in the oral cavity. The concentrations of the THC detected in the oral fluid were higher in the first six hours after smoking and the concentration in urine increased and exceeded the urine concentration after about 16 hours.

Immunological tests for drugs of abuse in urine are based on the following techniques: enzyme multiplied immunoassay technique (EMIT) such as EMIT® d.a.u.<sup>TM</sup> (Syva, UK), agglutination, such as Ontrak®, (Roche Diagnostics), fluorescence polarisation immunoassay, TDx® (Abbott Diagnostics) and radioimmunoassay, Abuscreen® (Roche Diagnostics). There are many different tests available for urine testing and these are also documented in the ROSITA reports. These urine screening tests are in

widespread use in analytical labs in institutions, hospitals, and clinics and are of an overall good standard. The limitations of the urine tests are that they can only be used for detection of drug metabolites in urine. The form of drug metabolites found in urine can differ significantly from the drug form found in saliva. Another significant limitation is that the cut off level of these tests (Table 1.2) is generally greater than the cut off level that would be acceptable for saliva testing. This therefore limits the use of these tests to urine screening for drugs of abuse.



## 1.8 *Summary of Introduction*

Assays for the detection of drugs of abuse range in complexity from immunoassays to more complicated analytical methods such as gas chromatography/mass spectroscopy (GC/MS). The development of immunoassays for the detection of illicit drug use that could be used in roadside testing would mean that screening could be performed in the same manner as current alcohol testing and the confirmatory test would be by GC/MS. Currently, the most popular biological media for quantitative measurement of illicit drugs is plasma and urine. However, saliva is now becoming common because of the obvious advantage of the non-invasive nature of collection and the correlation between psychological impairment and the level of detection of the illicit drugs and metabolites. In the development of the rapid roadside tests, the following considerations should be taken into account. The test must be:

- rapid
- specific and sensitive, with positive results correlating to the legal cut off level as determined by authorities such as the National Institute of Drugs of Abuse (NIDA)
- degree of cross reactivity with drug metabolites and other common legal substance needs to be established
- ‘user-friendly’ as it will be performed by non-lab personnel

The international scientific and legal communities must also begin to create guidelines for the establishment of such tests in order that all the essential qualities can be incorporated by research and development groups.

## *1.9 Aims of Thesis*

There is a huge demand in the scientific and security communities to establish rapid, robust, specific, sensitive assays for the detection of drugs of abuse in saliva samples. The aim of this project is the development of novel assays for the detection of drugs of abuse in saliva.

Chapter 3 describes the process of producing morphine and cocaine protein conjugates for the immunisation procedures. The drug-protein conjugates were used for the production of polyclonal antibodies to morphine and cocaine. THC-BSA was also used for the production of anti-THC polyclonal antibodies. The purified antibodies were then applied to an ELISA format for the detection of morphine, cocaine and THC.

Chapter 4 describes the production, purification and characterisation of the anti-amphetamine and anti-methamphetamine monoclonal antibodies. Successful clones of each antibody were produced and the antibodies were applied to an ELISA for the detection of amphetamine, methamphetamine, and the other common designer derivatives, MDA, MDMA, MDEA, MBDB. The ELISA was also established using saliva samples as the matrix. Particular attention was given to the cross reactivity studies of these antibodies during the cloning procedure, as many other legal 'amphetamine like' molecules, such as, ephedrine have shown cross reactivity with assays reported in the literature.

Chapter 5 expands the characterisation and application of the anti-amphetamine and anti-methamphetamine monoclonal antibodies using biosensor technology (BIAcore). Affinity constant measurements were determined using two different techniques, the classic ELISA based Friguet method and the solution-phase BIAcore assay. The antibodies were also applied for the development of a BIAcore-based inhibition assay for the detection of amphetamine and methamphetamine in saliva samples.

Chapter 6 describes the results of a pilot clinical study that was conducted to investigate the application of the antibodies produced for the detection of drugs of abuse. A number of different assays were used for the analysis of THC, and morphine. A

prototype of the Envitec device was used, and a rapid test for THC using the anti-THC polyclonal antibody was developed. BIAcore assays were also investigated for the detection of morphine in saliva samples. Real saliva sample samples were collected from drug users and analysed in the different formats. Specific issues relating to the stability of drugs in biological samples and the collection and storage of real saliva samples are also discussed.

## *Chapter 2*

### *Materials and Methods*

## 2.1 Materials

All chemicals and materials were obtained from Sigma Chemical Co. (Tallaght, Dublin 24). The exceptions are listed in Table 2.1.

**Table 2.1:** Chemicals that were obtained from companies other than Sigma-Aldrich.

Non-Essential Amino Acids	Gibco BRL
Fetal Calf Serum	Gibco BRL
Sodium Pyruvate	Gibco BRL
Briclone (Bioresarch Ireland)	Archport
Hydrochloric Acid (HCl) (Riedel de Haen)	Riedel de Haen
Acetic Acid	Riedel de Haen
Tween 20	Riedel de Haen
PBS Tablets	Oxoid
THC-BSA	Fitzgerald Industries
Morphine-BSA	Fitzgerald Industries
BEC-BSA	Fitzgerald Industries
BEC-BgG	Fitzgerald Industries
Amphetamine-BSA	Fitzgerald Industries
Methamphetamine-BSA	Fitzgerald Industries
THC-HRP	Fitzgerald Industries
Cocaine	McFarland Smith
Morphine	McFarland Smith
CM-Dextran	Fluka Chemicals
Bicinchoninic Acid Assay Kit	Pierce and Warriner

All licences necessary for importing and possessing illicit drugs were obtained.

### **Full Addresses of Suppliers**

**Archport**, Dublin City University Campus, Dublin 9.

**Fitzgerald Industries**, 34 Junction Square Drive, Concord, MA 01742, USA.

**Fluka Chemicals**, Gillingham, Dorset, UK.

**Gibco BRL**, Renfew Rd., Paisley, PA4 9RF, Scotland.

**McFarland Smith**, Wheatfield Road, Edinburgh, EH11 2QA, Scotland.

**Oxoid**, Basingstoke, Hampshire, RG24 8PW, UK.

**Pierce and Warriner**, Chester, UK.

**Riedel de Haen**, Wunstorfer Strabe, D-3106, Seelze, Hanover, Germany.

**Sigma Aldrich**, Tallaght, Dublin 24.

## 2.2 *Equipment*

**Table 2.2:** Equipment used and the supplier.

<b>Equipment</b>	<b>Supplier</b>
BIAcore 3000	BIAcore AB
3015 pH Meter	Jenway Ltd.
Titertek Multiscan Plate Reader	Medical Supply Company
Millipore Filtration Device	Medical Supply Company
Heraeus Labofuge 6000	Heraeus Instruments
Biofuge A Microcentrifuge	Heraeus Instruments
Ultrafiltration Cell	Amicon
Heraeus CO2 Incubator EG 115IR	Heraeus Instruments
Holten 2448K Laminar Flow Unit	Holten Laminar A/S
Nikon Diaphot Inverted Microscope	Nikon
UV 160A Spectrophotometer	Shimadzu
RM6 Lauda Waterbath	AGB Scientific
SB1 Blood Tube Rotator	Medical Supply Company
Atto dual minislabs system AE-6450	Atto

### **Full Addresses of Suppliers**

**AGB Scientific**, Dublin Industrial Estate, Glasnevin, Dublin 9.

**Amicon Inc.**, Beverly, MA 01915.

**Atto**, Bunhyo-Kui, Tokyo 113, Japan.

**BIAcore AB**, Uppsala, Sweden.

**Holten Laminar A/S**, Gydevang 17, DK 3450 Allerod, Denmark.

**Heraeus Instruments Inc.**, 111-a Corporate Boulevard, South Plainfield, NJ 07080.

**Jenway Ltd.**, Gransmore Green, Felsted Dunmow, Essex, CM6 3LB, UK.

**Medical Supply Company**, Damastown, Mulhuddart, Dublin 15.

**Nikon Corp.**, 2-3 Marunouchi 3-Chrome, Chiyoda-Ku, Tokyo, Japan.

**Shimadzu Corp.**, 1 Nishinokyo-Kuwabaracho, Nakagyo-ku, Kyoto 604, Japan.

### 2.3 Consumables

**Table 2.3:** Consumables used and the suppliers.

<b>Consumable</b>	<b>Source</b>
BIAcore sensor chips	BIAcore
Plastic labware, e.g., eppendorfs, centrifuge tubes, pipettes, pipette tips	Sarstedt
Maxisorb 96 well plates	Nunc
Tissue culture plasticware	Nunc
Ultrafiltration filters	Amicon
Saliva Collection Device	Trinity Biotech

#### **Full Addresses of Suppliers**

**Amicon**, Beverly, MA 01915.

**BIAcore AB**, Uppsala, Sweden.

**Nunc**, Kamstrup DK, Roskilde, Denmark.

**Trinity Biotech**, Southern Cross Business Park, Bray, Co. Wicklow.

**Sarstedt**, Sinnottstown Lane, Drinagh, Co. Wexford.



## **2.4 Standard Solutions**

### **Phosphate Buffered Saline (PBS)**

One PBS tablet was dissolved in 100mls deionised water per the manufacturers instructions. The final solution contains 10mM phosphate buffer with 0.14 M NaCl, pH 7.2-7.4.

### **PBS/Tween**

PBS containing 0.05% (v/v) Tween-20.

### **Hepes Buffered Saline (HBS)**

50 mM NaCl, 10 mM Hepes, 3.4 mM EDTA, and 0.05% (v/v) Tween-20. The pH was adjusted to pH 7.4 with 2M NaOH. The solution was filtered through a 0.2µm filter and degassed.

### **2.4.1 Cell culture media**

#### **DMEM**

DMEM (Dulbecco's modification of Eagle's medium) supplemented with 2mM L-glutamine, 25µg/ml gentamicin and 10% (v/v) fetal calf serum.

#### **HAT**

DMEM (as above) supplemented with non-essential amino acids, 1mM sodium pyruvate, 100uM hypoxanthine, 400nM aminopterin, and 16uM thymidine.

#### **HT**

HT prepared as for HAT without aminopterin.

## 2.4.2 SDS PAGE Solutions

### Stock Solutions

- 30% (w/v) acrylamide containing 0.8% (w/v) bis-acrylamide
- 1.5 M Tris-HCl, pH 8.8, containing 0.4% (w/v) SDS
- 0.5 M Tris-HCl, pH 6.8, containing 0.4% (w/v) SDS
- 10% (w/v) ammonium persulphate

**Table 2.4:** Volumes of stock solutions required for resolving and stacking gels for SDS-PAGE.

<i>Solution</i>	<i>Resolving Gel (10% (w/v) Acrylamide)</i>	<i>Stacking Gel (3% (w/v) Acrylamide)</i>
Distilled H <sub>2</sub> O	14.0 ml	5.625 ml
30% (w/v) Acrylamide	10.5 ml	1.875 ml
1.5 M Tris pH 8.8	6.3 ml	-
0.5 M Tris pH 6.8	-	0.9 ml
10% (w/v) SDS	0.18 ml	0.075 ml
TEMED	0.03 ml	0.075 ml
Ammonium Persulphate	0.15 ml	0.04 ml

### Electrophoresis Buffer

25 mM Tris (pH 8.8), 192 mM glycine, and 0.1% (w/v) SDS.

### Sample Loading Buffer

60 mM Tris (pH 6.8), 25% (v/v) glycerol, 2% (w/v) SDS, 14.4 mM 2-mercaptoethanol, and 0.1% (w/v) bromophenol blue.

### Coomassie Blue Stain

Coomassie blue stain was prepared containing 0.2% (w/v) coomassie blue R250 in 30:10:60 (v/v/v) (methanol:acetic acid:water).

## **Destain Solution**

Destain was made up with methanol:acetic acid:water in a 10:7:53 (v/v/v) ratio.

## **2.5 Production of drug-protein conjugates**

### **2.5.1 Conjugation of morphine-3-glucuronide to protein**

Morphine-3-glucuronide (25mgs) was dissolved in 0.5mls 0.15M HCl and the solution made up to 2.5mls in 0.05M phosphate buffer, pH 7.8. Solid NHS was added to the morphine-3-glucuronide to give a final molarity of 0.1M. EDC was dissolved in 1.25mls H<sub>2</sub>O and added to the mixture to give a final molarity of 0.4M. The solution was mixed for 10 minutes with stirring. Protein (OVA, BSA, or BTG) (30mgs) was dissolved in 2.5mls 0.05M phosphate buffer, pH 7.8. This was added to the mixture and stirred for 1-2 hours at room temperature. The mixture was dialysed extensively against PBS at 4°C overnight.

### **2.5.2 Conjugation of cocaine to protein**

A solution of cocaine was prepared in phosphate buffered solution as the phosphate ions could serve as a catalyst for the hydrolysis reaction, (Das Gupta, 1982). A 5mg/ml solution in PBS was prepared and stored at room temperature for one week to allow the conversion of cocaine to BEC. NHS was added to 1 ml of the solution to give a final molarity of 0.1M. EDC was dissolved in 0.25mls H<sub>2</sub>O and when added to the mixture gave a final molarity of 0.4M. The solution was mixed for 10 minutes at room temperature. 10mgs of protein (OVA, BSA or BTG) was dissolved in 0.5mls 0.05M phosphate buffer, pH 7.8. This was added to the mixture and stirred for 1-2 hours at room temperature. The mixture was dialysed extensively against PBS at 4°C overnight.

### **2.5.3 Commercial conjugates**

Morphine-bovine serum albumin (morphine-BSA) conjugate was obtained from Fitzgerald Industries International, MA, USA. The morphine was conjugated to the BSA at the -3 position. The molar ratio of morphine to BSA of the conjugate was 123:1.

Benzoylcegonine-bovine serum albumin (BEC-BSA) was obtained from Fitzgerald Industries International, MA, USA. The molar ratio of BEC to BSA of the conjugate was 60:1.

Tetrahydrocannabinol-bovine thyroglobulin (THC-BTG) was obtained from Fitzgerald Industries International, MA, USA. The THC was conjugated through the delta-8 position.

### **2.6 Immunisations for polyclonal and monoclonal antibody production**

The following protocols are employed for the production of polyclonal and monoclonal antibodies. Three rabbits were immunised with THC-BTG, BEC-BSA and morphine-BSA. Two groups of mice (4 in each group) were immunised with amphetamine-BSA and methamphetamine-BSA. Care of animals was in accordance with DCU guidelines. All appropriate licenses were obtained and adhered to for work involving animals.

#### **2.6.1 Immunisation Protocol for the production of Monoclonal Antibodies**

##### **Animals Used**

6 week old Balb/c female mice

##### **Day 1:**

Mice were immunized by sub-cutaneous injection with an emulsion (250ul) consisting of a 1 mg/ml solution of hapten - protein conjugate mixed 1: 1 with Freund's Complete Adjuvant

##### **Day 21:**

Re-immunized intraperitoneally

Note: Freund's Incomplete Adjuvant is used instead of Freund's Adjuvant.

**Day 28:**

A blood sample was collected (by least invasive method – tail bleed) and the antibody titre determined by ELISA against the respective antigen.

**Day 52:**

The mouse was boosted intraperitoneally using Freund's Incomplete Adjuvant.

**Antiserum Recovery:**

Animal was re-immunised and serum titred, when possible, until an acceptable titre was obtained and a schedule of immunisations of approximately six months or greater is completed.

Five days after the last immunisation, the animal was sacrificed and the blood and the spleen removed.

## ***2.6.2 Immunisation Protocol for the production of Polyclonal Antibodies***

**Animals Used**

Adult New Zealand white female rabbits

**Day 1:**

Rabbit immunised by sub-cutaneous injection with an emulsion (1ml) consisting of a 1 mg/ml solution of hapten - protein conjugate mixed 1: 1 with Freund's Complete Adjuvant.

**Day 14:**

Rabbit re-immunised as before.

Note: Freund's Incomplete Adjuvant was used instead of Freund's Complete Adjuvant.

**Day 28:**

5ml blood sample removed from ear vein and the antibody titre determined against the respective antigen.

**Day 35:**

Rabbits re-immunised as before.

Note: Freund's Incomplete Adjuvant was used instead of Freund's Complete Adjuvant.

**Day 42:**

A blood sample was collected, as before, and antibody titre determined against the respective antigen.

#### **Antiserum recovery:**

The animal was re-immunised and serum titred when possible, until an acceptable titre (>1/500,000) was obtained and a schedule of approximately six months was completed.

At this point the animal was sacrificed and the blood recovered by cardiac puncture.

#### **2.6.3 Preparation of rabbit serum**

For estimation of titre, the blood collected was allowed to clot for 2 hours at room temperature. It was stored overnight at 4°C to allow the clot to tighten and then centrifuged at 4,000 rpm for 20 minutes. The supernatant was removed and stored at -20°C.

#### **2.6.4 Preparation of mouse serum**

The procedure was as above with the exception of the centrifuge step; for mouse serum because of the small volume, it was centrifuged at 13,000 rpm for 20 minutes.

### **2.7 Production of monoclonal antibodies**

All cell cultures were grown in an incubator with 5% CO<sub>2</sub>, at 37°C. Cell counts were performed using a Neubauer Counting Chamber. The viability of cells was determined by mixing the cells at a 5:1 ratio with 0.4% (w/v) Trypan Blue Solution. Dead cells stained blue and viable cells remained white. The cells were visualised with a phase contrast microscope. The count was performed within five minutes of mixing with the Trypan Blue.

#### **2.7.1 Cell lines**

Sp2/0 (ATCC CRL 1581) cell line was cultured in DMEM. The cells were subcultured using a split ratio of 1:2 when the cells had grown to approximately 75% confluency.

### **2.7.2 Immunisation Schedule**

The mice were immunised as per the schedule detailed in Section 2.6.1. Five days before the fusion the mouse was re-immunised by an intraperitoneal injection of 0.25mls of 500µg/ml of drug-protein conjugate mixed 1:1 with Freund's Incomplete Adjuvant.

### **2.7.3 Fusion**

#### **Sp2/0**

Sp2/0 cells were grown for at least two weeks prior to the fusion. The cells were grown in T-75 flasks and on the day before the fusion they were subcultured at a split ratio of 1:2 as they should be in the logarithmic phase of growth for the fusion. On the day of the fusion, the cells were centrifuged at 2000 rpm for 10 minutes and resuspended in 5mls of DMEM lacking FCS. They were counted, as described above, and stored until needed.

#### **Splenocytes**

The immunised mouse was sacrificed and the spleen removed. The splenocytes were harvested by injecting DMEM slowly into the spleen and repeating this procedure until the spleen capsule is relatively clear. The splenocytes are then injected into a universal centrifuge tube and a cell viability count performed separately. The cells were centrifuged at 2000 rpm for 10 minutes and resuspended in 5mls of DMEM lacking FCS.

The splenocytes and Sp2/0 cells were mixed to give a cell ratio of 10 splenocytes per Sp2/0 cell. This cell mixture was pelleted and washed four times with 5mls of DMEM lacking FCS.

All of the supernatant from the final wash was removed except for 50-100µl, and the cells were resuspended by tapping. The suspension was placed in an ice/water bath and 1.5 mls of 50% (v/v) PEG was added to it over a one minute period, while the suspension was swirled. The swirling was continued for a further 90 seconds. The centrifuge tube was removed from the water bath and placed in the palm of the hand. DMEM was pre-warmed to 37°C and 20mls was added to the suspension over a five

minute period, with constant slow swirling. The suspension was placed in a 37°C water bath for 15 mins.

The mixture was centrifuged at 2000 rpm for 10 minutes and the cells resuspended at a cell density of  $1.2 \times 10^6$  cells/ml in HAT supplemented with 5% (v/v) Briclone. The suspension was plated in 96 well cell culture plates at 0.1ml per well. The plates were incubated for 7 days. On day 7, 50µl of HAT supplemented with 5% (v/v) Briclone was added to each well. On day 8, 50µl of medium was removed and fresh medium was added. This medium was then screened for antigen reactivity. Wells were fed as required.

#### ***2.7.4 Screening of hybridoma supernatants***

Supernatants from the wells were screened for antigen reactivity using a conventional ELISA format as described for the polyclonal antibody assays (Section 2.9). Positive wells were scaled up to 48 well plates with 0.5mls medium. Wells subsequently found to be positive were scaled up in 24, 12, and 6 well plates and finally in T-25 and T-75 flasks.

#### ***2.7.5 Storage of cell lines***

Cells were flushed off the surface of the tissue culture flasks using a pasteur pipette. The media was centrifuged at 2000 rpm for 10 minutes. The resulting pellet was resuspended in 2 mls of FCS and 2 mls of 10% (v/v) of DMSO in FCS. The mixture of 4mls was divided into four 1 ml cryovials. The freezing process was performed gradually over a 2.5 hours time period using a freezing tray, in the vapour phase of liquid nitrogen. The vials were then immersed in the liquid nitrogen and stored long-term.

#### ***2.7.6 Mycoplasma screening***

Mycoplasma testing was performed through the services of the National Cell and Tissue Culture Centre, Dublin City University. Cells were provided to them that had been grown in antibiotic free medium for three sub-culturing processes.



## ***2.8 Purification and Characterisation of antibodies***

### ***2.8.1 Purification of polyclonal rabbit serum***

The rabbits were immunised as per the schedule detailed in Section 2.6. Fourteen days pre-sacrifice, the rabbits were reimmunised by an intraperitoneal injection of 1.0ml of 500µg/ml of drug-protein conjugate mixed 1:1 with Freund's Incomplete Adjuvant. For the production of anti-THC antibodies, THC-BTG was used as the immunogen and THC-BSA was used as the screening conjugate. BEC-BSA was the immunogen and BEC-BgG was used as the screening conjugate for the production of anti-cocaine antibodies. Morphine-BSA was used as the immunogen and morphine-3-glucuronide-OVA was the screening conjugate for the production of the anti-morphine antibodies.

#### ***2.8.1.1 Ammonium sulphate precipitation***

A cold saturated ammonium sulphate solution was prepared and a volume added to an equal volume of serum by dropwise addition, with constant stirring on ice. The mixture was stirred on ice for one hour and then centrifuged at 3000 rpm for 20 minutes. The supernatant was discarded and the pellet washed twice in 10mls of 45% (v/v) ammonium sulphate. The final pellet was dissolved in 5ml of PBS and dialysed overnight at 4°C against 5 litres PBS (pH 7.3, 0.15M NaCl).

### ***2.8.2 Monoclonal Antibody Purification***

#### ***2.8.2.1 Concentration of tissue culture supernatant***

The hybridoma cell line was grown for one to two weeks. The cell culture supernatant was collected, and sodium azide was added at a concentration of 0.025% (w/v), final concentration. The supernatant was stored at 4°C. 50ml of supernatant was concentrated to 5 ml on a stirred ultracentrifugation apparatus with a 76mm diaflo ultrafilter membrane, with a molecular weight cut-off of 100 Kdaltons. The concentrate was stored at 4°C.

### **2.8.3 Protein G Affinity Column**

A protein G column was prepared by adding 1ml of protein G immobilised on Sepharose 4B Fast Slow into a 5 ml syringe. The column was equilibrated with 20ml PBS. The dialysate from the ammonium sulphate precipitation, in the case of polyclonal antibody purification, and concentrated supernatant, in the case of monoclonal antibody purification, was added to the column. 1ml of PBS was added to the eluate and it was reapplied to the column. The eluate was collected and 4ml PBS added and the mixture reapplied again. The collected eluate was applied to the column again. The column was washed with 25ml PBS. The bound immunoglobulin was then eluted by addition of 0.1M glycine/HCl, pH 2.5, to the column. The glycine was equilibrated in the column for about 15 minutes. 100ul of Tris/HCl, pH 8.7 was added to eppendorf collection tubes and the absorbance of the eluate was monitored in each at 280nm. The fractions containing protein were pooled and dialysed overnight at 4°C against 5 litres of PBS with two changes of buffer.

### **2.8.4 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis**

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to assess the purity of the purified antibody fractions from the Protein G purification step. The solutions used are outlined in Section 2.4.2. Samples were prepared in sample buffer (4:1, sample :buffer ratio), and boiled for 5 minutes before being loaded onto gels. The gels were run at 50 mA, using an ATTO vertical minigel electrophoresis system, until the blue dye of the sample buffer had reached the bottom of the gel. The gels were subsequently stained for 30 minutes using Coomassie Blue staining solution. The gels were destained by overnight incubation in destaining solution.

### **2.8.5 Determination of Protein Concentration - BCA**

A Bicinchoninic Acid assay kit (BCA Kit, Pierce & Warriner, UK) was used for the determination of protein content. Standard protein solutions were prepared in PBS using either BSA or immunoglobulin. 10 µl of the solution was mixed with 190 µl of

BCA kit working solution in a 96 well plate. The plate was incubated at 37 °C for 30 minutes. The absorbance was read at 562 nm on the Titretek plate reader. A standard curve of the protein standards was plotted and the concentration of the unknown solutions was determined.

## **2.9 Immunoassays**

### **2.9.1 Estimation of rabbit antibody titre**

Levels of antibody in serum from immunised rabbits were measured using ELISA. One series of wells were coated with 100 µl of the drug-protein conjugate (in the case of the serum from the rabbit immunised with THC, 1µg/ml of THC-BTG or THC-BSA was used; for the serum from the rabbit immunised with BEC, 10 µg/ml BEC-BSA or BEC-BgG was used; for the serum from the rabbit immunised with morphine-BSA, 10 µg/ml morphine-3-glucuronide-OVA was used) and a second series of wells were coated with the protein alone (BTG, BSA or OVA) at a concentration of 1µg/ml and incubated for 90 minutes at 37°C. The plates were washed five times in PBS/Tween and were blocked by adding 200 µl of PBS/Tween containing 2% (w/v) milk powder and incubated again for 90 minutes at 37°C. The serum was diluted in PBS/Tween and PBS/Tween containing 0.1% (w/v) protein (BTG in the case of the THC-BTG immunised rabbit, BSA in the case of the BEC-BSA and morphine-BSA immunised rabbits), from 1/200 to 1/51,200. The plates were incubated for 90 minutes at 37°C. The plates were washed five times with PBS/Tween. 100 µl of commercial goat anti-rabbit IgG antibody, labeled with horseradish peroxidase at a dilution of 1/5,000 in PBS/Tween, was then added to the wells. The plate was incubated for 90 minutes at 37°C. The plates were washed five times with PBS/Tween. The substrate for horseradish peroxidase, OPD, was then added. The plates were incubated for 30 minutes in the dark at room temperature. The absorbance was read at 450 nm using a microtitre plate reader.

### **2.9.2 Estimation of mouse antibody titre**

Levels of antibody in serum from immunised mice were measured using the above procedure with the following exception: the secondary antibody used was HRP-labelled rabbit anti-mouse IgG used at a dilution of 1/2000.

### **2.9.3 Non-Competitive Enzyme-linked Immunosorbent Assay for determination of antibody titre and optimal concentration of protein conjugates**

Microtitre plates were coated with a range of drug-protein concentrations by adding 100  $\mu$ l of drug-protein conjugate dissolved in PBS (pH 7.3, 0.15M NaCl) to each well. The plates were incubated for 60 minutes at 37°C. The plates were emptied and washed five times with PBS/Tween (0.05% (v/v) Tween 20). The wells were blocked by addition of 100  $\mu$ l PBS containing 2% (w/v) milk powder and incubated for 60 minutes at 37°C. The plates were emptied and washed five times with PBS/Tween as before. Serial dilutions of antibody in PBS (100  $\mu$ l) were added into the wells of each conjugate concentration on the plate. The plates were incubated for 60 minutes at 37°C. The plates were washed five times with PBS/Tween as before. Enzyme-conjugated IgG (anti-rabbit in the case of polyclonals, and anti-mouse in the case of monoclonal antibodies) were diluted in PBS/Tween containing 2% (w/v) milk powder (100  $\mu$ l) was added to the wells and incubated for 60 minutes at 37°C. The plates were washed five times with PBS/Tween and 100  $\mu$ l of substrate (OPD in case of HRP Conjugate, and p-NPP in case of AP conjugate) was added to each well. The plate was covered with foil and left at room temperature for 30 minutes for the colour to develop. The absorbance was measured on a microtitre plate reader at 450nm.

### **2.9.4 Competitive Enzyme-linked Immunosorbent Assay**

Microtitre plates were coated by adding 100  $\mu$ l of drug-protein conjugate dissolved in PBS (pH 7.3, 0.15M NaCl) to each well. The plates were incubated for 60 minutes at 37°C. The plates were emptied and washed five times with PBS/Tween (0.05% (v/v) Tween 20). The wells were blocked by addition of 100  $\mu$ l PBS containing 2% (w/v) milk powder and incubated for 60 minutes at 37°C. The plates were emptied and washed five times with PBS/Tween as before. Drug standard, 50  $\mu$ l, containing from

0.38 ng/ml to 100,000 ng/ml and mouse anti-drug antibody, 50  $\mu$ l, (diluted in PBS/Tween containing 2% (w/v) milk powder) were added into each well. The plates were incubated for 60 minutes at 37°C. The plates were washed five times with PBS/Tween as before. Horseradish-peroxidase conjugated IgG was diluted 1/2000 in PBS/Tween containing 2% (w/v) milk powder and 100  $\mu$ l was added to the wells and incubated for 60 minutes at 37°C. The plates were washed five times with PBS/Tween and 100  $\mu$ l of substrate (0.4 mg/ml o-phenylenediamine (OPD), in phosphate citrate buffer, pH 5, and 0.4 mg/ml of urea hydrogen peroxide) was added to each well. The plate was covered with foil and left at room temperature for 30 minutes for the colour to develop. The absorbance was measured at 450 nm on a microtitre plate reader. A schematic representation of the ELISA procedure used is shown in Figure 2.1.

### ***2.9.5 Isotyping of monoclonal antibodies***

ELISA plates were coated and blocked with the appropriate drug-protein conjugate and milk protein, respectively, for 60 minutes, as described in Section 2.9.3. The monoclonal antibody was added and incubated for 60 minutes. After washing, alkaline phosphatase-labelled goat anti-mouse immunoglobulins were added to the wells and the ELISA developed using para-nitrophenyl phosphate (p-Npp) provided in table form and dissolved in the required volume of deionised H<sub>2</sub>O, as described by the supplier's instructions. The absorbance of the reactive wells indicates the monoclonal antibody isotype.

### ***2.9.6 Affinity analysis ELISA – Friguet method***

Twelve hours before the Friguet assay was performed, a series of antibody-antigen mixtures were incubated in eppendorf tubes, to reach equilibrium. The solutions contained a constant, nominal, dilution of antibody, referred to as '1', and varying concentrations of antigen. In another set of eppendorfs, serial dilutions of the nominal concentration of antibody were prepared. These were used to construct the standard curve of nominal antibody concentration versus absorbance at 450nm. Twelve hours later, the ELISA was performed on these solutions, as per Section 2.9.3. Absorbance readings at 450nm of the antigen:antibody mixtures were related to the nominal concentration values by reference to the standard curve of nominal concentration versus

absorbance at 450nm. The fraction of total antibody bound by the antigen ( $v$ ) was calculated for each antigen:antibody mixture. The dissociation constant for the antigen:antibody interaction was defined by the slope of the plot of  $1/v$  versus  $1/[A]$ , (see Section 5.1.5).

### ***2.9.7 Determination of immunoglobulin concentrations by affinity capture ELISA***

Commercial goat anti-mouse immunoglobulin at a concentration of 10  $\mu\text{g/ml}$  was used to coat the wells of a microtitre plate, and it was subsequently blocked with PBS containing 2% (w/v) milk powder as described in Section 2.9.3. Dilutions of mouse IgG of known concentration were prepared in PBS. Dilutions of the purified antibody were also prepared in PBS. 100  $\mu\text{l}$  of the solutions (standards and unknowns) were added to the wells and the ELISA developed as described in Section 2.9.3. A standard curve of absorbance at 450nm versus mouse IgG concentration was used for the determination of the mouse IgG concentration in the purified antibody solutions.

## ***2.10 BIAcore Studies***

The CM5 sensor chip was used for all experiments, with the exception of the use of the F1 chip which is described in Chapter 6, for the optimisation of the BIAcore assay for the detection of morphine in saliva samples.

### ***2.10.1 Preconcentration studies***

An initial preconcentration step was carried out to determine the optimum pH for the immobilisation of the drug-protein conjugate. Proteins at pH values below their isoelectric point, pI, have a positive charge and will be electrostatically attracted to the negatively charged carboxy groups on the dextran matrix. The pI value of a protein is often changed by conjugation to a drug so the optimum pH is determined by the preconcentration study. Drug-protein was dissolved at a concentration of 50 µg/ml in 10 mM sodium acetate buffer, at a range of pH values between 3.8-5.0. These were passed sequentially over an underivatized chip surface and the pH giving the highest mass change in terms of response units (RU) was used for subsequent drug-protein immobilisation procedures.

### ***2.10.2 Immobilisation of drug-protein conjugates***

The carboxymethylated dextran was activated, by injecting 35 µl of a solution containing 0.05 M NHS and 0.2 M EDC over the chip surface at a flow rate of 5 µl/min. 35 µl of a solution of drug-protein in 10 mM acetate buffer, at the appropriate pH, was passed over the surface at a flow rate of 5 µl/min. Unreacted NHS groups were 'capped' (Section 4.2.2.), by passing 35 µl of a 1 M ethanolamine (pH 8.5) solution over the surface at a flow rate of 5 µl/min.

### ***2.10.3 Regeneration Studies***

The stability of the immobilised drug-protein conjugates surface, was assessed by passing a known concentration of antibody over the chip surface and the surface was

regenerated with mild acid/base solution as detailed in the results sections. The cycle of binding and regeneration was performed for approximately 50-100 cycles, and the binding signal measured to assess the stability of the immobilised surface for assay development.

#### ***2.10.4. Non-Specific binding Studies***

Purified monoclonal and polyclonal antibody solutions at the appropriate dilution were passed over the dextran matrix and the appropriate immobilised protein surface. Non-specific binding to either dextran or immobilised protein surface was eliminated by the addition of either dextran or protein, or in some cases both, to the antibody solution.

#### ***2.10.5. Competitive Assays***

Drug solutions was prepared at a series of concentrations ranging from 0.03 – 25,000 ng/ml by serial dilution, using HEPES Buffered Saline (HBS) as diluent. Antibody at a constant dilution was added to the various antigen concentrations. The antibody:antigen mixture was allowed to equilibrate for 15 minutes. The equilibrium mixtures were passed in random order over the chip surface at 5  $\mu$ l/min for 4 minutes, and the chip surface regenerated between cycles by pulses of the appropriate regeneration solution. The amount of bound antibody was measured in terms of response units (RU). The response units were divided by the response measured for the antibody:antigen mixture containing zero antigen to give normalised binding responses. A plot of antigen concentration (ng/ml) versus normalised binding responses could then be used to construct the calibration plot using BIAevaluation 3.1 software.

#### ***2.10.6. Solution Affinity Analysis using BIAcore***

Drug-protein conjugates were immobilised using the conventional EDC/NHS coupling chemistry. Serial dilutions of the monoclonal antibodies of known concentration (molarity) were passed over the immobilised surface, and a calibration curve was constructed of mass bound measured in terms of response units, versus antibody concentration. A known concentration of antibody was then incubated with varying



concentrations of free drug (molarity) and allowed to reach equilibrium overnight. The equilibrium samples were passed over the immobilised surface and the binding response calculated. The response values measured were used to calculate the amount of free antibody in the equilibrium mixtures, from the calibration curve. A graph was then plotted of drug concentration versus free antibody concentration. The solution phase interaction models in BIAevaluation 3.1 software, was used to determine the overall affinity constant, (see Section 5.1.6).

## ***2.11 Collection of saliva samples***

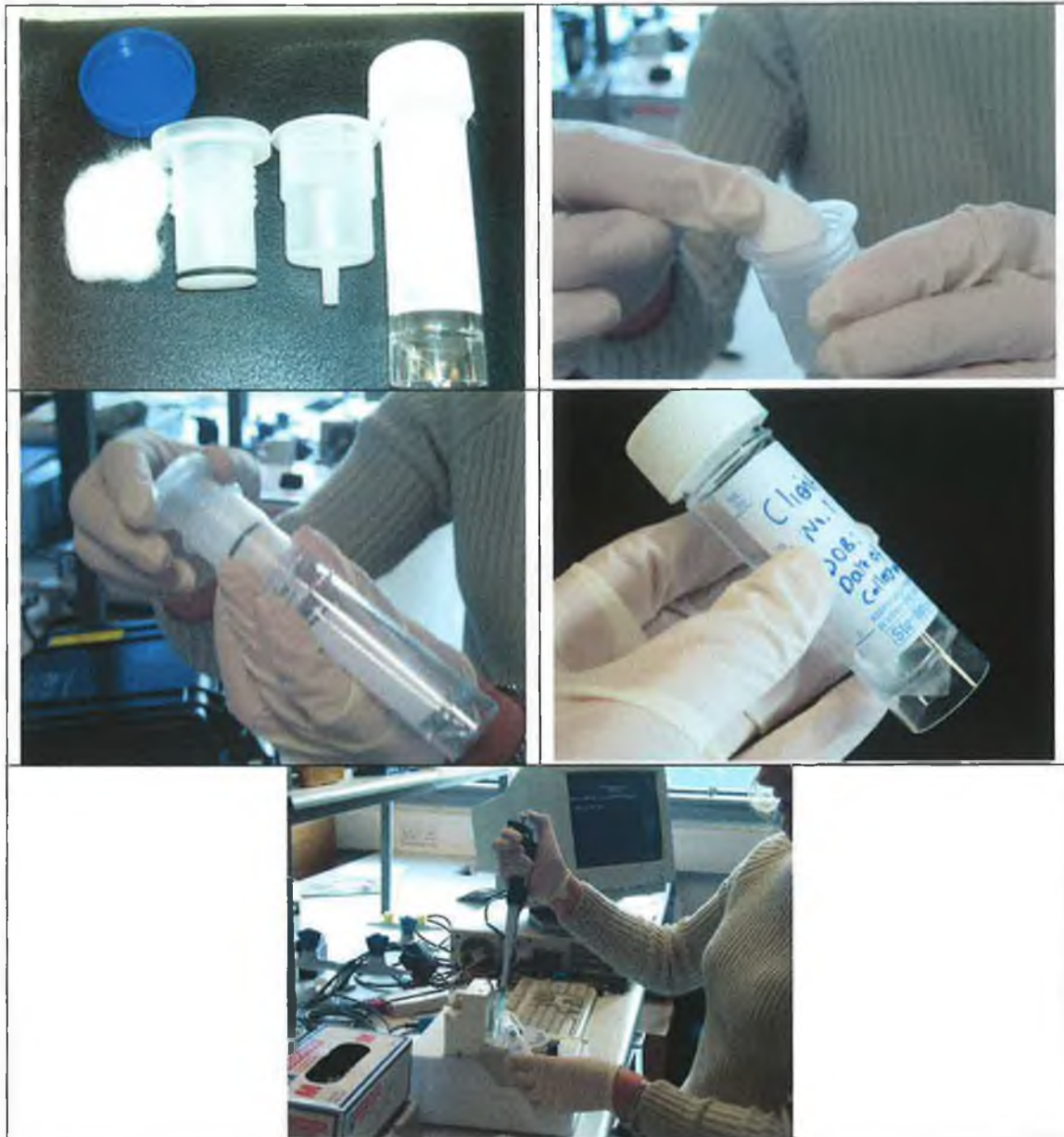
Saliva samples were collected using the saliva collection device from Trinity Biotech. The absorbant pad was removed and placed in the mouth for a couple of minutes. It was removed and placed into the plastic 'filter-like' component. A second part was screwed into this container and the saliva collected in an universal tube, through the pressure of the second part squeezing the saliva from the pad, see Figure 2.1.

## ***2.12 Development of Envitec Device Assay for detection of THC***

### ***2.12.1 Background to Envitec Device***

DCU collaborated with Envitec-Wismar GmbH on the European Commission Standards, Measurement and Testing Project, entitled, 'On-site measurement of drugs of abuse in a saliva sample'. The aim of the project was to develop a new solid-phase format for the rapid detection of drugs of abuse in a saliva samples. Envitec developed an automatic device that could be used for this purpose. To achieve a safe and easy to use assay, the critical steps of the laboratory procedures for completing an immunoassay have to be simplified and the incubation steps shortened in time.

DCU obtained a prototype of the Envitec device and worked on the development of an assay for detection of THC in saliva samples, using the anti-THC polyclonal antibody that was produced and characterised as described in Chapter 3.



**Figure 2.1:** Saliva sample were collected using a saliva collection device (Trinity Biotech, Dublin). The absorbant pad was removed and placed in the mouth for a couple of minutes. It was removed and placed into the plastic filter like component. A second part was screwed into this container and the saliva collected in an universal tube, through the pressure of the second part squeezing the saliva from the pad. The sample collected was diluted 1:1 with PBS and the sample applied to the Envice device for the detection of THC.



**Figure 2.2:** Envitec prototype device for rapid analysis of drugs of abuse in saliva samples.

The well positions are shown in Figure 2.3, and the schedule of the final assay is described below.

### **2.12.2 Envitec Assay**

#### **Well Preparation**

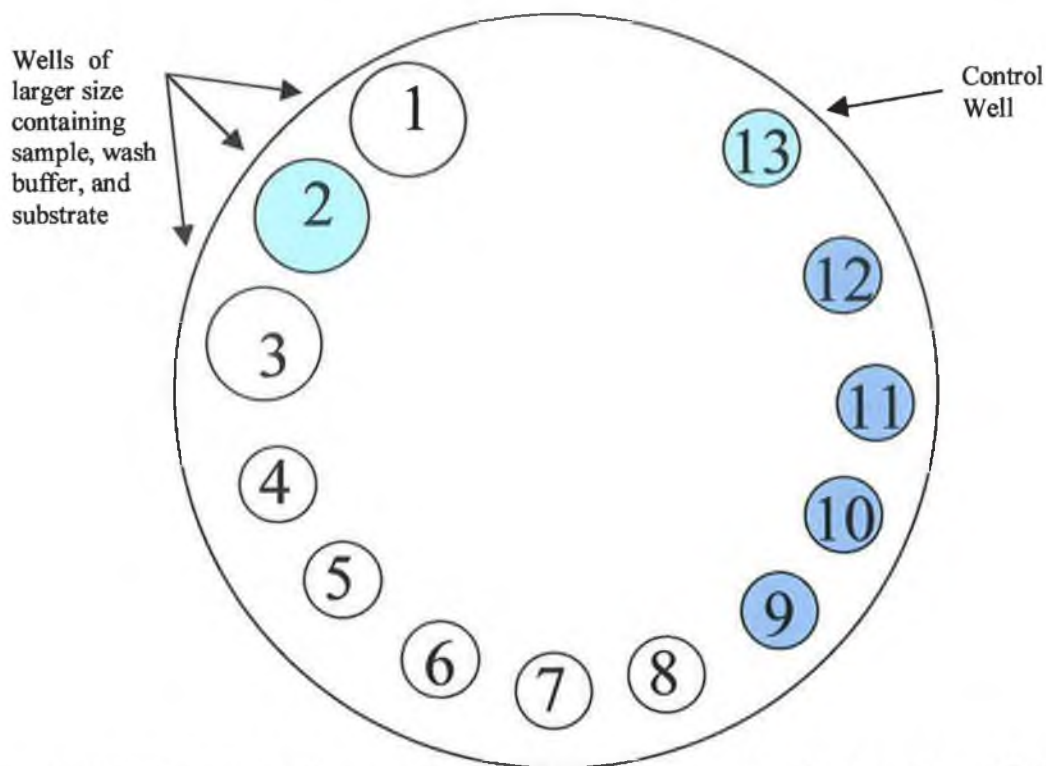
1. Nunc prototype wells were coated with 300 $\mu$ l/ml of 1/500 dilution of 1 mg/ml Sigma goat anti-rabbit immunoglobulin in PBS, pH 7.4, overnight at 4°C.
2. Wells were washed four times with PBS.
3. Wells were coated with 250 $\mu$ l/ml of 1/100 dilution of anti-THC polyclonal antibody for 4 hours at room temperature on orbital shaker.
4. Wells were washed four times with PBS.
5. Wells were blocked with 300 $\mu$ ls of 2% (v/v) milk protein in PBS, 30 minutes at 37 °C.
6. Wells were washed four times with PBS containing 0.05% (v/v) Tween.

#### **Assay**

- Saliva sample was diluted 1:1 with PBS and added to well 1 of the device.
- A 1/500 dilution of THC-HRP in PBS was prepared and added to well 2 of the device

### *Automated Assay Schedule*

- 100µl of the saliva sample was transferred to wells containing 100µl THC-HRP.
- The mixture was transferred to the reaction wells.
- The mixture was incubated for 4 minutes.
- The mixture was sent to waste compartment and reaction wells were washed three times with Tris Buffer.
- TMB was transferred to reaction wells and the first optical measurement recorded.
- The wells were incubated with TMB for five minutes.
- A second optical measurement recorded.
- The results were displayed.



**Figure 2.3:** Schematic diagram of the carousel of the Envitec device and the different wells.

**Well 1:** Saliva sample mixed 1:1 with PBS (minimum 500 $\mu$ l needed)

**Well 2:** TMB Substrate (1ml)

**Well 3:** Waste well

**Wells 4-8:** 100 $\mu$ ls THC-HRP (Saliva sample is transferred to these wells for mixing with THC-HRP)

**Wells 9-13:** Reaction wells (coated with anti-THC antibody, after incubation step with sample and THC-HRP mixture, the TMB is transferred here and the transmission read

## ***Chapter 3***

### ***Production and Characterisation of Polyclonal Antibodies to Tetrahydrocannabinol, Cocaine and Morphine***

## **3.1 Introduction**

### **3.1.1 The Immune System**

The immune system is composed of two levels, the innate and the acquired systems. The innate system acts as the body's first line of defence against pathogens. Basic mechanisms of the innate response include physical barriers such as skin and mucous membranes and internal mechanisms include phagocytosis, and inflammation. Phagocytosis involves the internalisation and destruction of foreign matter by cells of the mononuclear phagocyte system. Natural killer cells are lymphocytes that can recognise the Class I Major Histocompatibility Complex (MHC) molecules on a cell surface. Cells with reduced MHC molecule expression such as cells that are virally infected, or cancerous cells, are susceptible to attack by the natural killer cells. In addition to killing cells, NK cells can also secrete cytokines such as anti-viral cytokine IFN- $\gamma$  and the inflammatory cytokine TNF- $\alpha$ . The important differentiation between the innate system and the acquired system is the non-specific nature of the innate response. The acquired immune system is further divided into humoral immunity and cell-mediated immunity. The defining characteristics of the acquired immune system are:

- Specificity
- Inducibility
- Diversity
- Memory
- Distinguish self from non-self
- Downregulation (Elgert, 1996)

The principle components of the humoral immune system are the B lymphocytes and their products, the antigen-specific antibodies. Cell mediated immunity protects against intracellular pathogens and release immune system messengers such as cytokines ( $T_H$  cells) and kill target cells, ( $T_C$  cells).



### **3.1.2 *The Lymphoid System***

Lymphoid organs are composed of lymphocytes at different stages of development. They are classified as primary or secondary lymphoid organs. Primary lymphoid organs are the sites where immune cells, lymphocytes, can mature into functional effector cells. Generally, T cells are responsible for cell mediated immunity, and B cells are responsible for the humoral response, although it is critical that there is interaction between T cells and B cells for antibody production. In humans, the primary lymphoid organs are the bone marrow and thymus. B cells are produced and mature in the bone marrow. The precursors of T cells, produced also in the bone marrow transfer to and mature in the thymus. The secondary lymphoid organs include the spleen, lymph nodes, and mucosal-associated lymphoid tissue, (MALT), and it is at these sites that the lymphocytes can interact with antigens and undergo differentiation. The lymph nodes primarily respond to antigens in the tissue that they serve. The spleen acts as a filter for the circulatory system. The MALT system organises antibodies at major entry points of antigen entry (Roitt, 1994; Kimball, 2002).

### **3.1.3 *Antibody production and the Humoral immune system***

The specificity, diversity and memory are the key characteristics of the acquired immune response. As described below haptens less than 5Kda in size are usually unable to illicit an immune response. Adjuvants are oil/water emulsions with microbial components, e.g., heat killed *Mycobacterium tuberculosis*, in Freund's complete Adjuvant. They are used to increase the immunogenicity of the substance, by localising the injection in the emulsion, and the microbial components cause an increase in the initial response involving the macrophages. The primary response of the body to a foreign agent primes the immune system for subsequent immunisations.

B cell receptors bind antigens and engulf them by endocytosis. The antigen is digested into fragments and they are displayed at the cell surface in conjunction with a class II MHC molecule. Helper T cells ( $T_H$  cells) specific for this structure bind the B cell and secrete lymphokines that stimulate the B cell to develop into a clone of cells with identical antibodies and differentiate into plasma cells that secrete these antibodies.

There are two kinds of T<sub>H</sub> cells: T<sub>H</sub>1 cells that participate in cell mediated immunity and T<sub>H</sub> 2 cells that are essential for antibody-mediated immunity.

When the precursors to T<sub>H</sub> cells are presented with an antigen, by an antigen presenting cell, they proliferate and become activated. Depending on the origin of the APC, the T<sub>H</sub> cell will develop into T<sub>H</sub> 1 or T<sub>H</sub> 2 cells.

T<sub>H</sub> 1 cells are produced when the APC presents antigen to the Tcell receptor for antigen in combination with the activation by IL-12. The T<sub>H</sub> 1 cells then secrete tumor-necrosis factor-beta (TNF-β ) and interferon-gamma (IFN-γ )

These stimulate phagocytosis by macrophages and recruit other lymphocytes to the site producing inflammation.

T<sub>H</sub> 2 cells are produced when another type of APC present antigen to the T cell's receptor for antigen.

The major lymphokines secreted by T<sub>H</sub> 2 cells are IL-4, IL-5, IL-10 and IL-13.

**Interleukin 4 (IL-4):** Stimulates class-switching in B cells and promotes synthesis of IgE antibodies. It also acts as a positive-feedback device promoting more T<sub>H</sub> cells to enter the T<sub>H</sub> 2 pathway. It also inhibits expression of the IL-12 receptor thus inhibiting cells from entering the Th1 path.

**Interleukin 5 (IL-5):** Attracts and activates eosinophils

**Interleukin 10 (IL-10):** Inhibits IL-12 production by APCs. This inhibits cells from entering the T<sub>H</sub> 1 pathway.

**Interleukin 13 (IL-13):** Promotes the synthesis of IgE antibodies.

The foreign material is engulfed by macrophages and displayed on antigen presenting cells in conjunction with the Class II MHC receptor. These are presented and bind to T<sub>H</sub> cells and initiate a series of immune responses leading to T cell proliferation and release of interleukin-1 (IL-1). This results in subsequent release of IL-2. The activated T<sub>C</sub> cells respond directly in the cell-mediated immune response by acting as cytotoxic cells. The antigen also binds specifically to the B lymphocytes, and after the activation and presentation of the antigen to T<sub>H</sub> cells, the B lymphocytes convert to plasma cells through the critical interaction of T<sub>H</sub> cells and IL-4 and IL-5. The plasma

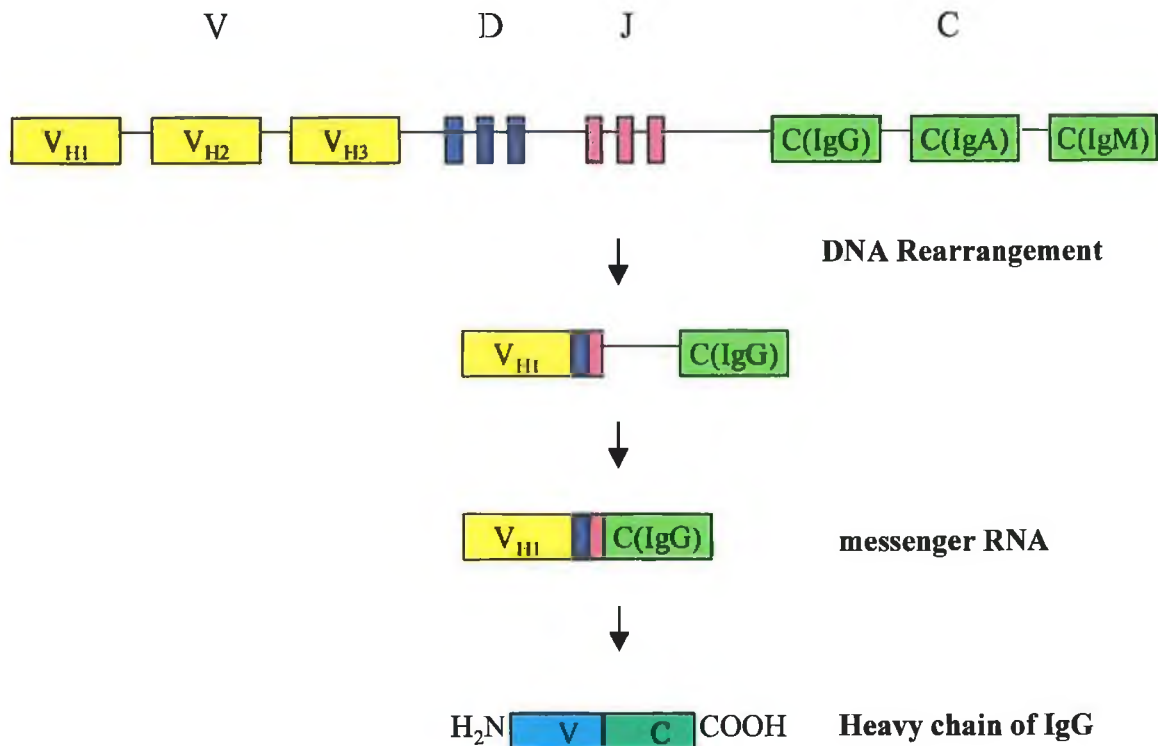
cells secrete the specific antibodies. Some of the cells remain as memory B cells that are ready in case of future exposure to the specific antigen (Kimball, 2002).

#### ***3.1.4 Antibody Diversity***

The range of antigens that are presented to lymphocytes is huge and so the immune system must be capable of responding through its ability to reorganise the DNA material responsible for the production of immunoglobulins. The human genome has the DNA information to encode for all the immunoglobulins, however they are not organised into genes, but rather the genes are assembled from different sections of DNA.

For the antibody chains the gene segments are composed of variable (V) segments. Each of these encodes most of the N-terminal of the antibody, including the first two (but not the third) hypervariable region. The diversity (D) gene segments encode part of the third hypervariable region. The joining (J) gene segments encodes the remainder of the V region including the remainder of the hypervariable region. The constant (C) regions encode the remaining constant region of the antibody.

Four mechanisms contribute to this antibody diversity. The obvious processes to contribute to this diversity, are the many different V, D, and J germline gene sequences, and secondly the combinatorial recombination of these gene segments and chain association (Figure 3.1). The different combinations of V and J segments combining for constant light chains and V, D, and J segments joining for heavy chains, and then subsequent random association of the different light and heavy chains leads to a large diverse range of immunoglobulins. Another process contributing to the diversity is junctional diversity. This happens when there is imprecise DNA rearrangement involved in the joining of V with J, D with J, or V with D. Another contributor to junctional diversity is the insertion of random nucleotide regions between V, or J and D DNA segments in heavy chain genes. Finally the other contributory factor of overall antibody diversity is somatic mutation. The mutants created as the B cells divide allow for the selection by antigen of antibodies that provide better binding (Elgert, 1996).



**Figure 3.1:** Recombinational arrangement of the DNA encoding variable, (V), diversity, (D), junction, (J) and constant (C) regions of an immunoglobulin heavy chain and the subsequent transcription to messenger RNA and translation into the heavy chain.

### 3.1.5 Antibody Structure

The structural characterisation of antibodies began in the 1930s with work performed by Tiselius & Kabat. They did electrophoretic studies on non-immunised and post-immunisation rabbit serum, and found that there was an increase in the gamma-globulin fraction following immunisation. This led to one characterisation of them as gamma-globulin. The chemical structure was further investigated by Porter, Edelman, and Nisonoff in the 1950s and 60s, (Elgert, 1996). Porter digested the immunoglobulin with the proteolytic enzyme, papain, to cleave the peptide bonds, producing three fragments, two antigen binding Fab fragments and a non-antigen-binding Fc fragment. Edelman disrupted the disulphide bonds with dithiothrietol, iodoacetamide, and a denaturing agent, producing the two heavy chains and two light chains. Nisonoff used pepsin to hydrolyse the antibody at different sites to the papain and this hydrolysis resulted in one

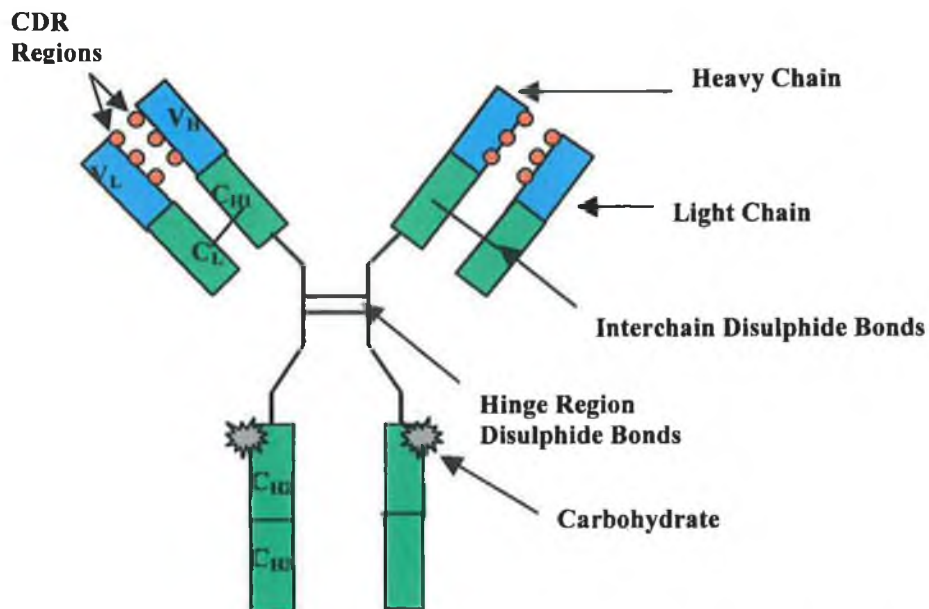
large fragment called F(ab)', that could bind antibody, and other smaller fragments. It could be further reduced to yield two Fab-like fragments called Fab'.

The basic structure of an antibody is shown in Figure 3.2. The heavy and light chains are made up of repeated domains, each about 110 amino acids in length. The heavy chains have one variable region and three constant domains. The light chain has one variable domain and one constant domain. The variability of the antigen binding site is located in the complementarity-determining regions (CDRs), sub-divided into CDR1, CDR2, and CDR3. The variable regions of the chains are responsible for the antigen recognition and the constant regions are central to the biological effector functions. Binding to antigen is also facilitated by the flexible movement of the two Fab portions, which can change angle of between 60 to 180 degrees.

Immunoglobulins are divided into five groups based on their isotype. Isotypic determinants distinguish C-region sites on a heavy chain. The five groups are IgG, IgA, IgM, IgD and IgE. Subdivisions of these classes exist also. Antigenic determinants on light chains distinguish them as either  $\kappa$  or  $\lambda$ . Different isotypes have different functions. IgG is the major immunoglobulin in the blood and is primarily induced by antigens. IgA is dimeric and is usually found in body fluids such as saliva and tears, and acts to guard these areas of the body. IgM is a pentamer and is the activator of complement. IgD is found on the surface on B cells where it is thought to be involved in regulation of B cell activity. IgE is found in hypersensitivity allergic reactions (Roitt, 1994; Kimball, 2002).

**Table 3.1:** Classification and characteristics of immunoglobulins.

Class	H chain	L chain	Characteristic
IgG	gamma	kappa or lambda	Most common antibody seen, transferred across placenta
IgM	mu	kappa or lambda	Pentamer antibody, appears in primary response after immunization
IgA	alpha	kappa or lambda	Dimer antibody, found in secretions such as saliva, tears
IgD	delta	kappa or lambda	Uncertain function
IgE	epsilon	kappa or lambda	Involved in allergic reactions by binding to mast cells and sensitizing them



**Figure 3.2:** Structure of the immunoglobulin molecule. The antibody is composed of two light chains and two heavy chains. The variable regions are located at the amino acid terminal end of the molecule. The light chain is composed of one variable region and one constant region. The heavy chains are composed of one variable region and three constant regions. The hinge region allows flexibility in the molecule for antigen binding. The antigen binding sites are specific and are represented by the complementarity-determining regions (CDR) regions. The heavy and light chains are connected via disulphide bonds, and there are disulphide bridges at the hinge region also between the two heavy chains. Disulphide bonds are also present in the constant and variable regions.

### **3.1.6 Drug protein conjugation**

Haptens are small chemical compounds, less than 5 KDa in size. They must be conjugated to a large carrier protein to be rendered immunogenic. To elicit an immune response the drugs examined in this study need to be conjugated to protein. The use of a drug-protein conjugate as an immunogen results in antisera containing a mixture of antibodies specific for the drug, protein and linking region between the drug and protein.

In the design of the conjugate, several factors need to be considered. The reactive groups and the positions on the drug provide a starting point for the design. The drugs under study were coupled through a carboxyl group to the amine groups on the proteins. This proceeded through EDC/NHS coupling chemistry. For the application of polyclonal antibodies in an ELISA format it is necessary to use conjugates differing from the immunogens with regard to the protein used and if possible the linkage between the drug and hapten. Bovine thyroglobulin, bovine serum albumin and keyhole limpet haemocyanin are proteins that could be used for the production of hapten-protein conjugates. For the purposes of screening a more soluble protein such as bovine serum albumin is suitable for the ELISA format, as it is water soluble. Dextran has a low immunogenicity, (P. Dillion, Personal Communication) and when conjugated to a hapten can be used as a screening conjugate for the identification of hybridomas specific for the drug of interest. The likelihood of the antibodies produced recognising the dextran part of the conjugate is small therefore eliminating the occurrence of positives that do not recognise the free drug. This is true of many 'polymer-type' substances, in that they do not make good immunogens (Hermanson, 1996). Ethylenediamine can be used as a linker between the drug and protein.

The procedures for the conjugation of a drug to a protein are well documented, (Hermanson, 1996). The usual method involves linking the drug to a carrier protein via a peptide bond. To perform this conjugation the drug must have suitable carboxyl or primary amino groups. If they are not present, the drug must first be derivatised to synthetically produce a derivative that contains those groups. The choice of derivatisation site on the hapten is of utmost importance in the design of an immunogen. Care must be taken not to derivatise those groups that distinguish a molecule from its relatives. The hapten should be linked to the carrier protein according to Landsteiner's

principle which states that 'antibody specificity is directed primarily at the portion of the hapten furthest removed from the functional group that is used to link the hapten to the carrier protein' (Erlanger, 1980). Exposed sites act as antigenic determinants and are available to circulating lymphocytes, so antibodies to these are produced in numbers. The ideal epitope density per molecule is in the range 8-25 haptens per protein. This ratio seems to affect only the time taken for a suitable immune response to be generated. As little as two haptens per protein can generate a response but it will be delayed, (Erlanger, 1980).

By preparing a conjugate that has a structure common to the parent drug and its metabolites, antibodies with a general specificity for a drug and its metabolites will be produced. Fasciglone *et al.* (1996), reported that the immunogenicity of a conjugate is related to the hydrophobicity of the carrier. They concluded that hydrophobic haptens hide inside carrier proteins by interactions with the hydrophobic segments, resulting in no immunogenic response. It would, therefore, follow that for the generation of an immunogenic response against a hydrophobic hapten, it would be advisable to use a hydrophilic carrier protein.

Ethylenediamine can be as a means of introducing a linker into the drug-protein conjugate. The ethylenediamine initially cationises the ovalbumin. The carboxylate groups of the protein are modified by the ethylenediamine by the formation of amide bonds with an alkyl spacer containing a terminal primary amine group. This blocking of the carboxyl groups on the protein and the addition of terminal primary amines raises the pI value. The highly positive charge of the cationised protein has been shown to significantly increase its immunogenicity. (Hermanson, 1996) When haptens are coupled through the cationised protein amine residues, the charge still remains high and produces a greater immune response. The positive charge assists in its binding to the antigen presenting cells and gets processed at an increased rate.

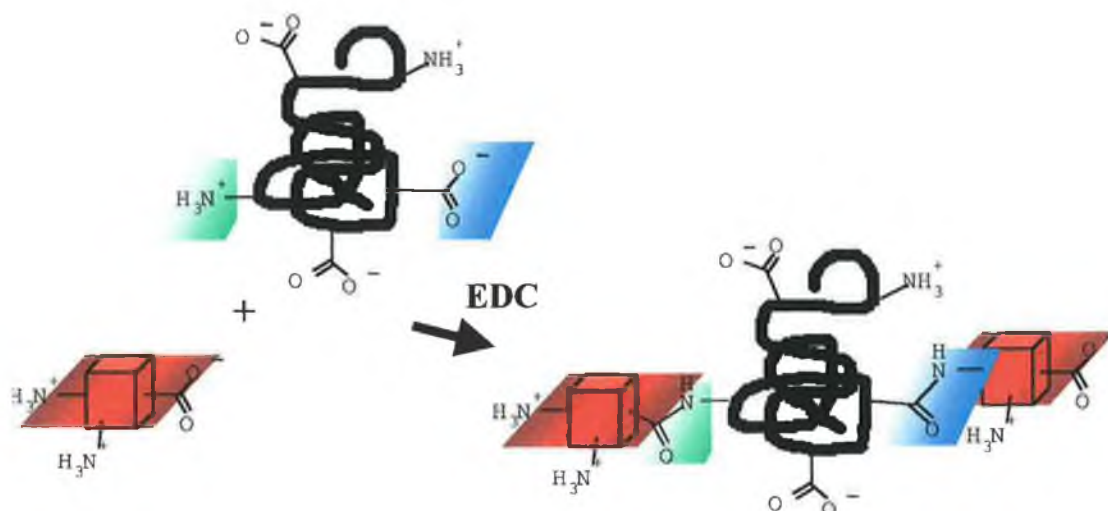
The production of antibodies to the main metabolite of heroin, morphine, provides a challenge regarding antibody production, due to its closeness in structure to the legal medication codeine. Findlay *et al.* (1981) investigated the relationships between immunogen structures and the resulting antibodies in the area of opioids. They found that conjugates of codeine-6-hemisuccinate, ethylmorphine-6-hemisuccinate or oxycodone-6-carboxymethyloxime had greater recognition of structural changes around the piperidine ring nitrogen atom and the 14-position. N-carboxypropylnormorphine-BSA, N-carboxypropylnorcodeine-BSA and norcodeine-BSA elicited antibodies that



recognised changes in the 14-substituent. Codeine conjugated through the 8 position elicited antibodies similar to those elicited by N-carboxypropylcodeine-BSA.

Salamone *et al.* (1998) reported the use of a non-cannabinoid immunogen used to generate antibodies with broad cross reactivity to the cannabinoid metabolites. They derivatised a benzpyran structure to elicit antibodies that were directed towards the conserved epitopes of cannabinoid metabolites. These antibodies showed two to three times higher cross reactivity with the cannabinoids than traditional phenolic-linked or 9-position-linked immunogens.

The design of a immunogen can be assessed by molecular modeling studies, however, the success of the immunogen can only be measured by the resulting titre of the antiserum produced.



**Figure 3.3:** The carbodiimide method for conjugating haptens and proteins through their carboxyl and amine groups, respectively. The process is mediated by EDC and NHS.

In the following results section, the production of morphine-protein and cocaine-protein conjugations are outlined. The immunisations and resulting titres of rabbit serum are presented. The purification process of the antibodies and the subsequent characterisation of the anti-THC, anti-morphine and anti-cocaine polyclonal antibodies are described. These antibodies were applied to an ELISA format and an assay developed and optimised for the detection of THC, morphine and cocaine.

## 3.2 *Results*

### 3.2.1 *Drug protein conjugate production*

The following schemes outline the process for the conjugation of cocaine and morphine to proteins, through EDC/NHS chemistry. Commercial conjugates of THC were obtained for the purposes of this project as there were difficulties encountered sourcing sufficient quantities of these drugs.

#### 3.2.1.1 *EDC/sulfo-NHS coupling chemistry*

EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride)/NHS((N-hydroxysulfosuccinimide) coupling chemistry is used to conjugate the drug and protein. The scheme used is outlined in Figure 3.3 (Hermanson, 1996). EDC reacts with the carboxylic group to form an active o-acylisourea ester intermediate. To stabilize the intermediate, sulfo-NHS is added and a stable sulfo-NHS ester intermediate is formed. The sulfo-NHS esters are hydrophilic active groups that react rapidly with the amines on the protein.

#### 3.2.1.2 *Conjugation of morphine to protein*

Morphine contains the following groups

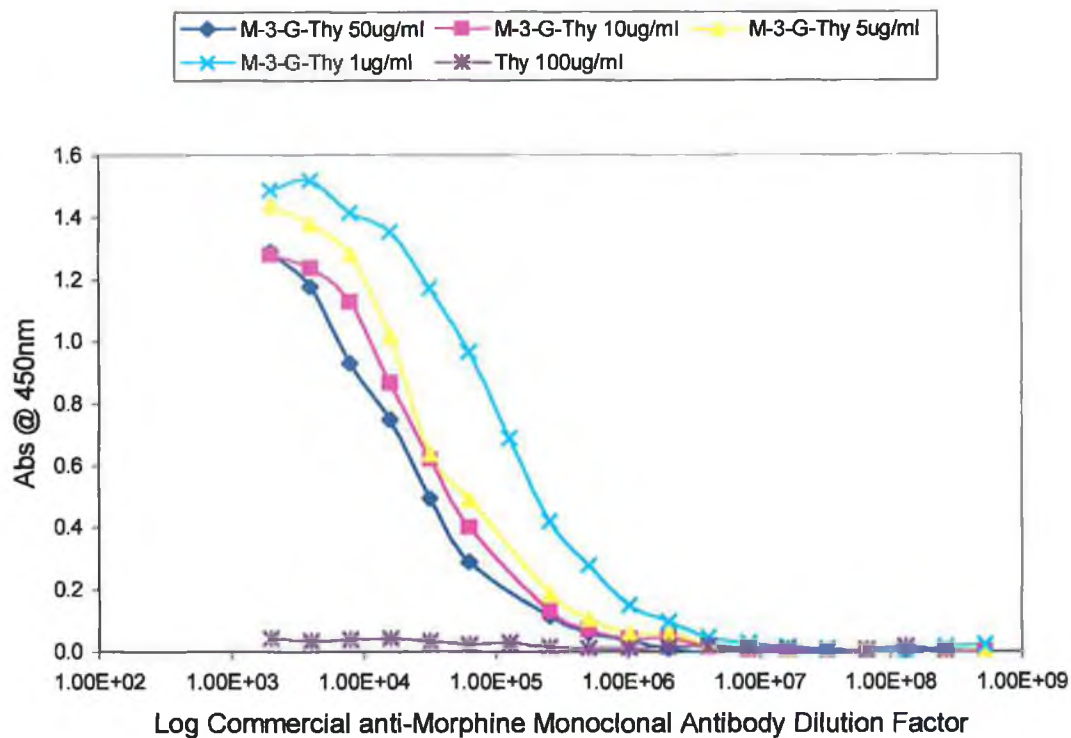
- Tertiary amino group
- Phenolic group (crucial to analgesic activity)
- Alcohol group
- Aromatic ring (Receptor sites in the brain)
- Ether bridge
- Double bond

Heroin is a powerful analgesic with twice the effects of morphine. Heroin differs from morphine in two areas. The 3-OH (Phenolic OH group) has become acetylated and the 6 alcohol has also been acetylated. Morphine has three polar groups (phenol, alcohol and an amine) whereas analogues have either lost the polar alcohol group or have masked it by an alkyl or acyl group. Heroin has two polar groups. The brain barrier is fatty, and

the significance of the polar groups becomes clear, the more polar morphine molecule is prevented from entering the brain, whereas the less polar heroin can enter easily.

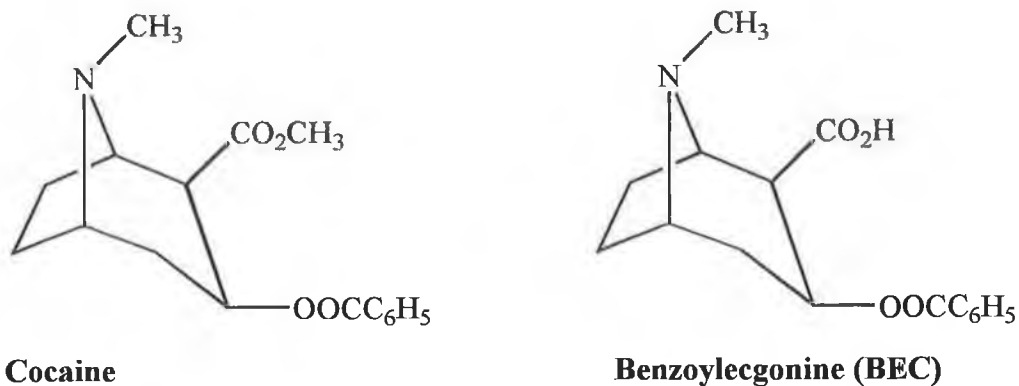
For the purposes of generating a morphine-protein conjugate, morphine-3-glucuronide was used as the starting material. The glucuronide group provides an ideal reactive group for conjugation through EDC/NHS chemistry, as described later. It also provides an excellent linker region for the purposes of screening for antibodies specific for morphine and not the area between the morphine and BSA. The utilisation of the conjugate in the ELISA format was validated by performing an ELISA using the morphine-3-glucuronide-OVA as the coating conjugate and the commercial morphine antibody as the primary antibody.

An ELISA was performed to assess the success of the above conjugation. The morphine-3-glucuronide-thyroglobulin was used as the coating conjugate and commercial anti-morphine monoclonal antibody was used as the primary antibody. The ELISA was performed as described in Section 2.9. The results can be seen in Figure 3.4.



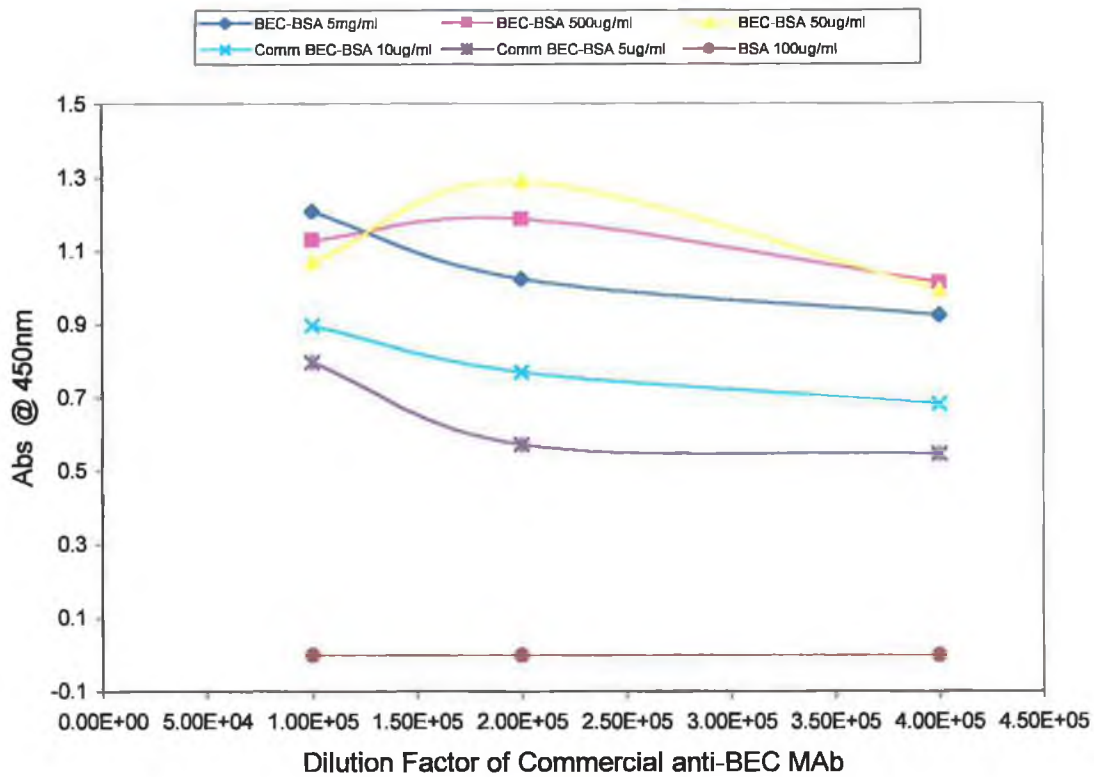
**Figure 3.4:** ELISA to confirm the conjugation of morphine to thyroglobulin. ELISA plates were coated with different concentrations of 'lab-produced' morphine-thyroglobulin conjugate. Commercial anti-morphine monoclonal antibody was used in the ELISA. The response to the thyroglobulin part of the conjugate was also measured by coating another series of wells with thyroglobulin alone. The response to the 'lab-produced' conjugate was positive, indicating a successful conjugation.

### 3.2.1.3 Conjugation of cocaine to protein



Cocaine and the main metabolite, BEC, are shown above. The original design for the conjugate was to use cocaine as the hapten and to derivatise the  $-\text{CO}_2\text{CH}_3$ , to create a linker and reactive carboxylic group for EDC/NHS coupling. The availability of the  $-\text{COOH}$  group on the BEC allows direct conjugation through EDC/NHS chemistry. Due to the time involved in obtaining import licenses for the BEC and the supply of cocaine available in the lab, an attempt was made to convert the cocaine to BEC. Cocaine is used topically as a local anesthetic and work has been carried out to determine the stability of an aqueous solution over time and over a range of pH, (Das Gupta, 1982). Other researchers have suggested that the levels of BEC found in blood and urine may not be an actual metabolite of cocaine but instead are the result of non-enzymatic hydrolysis and so this would lead to errors in measurements of both substances, (Fletcher & Hancock, 1981). The conditions under which the hydrolysis occurs optimally are at alkaline pH. It was with this in mind that an attempt was made to convert the cocaine available in the lab to BEC.

An ELISA was developed, as described in Section 2.9, to determine the degree of conjugation. The 'lab-produced' conjugate coated the wells at concentrations of 5mg/ml, 500 $\mu\text{g}/\text{ml}$  and 50 $\mu\text{g}/\text{ml}$  and the commercial BEC monoclonal antibody was used at dilutions of 1/100k, 1/200k, and 1/400k. Dilutions of the monoclonal antibody were made with and without BSA in the diluent. Controls included rows coated with the commercial BEC-BSA conjugate that had been used in previous competitive ELISAs and BSA alone. The ELISA results (see Figure 3.5) showed that the conjugation procedure used was successful using the protocol described above.



**Figure 3.5:** ELISA to confirm the conjugation of cocaine to bovine serum albumin (BSA). ELISA plates were coated with different concentrations of 'lab-produced' BEC-BSA conjugate and commercial BEC-BSA conjugate. Commercial anti-BEC monoclonal antibody was used in the ELISA. The response to the BSA part of the conjugate was also measured by coating another series of wells with BSA alone. The response to the 'lab-produced' conjugate was positive, indicating a successful conjugation.

### 3.2.2 *Determination of rabbit antibody titres*

Rabbits were immunised with the following drug-protein conjugates for the production of polyclonal antibodies. For the production of anti-THC antibodies, the immunogen used was THC-BTG, (Fitzgerald Industries). For the production of anti-BEC antibodies, the initial immunogen used was BEC-BSA, (Fitzgerald Industries) and later immunisations were prepared with the BEC-BSA that was produced as described in Section 2.5.2. The immunogen used to induce anti-morphine antibodies was initially morphine-BSA, (Fitzgerald Industries), and following the initial boosts, morphine-3-glucuronide-BSA was used.

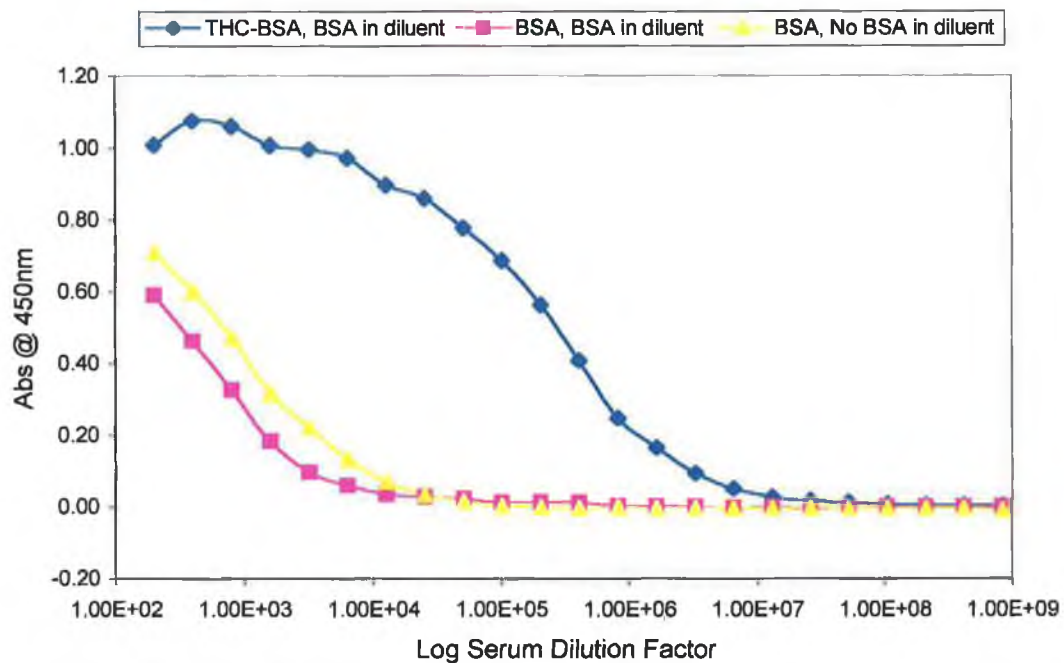
Figure 3.6 show the antibody titre from a rabbit immunised with THC-BTG. The serum was diluted in PBS/Tween containing 0.1% (w/v) BSA to remove non-specific interactions with the protein part of the conjugate. It was also titred against BSA, with and without BSA in the diluent. This was to detect any immune response to the protein part of the conjugate, and to ensure that 0.1% (w/v) was sufficient to remove the non-specific interactions. Although there was a response to the protein, it could be eliminated by the addition of the protein to the diluent. As can be seen from Figure 3.6, a very good response was obtained, the rabbit immunised with THC-BTG had a final titre of approximately 1/6 million.

Figures 3.7 and Figure 3.8 show the antibody titre from a rabbit immunised with BEC-BSA. The serum was diluted in PBS/Tween containing 0.1% (w/v) BSA to remove non-specific interactions with the protein part of the conjugate. It was also tested against BSA, with and without BSA in the diluent. This was designed to detect any immune response to the protein part of the conjugate, and to ensure that 0.1% (w/v) of BSA was sufficient to remove non-specific interactions. The response was greater to the protein carrier than it was to the drug, as can be seen from the figures. Figure 3.7 shows the final titre that was obtained and screened using BEC-BgG as the screening conjugate. The rabbit immunised with BEC-BSA had a disappointing final titre of approximately 1/50,000. It was decided to discontinue the immunisation schedule at that point as all titres had shown a much greater response to the BSA protein carrier than to the BEC drug hapten.

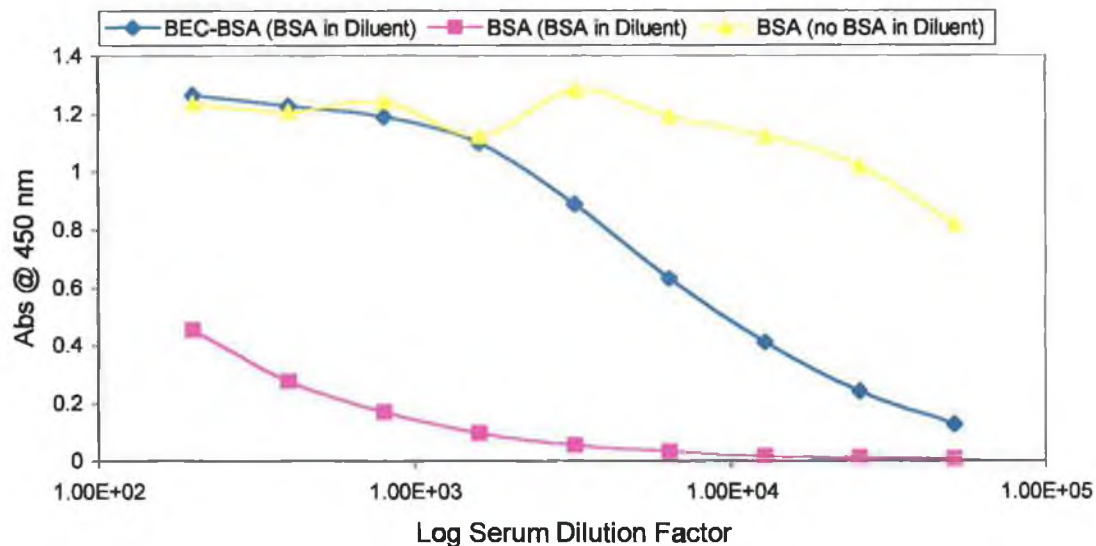
Figures 3.9 show the antibody titre from a rabbit immunised with morphine-BSA. It was screened against the morphine-3-glucuronide-ovalbumin conjugate. The rabbit immunised with morphine-BSA had a final titre of 1/400,000.



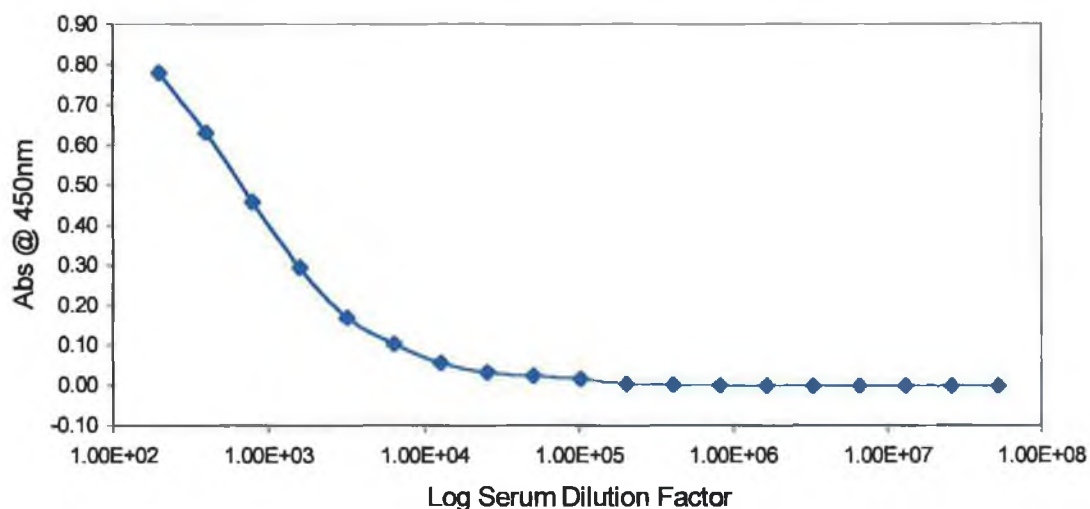
A final titre from the rabbit serum should be preferably in the region of 1/500,000. Experience by our research group and others have shown that a prolonged immunisation schedule of about six months is preferable (Danilova, 1994). This leads to greater affinity of the antibodies.



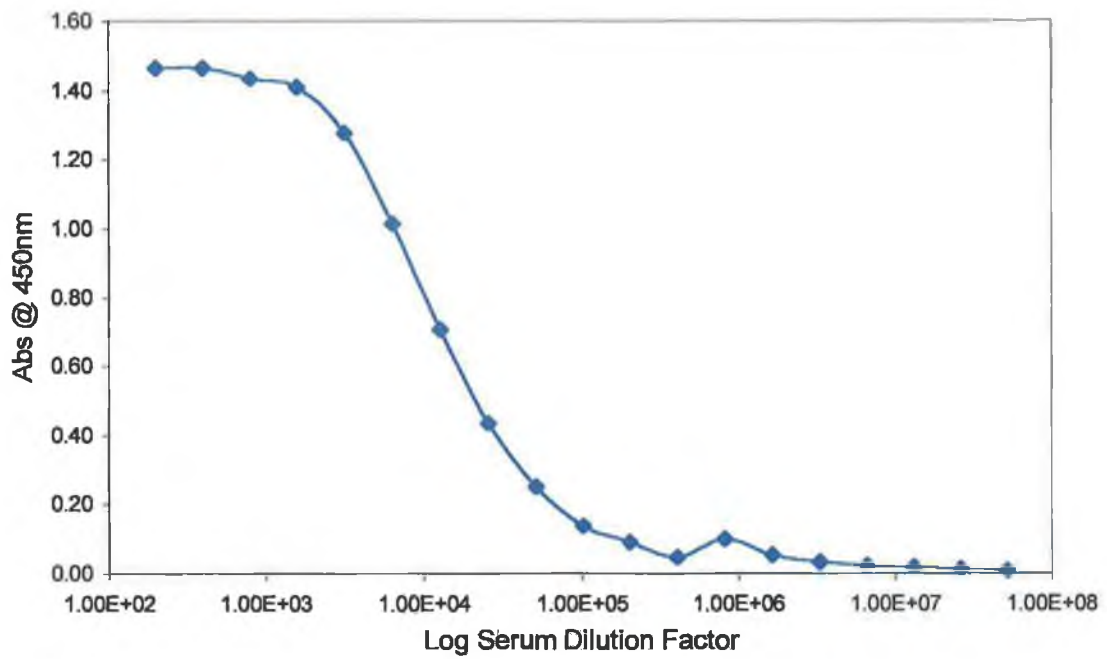
**Figure 3.6:** Titre of serum from rabbit immunised with THC-BTG (tetrahydrocannabinol-bovine thyroglobulin). BSA was incorporated into the diluent buffer to eliminate the binding interaction between the antibody and the protein carrier. The serum was also titered against BSA, with and without BSA in the diluent. This showed that the response to the protein could be eliminated by incorporating the protein into the diluent.



**Figure 3.7:** Titre of serum from rabbit immunised with BEC-BSA (benzoylcegonine-bovine serum albumin). BSA was incorporated into the diluent buffer to eliminate the binding interaction between the antibody and the protein carrier. The serum was also titered against BSA, with and without BSA in the diluent. This showed that the response to the BSA could be eliminated by incorporating the BSA into the diluent.



**Figure 3.8:** Titre of serum from rabbit (2A) immunised with BEC-BSA (benzoylcegonine-bovine serum albumin) and screened against BEC-BgG.

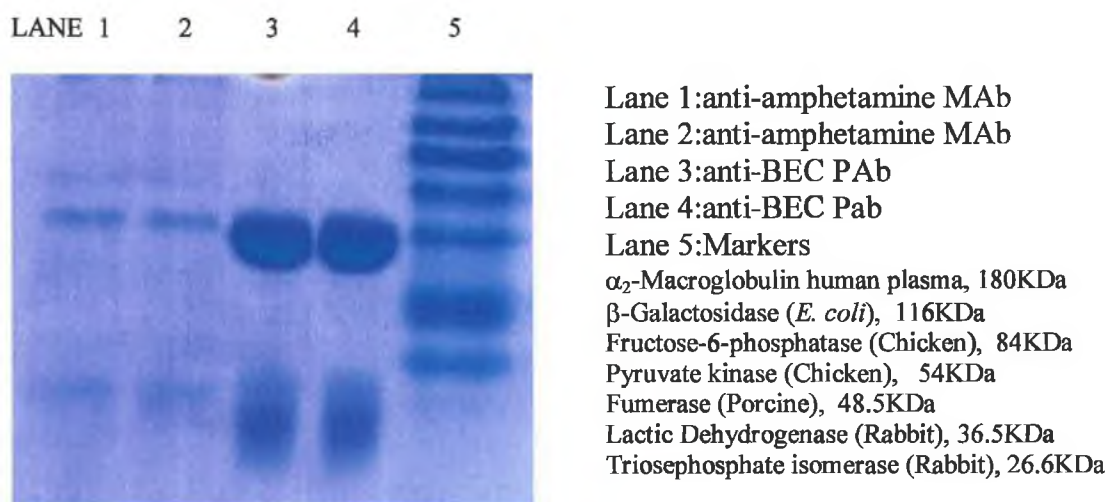


**Figure 3.9:** Titre of serum from rabbit immunised with morphine-BSA and screened against morphine-3-glucuronide-ovalbumin.

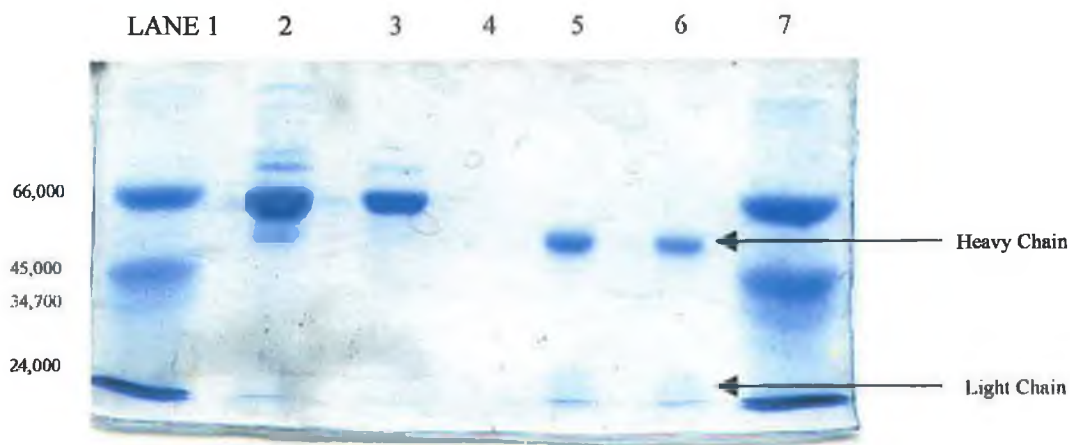
### 3.2.3 Purification and characterisation of polyclonal antibodies

The anti-THC, anti-BEC and anti-morphine polyclonal antibodies were purified by applying the dialysate from the ammonium sulphate precipitation to a Protein G immobilised Sepharose 3B column. The polyclonal antibodies were eluted from the column with 0.1M glycine, pH 2, as described in Section 2.8.3. The fractions collected were neutralised with 2M Tris, pH 8.6. The fractions were then read spectrophotometrically at 280 nm to determine the protein content. The fractions containing protein were pooled and dialysed in PBS overnight at 4°C with two of changes of PBS.

The purified antibodies were run on an SDS-PAGE to determine purity, as shown in Figure 3.10 and 3.11.



**Figure 3.10:** Characterisation by SDS-PAGE gel of the anti-BEC polyclonal antibody. Two bands can be seen, the top one at 50KDa representing the heavy chain and the lower band at 25KDa representing the light chains.



- 1 & 7: Molecular weight markers
- 2: Morphine antiserum
- 3: Supernatant from first SAS cut wash
- 4: Supernatant from second SAS cut wash
- 5: Purified anti-morphine polyclonal antibody
- 6: Purified anti-THC polyclonal antibody

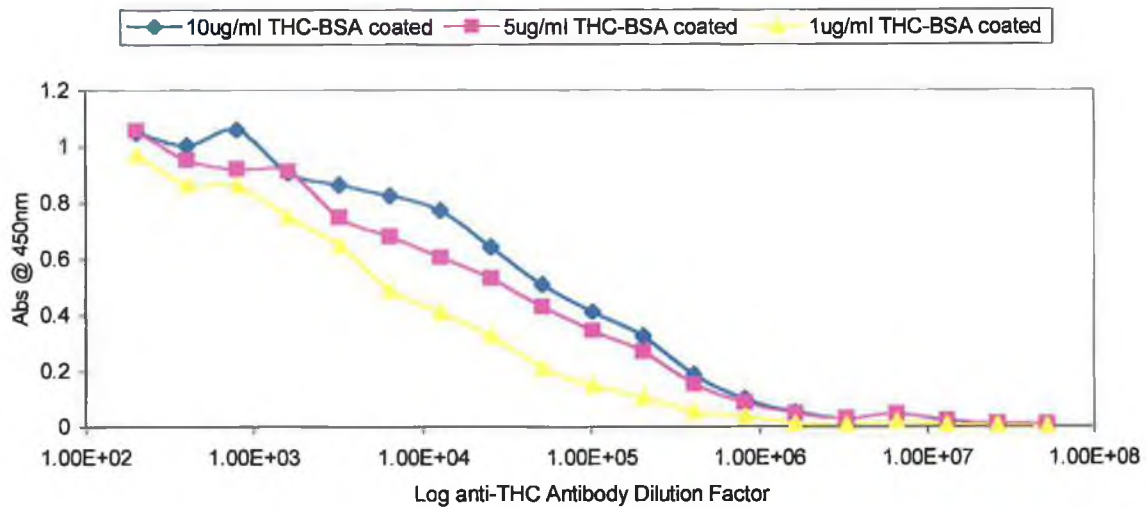
**Figure 3.11:** Characterisation by SDS-PAGE gel of the anti-morphine and anti-THC polyclonal antibody. Two bands can be seen, the top one at 50KDa representing the heavy chain and the lower band at 25KDa representing the light chains.

### ***3.2.4 Development of ELISAs for the detection of THC, morphine and cocaine using the polyclonal antibodies***

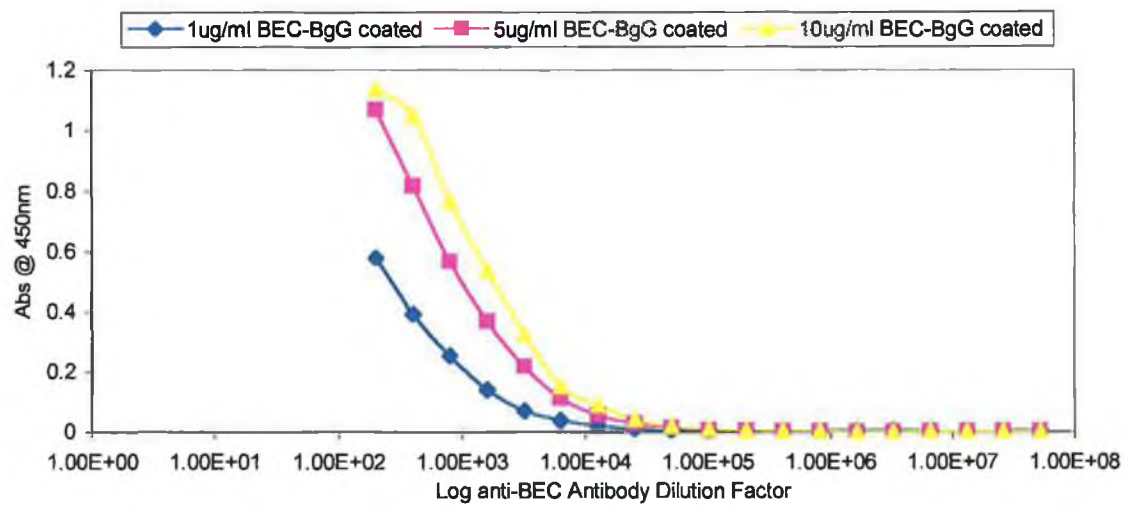
#### ***3.2.4.1 Anti-THC polyclonal antibody***

For the development of an ELISA for the detection of THC, the optimal coating concentration of THC-BSA and the optimal antibody dilution was determined by an indirect checkerboard ELISA. The results can be seen in Figure 3.12, the conjugate coating concentration ranged from 1  $\mu\text{g/ml}$  to 10  $\mu\text{g/ml}$ . The coating concentrations gave similar sensitivities and due to the expenses and availability of the conjugate, 1  $\mu\text{g/ml}$  was chosen as the concentrations for ELISAs. The optimal antibody dilution was approximately 1/5000, as this gave an absorbance in the 0.5 range and this is considered to be the sensitive region of the curve. However, for the purposes of optimisation of the assay with regard to sensitivities and cut off levels, the competitive assay was performed using a 1/10000 dilution of the polyclonal antibody and a less dilute secondary antibody dilution of 1/2000. Figure 3.6, the titre of the serum from this rabbit showed that at this concentration the response to the BSA carrier protein was negligible.

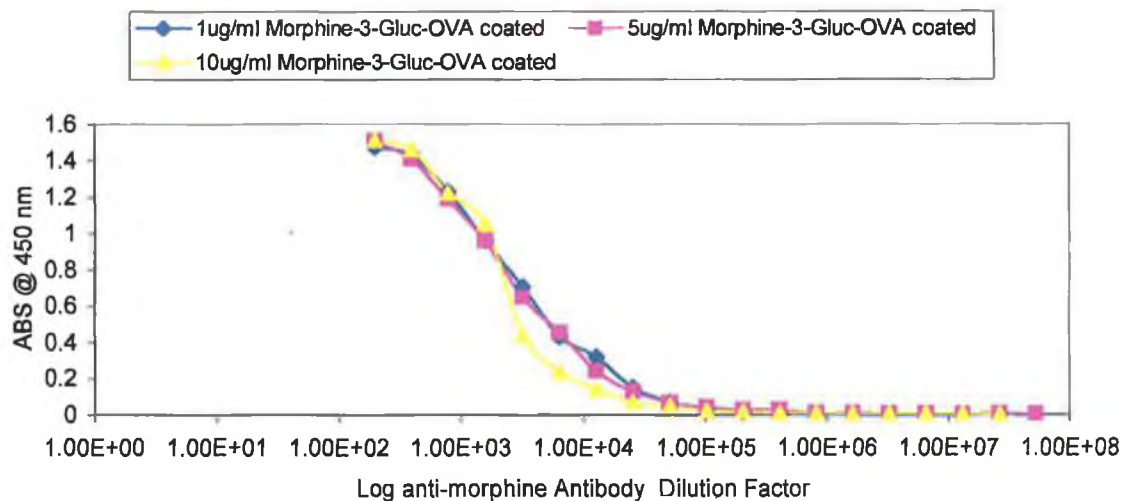
Figure 3.15 shows the relationship between the absorbance at 450nm and the concentration of free THC as determined by the competitive ELISA format. The range of detection of the assay was found to be between 24 and 50000 ng/ml. The intra-assay variation was determined from three replicates in an assay while the inter-assay variation was determined over five days of performing the assay. The intra-assay and inter-assay coefficients of variation are listed in Table 3.2 and 3.3.



**Figure 3.12:** Indirect checkerboard ELISA for the determination of optimal concentration of coating conjugate, THC-BSA, and optimal determination of anti-THC polyclonal antibody. THC-BSA was coated at 1, 5, and 10µg/ml. Doubling dilutions of the polyclonal antibody from 1/200 to 1/52,428,800 were carried out.

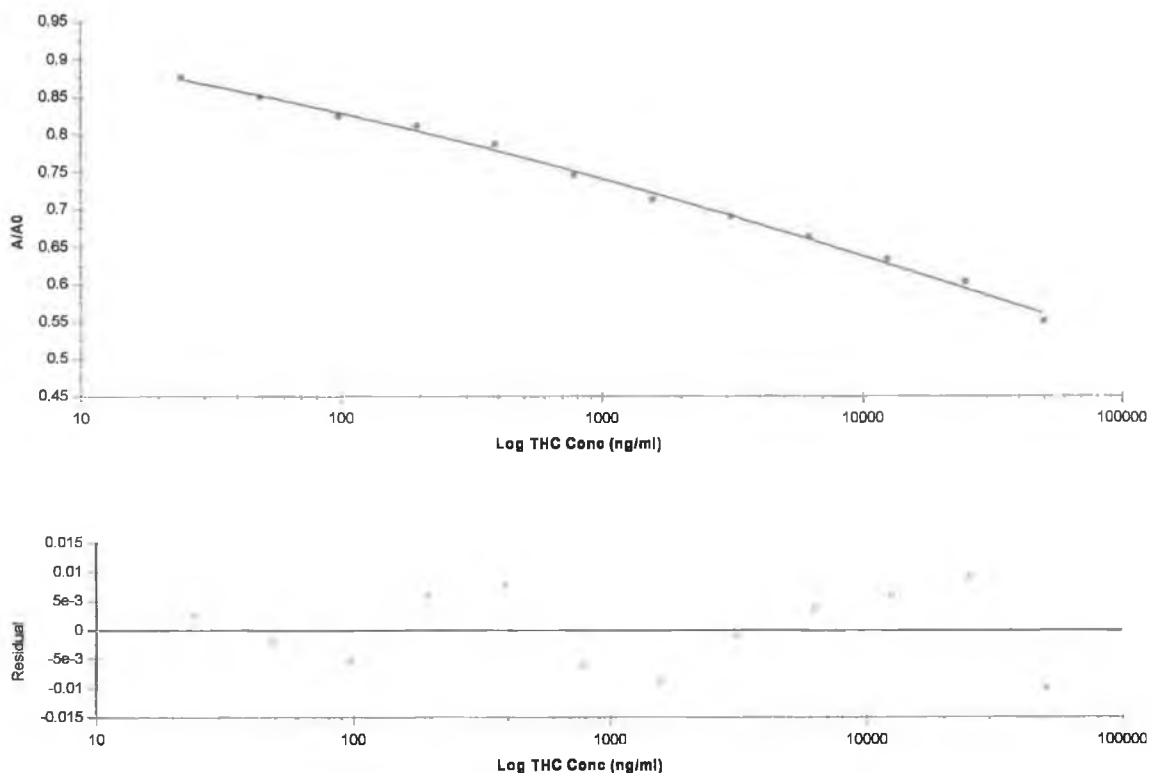


**Figure 3.13:** Indirect checkerboard ELISA for the determination of optimal concentration of coating conjugate, BEC-BgG, and optimal determination of anti-BEC polyclonal antibody. BEC-BgG was coated at 1, 5, and 10µg/ml. Doubling dilutions of the polyclonal antibody from 1/200 to 1/52,428,800 were carried out.



**Figure 3.14:** Indirect checkerboard ELISA for the determination of optimal concentration of coating conjugate, morphine-3-glucuronide-OVA, and optimal determination of anti-morphine polyclonal antibody. Morphine-3-glucuronide-OVA was coated at 1, 5, and 10 µg/ml. Doubling dilutions of the polyclonal antibody from 1/200 to 1/52,428,800 were carried out.





**Figure 3.15:** Inter-day curve for the detection of THC using the anti-THC polyclonal antibody on a THC-BSA immobilised surface. The data was correlated to a four-parameter model fit and the plot constructed using BIAevaluation 3.1 software. Each point on the graph is the average of five results obtained on five different days from a set of three replicates. Each value was normalised for that intra-assay by dividing the RU obtained by the RU for the positive control that only contained antibody and no morphine. The coefficient of variation, back-calculated amphetamine concentration and the percentage recovery for intra-day and inter-day assays are shown in Table 3.2 and 3.3. The range of detection of the assay is 24.4 – 50000ng/ml.

**Table 3.2:** Intra-assay variation (degree of precision) for the detection of THC in the ELISA using the anti-THC polyclonal antibody. The results presented are the mean of three replicates.

<b>Actual THC Conc (ng/ml)</b>	<b>Back-Calculated THC Conc (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
24.4	25.1	2.97%	102.75%
48.8	38.0	5.98%	77.83%
97.7	132.8	4.44%	136.03%
195.3	180.4	3.17%	92.35%
390.6	361.1	0.87%	92.44%
781.2	670.2	2.57%	85.78%
1562.5	1661.3	5.00%	106.32%
3125.0	4330.2	3.75%	138.57%
6250.0	5830.5	4.62%	93.29%
12500.0	9510.8	6.30%	76.09%
25000.0	20316.6	3.12%	81.27%
50000.0	73771.0	1.42%	147.54%

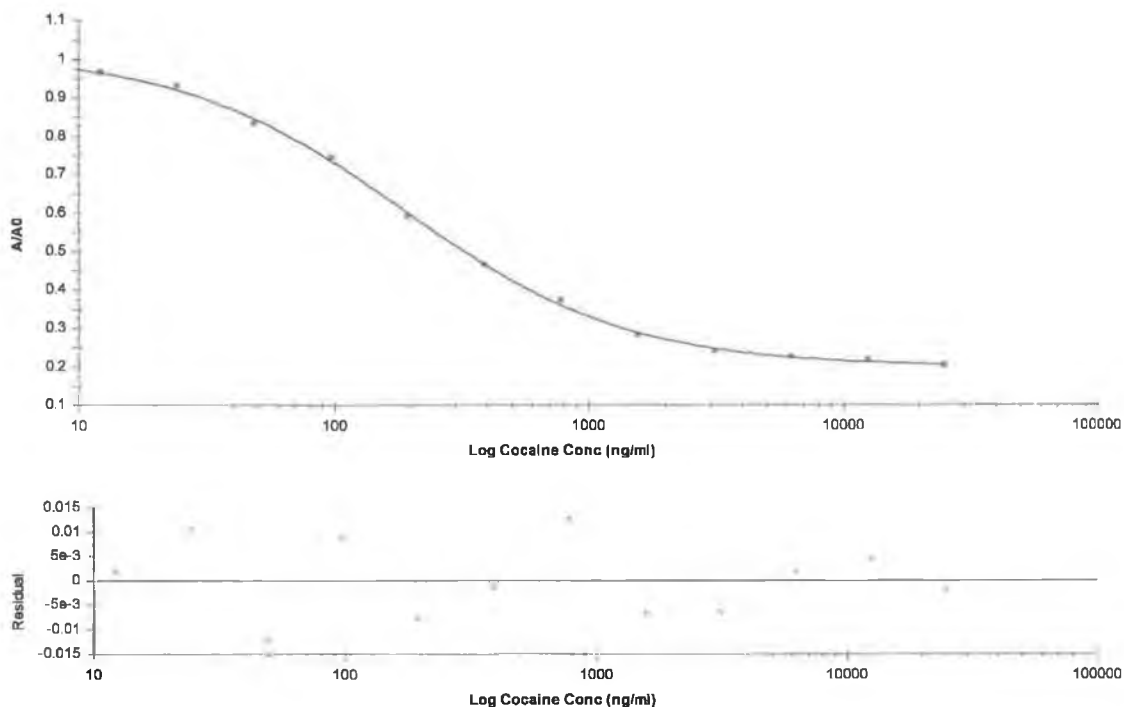
**Table 3.3:** Inter-assay variation (degree of accuracy and reproducibility) for the detection of THC in the ELISA using the anti-THC polyclonal antibody. The results presented are the mean values obtained from five intra-day assays, each assay had three replicates. The range of detection of the assay is 24.4 – 50000.0 ng/ml.

<b>Actual THC Conc (ng/ml)</b>	<b>Back-Calculated THC Conc (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
24.4	22.3	9.34%	91.46%
48.8	51.9	8.06%	106.27%
97.7	114.0	6.53%	116.71%
195.3	164.8	7.55%	84.37%
390.6	318.9	8.96%	81.64%
781.25	902.6	5.54%	115.54%
1562.5	1897.6	4.10%	121.45%
3125.0	3193.9	5.25%	102.21%
6250.0	5764.3	4.11%	92.23%
12500.0	11013.2	2.71%	88.11%
25000.0	20677.2	3.63%	82.71%
50000.0	61160.4	2.46%	122.32%

### ***3.2.4.2 Anti-cocaine polyclonal antibody***

The optimal coating concentration of BEC-BgG and the optimal antibody dilution was determined by an indirect checkerboard ELISA. The results can be seen in Figure 3.12, the conjugate coating concentration ranged from 1 µg /ml to 10 µg /ml. The coating concentrations gave similar sensitivities and due to the expenses and availability of the conjugate, 5 µg /ml was chosen as the concentrations for ELISAs. The optimal antibody dilution was 1/1000, as this gave an absorbance in the 0.5 range. However, in order to develop the competitive assay to detect free cocaine it was necessary to use a dilution of antibody at 1/500. The relationship between the absorbance at 450 nm and the concentration of cocaine is shown in Figure 3.16. This shows that a satisfactory competitive ELISA can be used with an antibody dilution of 1/500. The range of detection of the assay was between 6.1 to 25000 ng/ml. The intra-assay variation was determined from three replicates in an assay while the inter-assay variation was determined over five days of performing the assay. The intra-assay and inter-assay coefficients of variation are listed in Table 3.4 and 3.5.

Another series of experiments were devised to look at an assay format using the immunogen, BEC-BSA, as the coating conjugate with and without BSA in the diluent. Figure 3.7 shows that the response to the BSA-coated plate was greater than that to the BEC-BSA-coated plate, although this could be relieved by incorporating 1% (w/v) BSA into the diluent. Prior titres performed on the serum from animals immunised with this BEC-BSA conjugate also showed greater binding to the BSA. This means that there was a greater immune response towards the BSA part rather than the hapten. When a competitive assay format was investigated using the BEC-BSA as the coating conjugate, it was found that competition did not occur between the conjugate and the free cocaine.



**Figure 3.16:** Inter-day curve for the detection of morphine using the anti-BEC polyclonal antibody on an BEC-BgG (benzoylecgonine-bovine gamma globulin) immobilised surface. The data was correlated to a four-parameter model fit and the plot constructed using BIAevaluation 3.1 software. Each point on the graph is the average of five results obtained on five different days from a set of three replicates. Each value was normalised for that intra-assay by dividing the RU obtained by the RU for the positive control that only contained antibody and no morphine. The coefficient of variation, back-calculated amphetamine concentration and the percentage recovery are shown in Table 3.4 and 3.5. The range of detection of the assay is 6.1 – 25000ng/ml.

**Table 3.4:** Intra-assay variation (degree of precision) for the detection of cocaine in the ELISA using the anti-BEC polyclonal antibody. The results presented are the mean values of three replicates.

<b>Actual Cocaine Conc (ng/ml)</b>	<b>Back-Calculated Cocaine Conc (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
6.10	7.97	5.02%	130.69%
12.20	9.70	1.35%	79.50%
24.41	22.85	1.44%	93.62%
48.83	52.47	2.82%	107.45%
97.65	91.64	5.82%	93.85%
195.31	219.05	1.99%	112.16%
390.62	417.69	0.84%	106.93%
781.25	543.01	0.45%	69.51%
1562.50	1755.70	2.44%	112.36%
3125.00	5632.17	0.00%	180.23%
6250.00	7442.83	1.72%	119.09%
12500.00	7928.00	4.36%	63.42%
25000.00	18029.05	4.42%	72.12%

**Table 3.5:** Inter-assay variation (degree of accuracy and reproducibility) for the detection of cocaine in the ELISA using the anti-BEC polyclonal antibody. The results presented are the mean values obtained from five intra-day assays, each assay had three replicates.

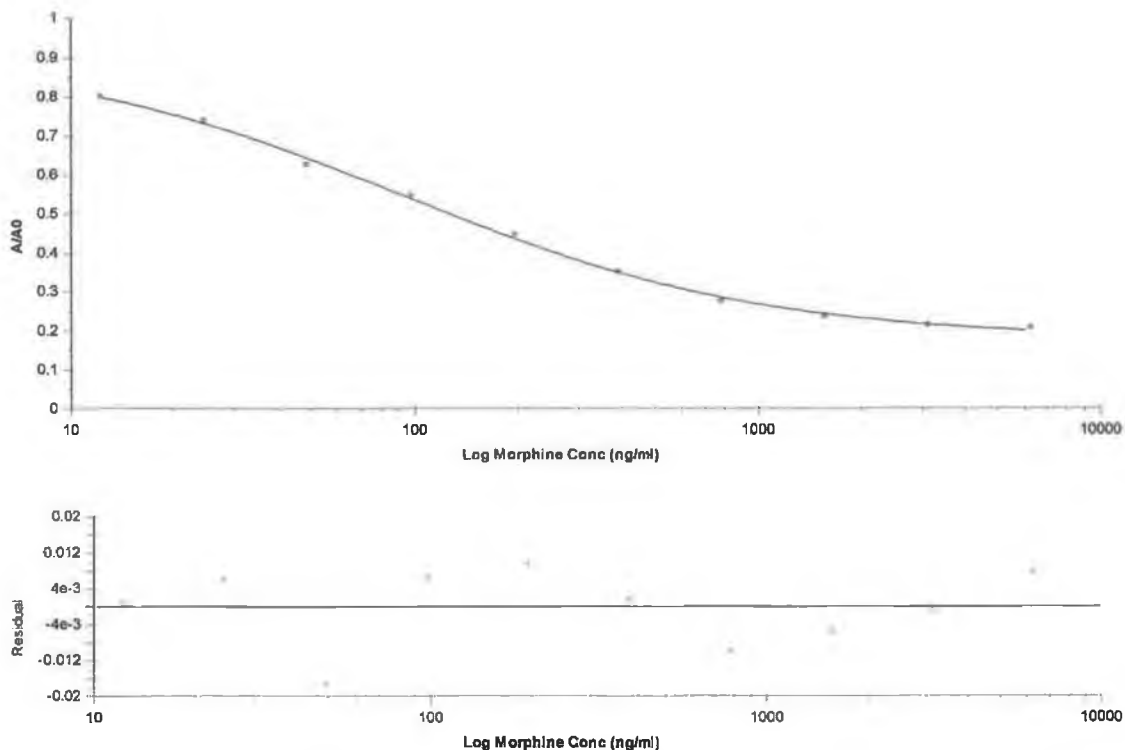
<b>Actual Cocaine Conc (ng/ml)</b>	<b>Back-Calculated Cocaine Conc (ng/ml)</b>	<b>% CV</b>	<b>% Recovery</b>
6.10	7.31	6.39%	119.85%
12.20	11.69	6.29%	95.84%
24.41	21.36	9.71%	87.48%
48.83	53.34	12.84%	109.24%
97.65	92.95	13.01%	95.18%
195.31	202.95	10.48%	103.91%
390.62	393.24	10.01%	100.67%
781.25	709.07	5.88%	90.76%
1562.50	1704.25	7.30%	109.07%
3125.00	3647.00	10.60%	116.70%
6250.00	5803.12	13.28%	92.85%
12500.00	9060.86	16.26%	72.49%
25000.00	33737.83	13.94%	134.95%

### ***3.2.4.3 Anti-morphine polyclonal antibody***

The optimal coating concentration of morphine-3-gluc-OVA and the optimal antibody dilution was determined by an indirect checkerboard ELISA. The results can be seen in Figure 3.13, the conjugate coating concentration ranged from 1 µg/ml to 10 µg/ml. The coating concentrations gave similar sensitivities and 1 µg/ml was chosen as the concentrations for ELISAs. The optimal antibody dilution was approximately 1/3200. However, for the purposes of optimisation of the assay with regard to sensitivities and cut off levels, the competitive assay was performed using a 1/1500 dilution of the polyclonal antibody.

Figure 3.17 shows the relationship between the absorbance at 450nm and the concentration of free morphine as determined by the competitive ELISA format using the antibody dilutions listed above. The range of detection of the assay was found to be between 0.38 and 6250 ng/ml morphine. The intra-assay variation was determined from three replicates in an assay while the inter-assay variation was determined over five days of performing the assay. The intra-assay and inter-assay coefficients of variation are listed in Table 3.6 and 3.7, and show the assay to be reproducible and accurate, as determined by the acceptable percentage recoveries and coefficient of variations.





**Figure 3.17:** Inter-day curve for the detection of morphine using the anti-morphine polyclonal antibody on an morphine-3-glucuronide-OVA immobilised surface. The data was correlated to a four-parameter model fit and the plot constructed using BIAevaluation 3.1 software. Each point on the graph is the average of three results obtained on three different days from a set of three replicates. Each value was normalised for that intra-assay by dividing the RU obtained by the RU for the positive control that only contained antibody and no morphine. The coefficient of variation, back-calculated amphetamine concentration and the percentage recovery are shown in Table 3.6 and 3.7. The range of detection of the assay is 0.38 – 6250ng/ml.

**Table 3.6:** Intra-assay variation (degree of precision) for the detection of morphine in the ELISA using the anti-morphine polyclonal antibody. The results are obtained from three replicates.

<b>Actual Morphine Conc (ng/ml)</b>	<b>Back-Calculated Morphine Conc (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
0.38	0.23	1.63%	60.00%
0.76	1.02	3.96%	133.82%
1.52	2.42	2.30%	159.47%
3.05	2.66	1.51%	87.28%
6.10	5.90	0.00%	96.64%
12.20	11.62	1.39%	95.26%
24.41	22.55	0.83%	92.39%
48.83	57.16	3.66%	117.06%
97.65	96.22	0.00%	98.54%
195.31	176.62	0.55%	90.43%
390.62	364.64	1.81%	93.35%
781.25	894.13	1.40%	114.45%
1562.50	2056.40	0.97%	131.61%
3125.00	3390.73	2.05%	108.50%
6250.00	3744.29	2.76%	59.91%

**Table 3.7:** Inter-assay variation (degree of accuracy and reproducibility) for the detection of morphine in the ELISA using the anti-morphine polyclonal antibody. The range of detection of the assay is 0.38 – 6250.0 ng/ml. The results presented are the mean values obtained from five intra-day assays, each assay had three replicates.

<b>Actual Morphine Conc (ng/ml)</b>	<b>Back-Calculated Morphine Conc (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
0.38	0.35	6.19%	91.05%
0.76	1.02	5.82%	134.74%
1.52	1.99	5.32%	131.05%
3.05	3.60	4.74%	118.00%
6.10	6.87	5.17%	112.54%
12.20	12.24	5.44%	100.30%
24.41	21.93	5.31%	89.85%
48.83	51.99	10.77%	106.46%
97.65	90.61	7.81%	92.79%
195.31	184.08	9.04%	94.25%
390.62	406.15	8.91%	103.98%
781.25	961.24	8.85%	123.04%
1562.50	1820.32	8.56%	116.50%
3125.00	3033.26	9.62%	97.06%
6250.00	3740.58	9.82%	59.85%

#### 3.2.4.4 Cross reactivity studies: Anti-morphine polyclonal antibody

The cross reactivity of the anti-morphine polyclonal antibody was determined against morphine-3-glucuronide, 6-MAM, norcodeine and codeine. The degree of cross reactivity was determined as per the competitive ELISA described in Section 2.9.4. The standards were obtained from a stock solution of 1mg/ml standard in ethanol. The degree of cross reactivity was determined as the concentration of cross reactant that gives a response of 50% or one-half of the observed maximum binding, (EC<sub>50</sub> – Cross Reactant) expressed a percentage of the specific analyte concentration that gives a 50% response, (EC<sub>50</sub> – Specific Analyte).

$$\% \text{ Cross Reactivity} = \frac{\text{Concentration of Analyte (EC}_{50} - \text{SA)}}{\text{Concentration of Cross Reactant (EC}_{50} - \text{CR)}} \times 100\%$$

The degree of cross reactivity of the anti-morphine antibody is expressed in Table 3.8.

**Table 3.8:** Cross reactivity of anti-morphine polyclonal antibody.

<b>Drug</b>	<b>% Cross Reactivity</b>	<b>Range of Detection (ng/ml)</b>
Morphine-3-glucuronide	100%	97.7-6250.0
6-monoacetylmorphine (6-MAM)	30.39%	48.8 –1562.5
Norcodeine	0.78%	195.3-12500
Codeine	100%	390.1

### **3.3 Discussion**

The conjugation of morphine and cocaine to proteins for the production of drug-protein conjugates was described. The resulting immunogens were used to induce antiserum in rabbits to the drugs of interest. Three different rabbits were induced with THC-BtG, morphine-BSA and BEC-BSA. Once a suitable titre was achieved, the animals were sacrificed and the serum collected and the polyclonal antibodies, anti-THC, anti-BEC and anti-morphine were purified and characterised. The antibodies were applied to an ELISA format for the detection of THC, cocaine and morphine.

A competitive ELISA was developed for the detection of THC with the anti-THC polyclonal antibody, and a 4-parameter fit was applied to the data. This antibody showed a range of detection between 24.4 to 50,000 ng/ml THC. The assay showed very good precision as determined by the intra-assay coefficients of variation (0.87% to 5.98%), and very good reproducibility as determined by the inter-assay coefficients of variations (2.46 – 9.34%). The degree of accuracy was also determined by a calculation of the percentage recovery. As described by Findlay, 2000, this is a concept that expresses the closeness of agreement between a measured test result and its theoretical true value. The percentage recoveries for the inter-assay were very good, between 81.64% and 122.32%. Overall, the anti-THC polyclonal antibody competitive ELISA for the detection of THC was a very good, accurate, reproducible assay.

The competitive assay for the detection of cocaine had a range of detection of 6.1 to 25,000ng/ml. The assay showed very good precision as determined by the intra-assay coefficients of variation (0% to 5.02%), and good reproducibility as determined by the inter-assay coefficients of variations (5.88% – 16.26%). The high %CV of 16.26% was obtained for the value of 12500 ng/ml THC. This larger CV value could have been a result of the fact that it is at the high end of the range of detection, and so there is more variability. The degree of accuracy was also determined by a calculation of the percentage recovery. The percentage recoveries for the inter-assay were very good, between 72.49% and 134.95%. These two outer ranges were obtained for the high cocaine concentrations of 12500 and 25000 ng/ml. All other CVs were within an acceptable range of 92.85% to 119.85%. Overall, the anti-BEC polyclonal antibody competitive ELISA for the detection of cocaine is a very good, accurate, reproducible assay. One interesting point found with this antibody is that it is only competitive in an

ELISA format when the BEC-BgG was used as the coating conjugate. The use of BEC-BSA as the conjugate resulted in an ELISA that did not detect free drug. This would imply that the orientation of the BEC in the BEC-BSA conjugate is such that the BEC is not sufficiently exposed for recognition by the antibodies. Whereas in the case of the BEC-BgG conjugate, the orientation of the BEC on the conjugate allows for it to be recognised by the anti-BEC antibodies.

A competitive ELISA was developed for the detection of morphine, the main metabolite of heroin, with the anti-morphine polyclonal antibody, and a 4-parameter fit was applied to this data, also. This antibody showed a range of detection between 0.38 to 6250.0 ng/ml morphine. The assay showed very good precision as determined by the intra-assay coefficients of variation (0% to 3.96%), and very good reproducibility as determined by the inter-assay coefficients of variations (5.17% – 10.77%). The percentage recoveries for the inter-assay were very good, between 89.85% and 123.04%. The percent recovery for the highest standard concentration of the range, 6250ng/ml, was 59.85%, which is considered to be outside of an accurate assay. This is probably due to the characteristic inaccuracies that are inherent in the asymptotes of such a model fit. Overall, the anti-morphine polyclonal antibody competitive ELISA for the detection of morphine was a very good, accurate, reproducible assay. The degree of cross reactivity of the assay with morphine-3-glucuronide, 6-MAM, norcodeine, and codeine was also examined. The degree of cross reactivity was 100% for the main metabolite found in urine, morphine-3-glucuronide, and for the medication, codeine. This was to be expected as the point of conjugation of the morphine to the protein, through the glucuronide group was the –3 position. It is at this position that codeine is distinguished from morphine, by the presence of an acetyl group. There is a 30.4% cross reactivity between 6-MAM, which again is expected as the 6-MAM molecule differs from the morphine molecule only at the 3-position, by the presence of a  $-C_2H_3O_2$  group. The degree of cross reactivity to norcodeine, a minor metabolite, was also examined and showed a 0.78% cross reactivity.

The characterisation and application of the anti-THC and anti-morphine polyclonal antibodies is continued in Chapter 6. The ELISAs described above were applied to saliva samples spiked with THC and morphine. Real samples were analysed using these assays. The antibodies were also applied to the BIAcore and competitive assays

were established for the detection of the drugs. Chapter 6 also describes the application of the anti-THC antibody to the Envitec Device for the development of a novel rapid assay.

## *Chapter 4*

### *Production and Characterisation of Anti-Amphetamine and Anti-Methamphetamine Monoclonal Antibodies*



## **4.1 Introduction**

### **4.1.1 Monoclonal Antibodies – Background**

The 1984 Nobel Prize in Physiology and Medicine was awarded to Georges Kohler and Cesar Milstein for their pioneering work to produce an immortalised monoclonal antibody producing cell (Kohler & Milstein, 1975). Their work revolutionised antibody production and the associated areas where the antibodies can be applied. Monoclonal antibodies are antibodies of a single idio type produced by immortalised B cells. Normal B cells are the end products of a differentiation pathway and cannot be maintained in culture. Myeloma cells are immortal, but the antibodies produced are of unknown specificity. Kohler and Milstein harnessed the pertinent qualities of each of the cells, and fused the B cells producing antibody of desired specificity with the myeloma cells. The result is a *hybrid-myel-oma*, called a hybridoma.

Interestingly, at the time of publication of the original work, the National Research Development Council, the organisation through which the Medical Research Council scientists could commercially exploit their work, wrote ‘It is certainly difficult for us to identify any immediate practical application which could be pursued as a commercial venture’ (Austin, 1989).

### **4.1.2 Production of monoclonal antibodies**

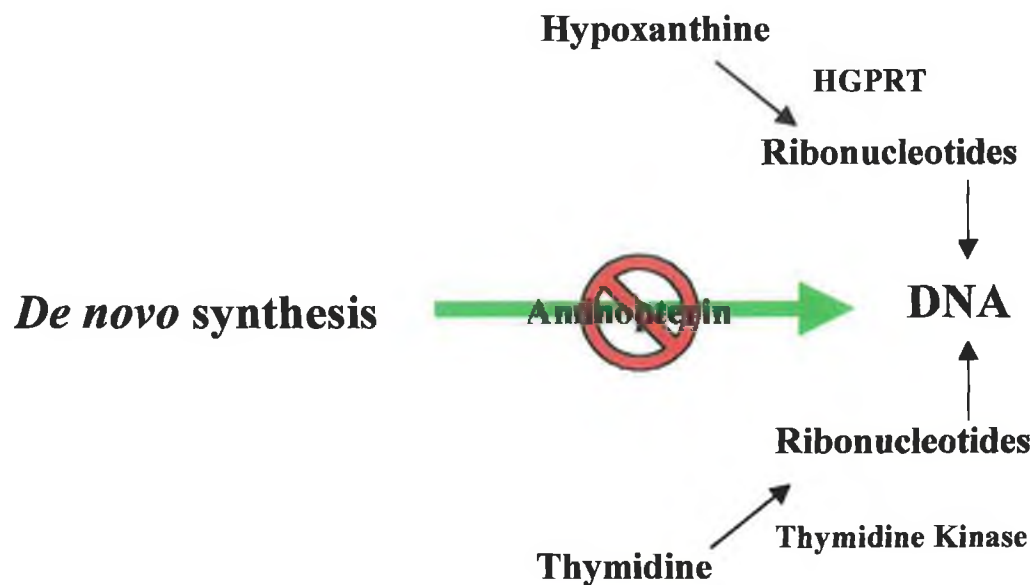
The production of monoclonal antibodies begins with immunisation of mice by either *in-vivo* or *in-vitro* immunisations. *In-vivo* immunisations are carried out at regular time intervals, usually at least 4-6 weeks apart for several months. The success of the immunisations can be monitored by taking samples of serum and following the titre of the antibodies produced. There are publications detailing shorter immunisation periods by more frequent immunisations, (Wring et al., 1999). Normally, for the isolation of spleenocytes, a longer time-scale is more beneficial, with regard to the affinity of the antibodies produced. It is also possible to produce an hybridoma from other lymphoid tissue such as lymph nodes.

The fusion between the spleenocytes and the myeloma cells e.g., Sp2/0-Ag14, is usually achieved through the use of polyethylene glycol, which causes a change in membrane

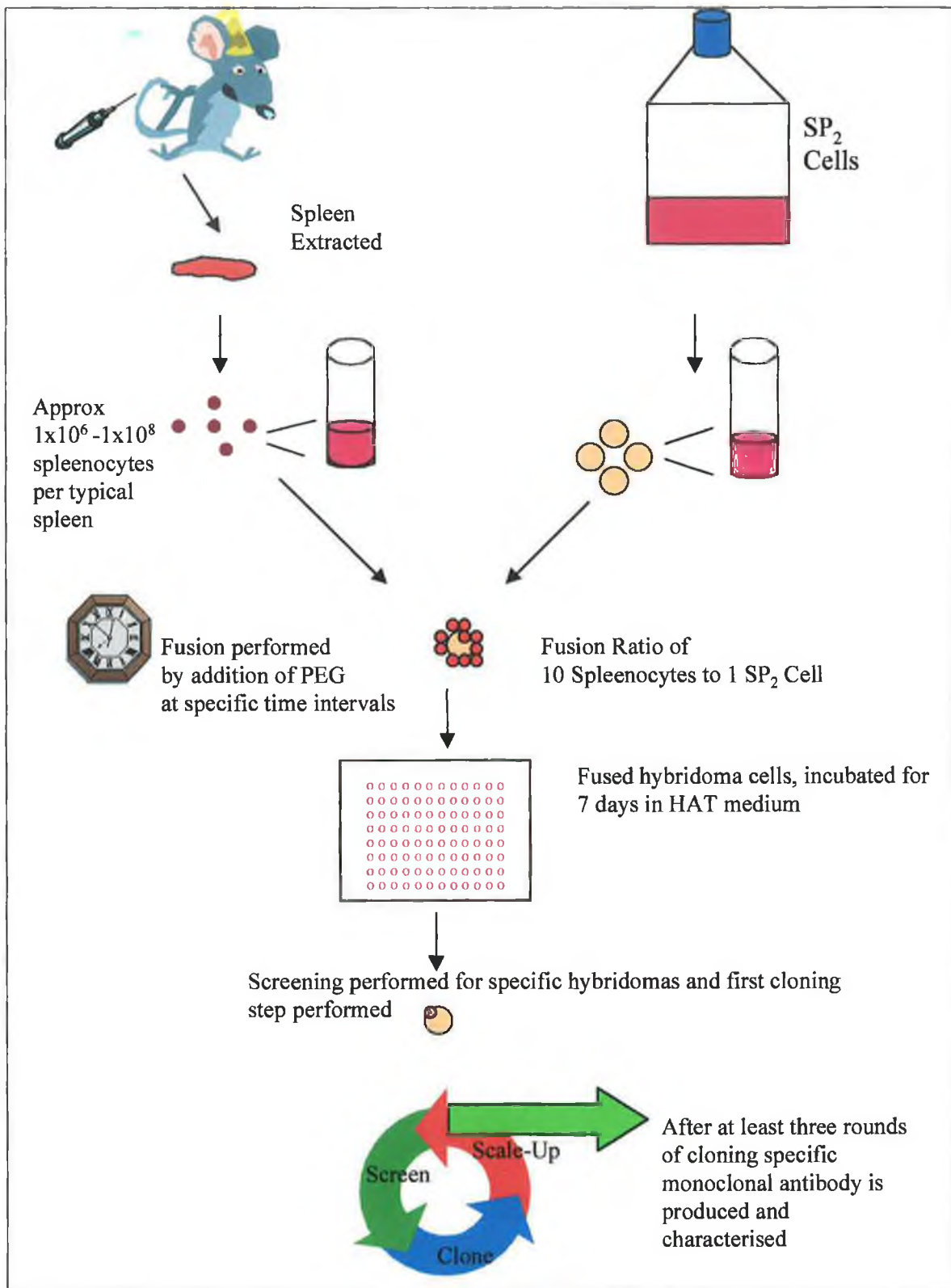
permeability. The original method for fusion was inactivated Sendai virus, which induces intercellular fusion in activated cells. However, the receptors for the Sendai virus fusion protein are needed and since some cells lack these proteins the fusion agent used now is PEG. Electroporation is another method that is used to promote fusion, though to a lesser extent (McCullough and Spier, 1990).

The fusion process is a relatively random process and the fused hybridoma cells must be selected from the unfused B cells and myeloma cells. The selection process used by Kohler and Milstein is accomplished by culturing the hybridoma cells in hypoxanthine-aminopterin-thymidine medium (HAT). Aminopterin blocks the *de novo* biosynthesis of the purines and pyrimidines that are required for DNA synthesis. When this pathway is blocked the cells can use the salvage pathway using the exogenous hypoxanthine and thymidine, however they need the enzymes hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and thymidine kinase (TK) to do this (Figure 4.1). The myeloma cells chosen for a fusion deliberately lack the hypoxanthine-guanine phosphoribosyl transferase enzyme (HGPRT). So, in the HAT medium the unfused myeloma cells die, as do any myeloma cells fused to other myeloma cells. The spleenocytes possess the HGPRT enzyme, however they have a limited time in culture and will eventually die after about 2 weeks. The myeloma cells that fused with spleenocytes now possess the HGPRT enzyme and can grow in the HAT medium.

The hybridoma cells are grown in HAT medium for about two weeks. This ensures that all hybridomas that revert to a myeloma phenotype are eliminated. The media is then changed to HT media for at least another seven feedings and sub-cultures at which time any traces of aminopterin should have been eliminated (McCullough and Spier, 1990). The process for producing a monoclonal antibody is outlined in Figure 4.2 and described in detail in Chapter 2.



**Figure 4.1:** In the presence of aminopterin, the *de novo* biosynthesis of purine and pyrimidines is blocked. Hypoxanthine and thymidine are needed for the salvage pathway, as are the enzymes HGPRT and thymidine kinase. Cells lacking these enzymes will die in HAT medium because they are incapable of producing the nucleic acids. It is through this process that fused myeloma cells are selected. The myeloma cells are HGPRT<sup>-</sup> and so only those fused with the HGPRT<sup>+</sup>-B cells survive in the HAT medium.



**Figure 4.2:** Principle of monoclonal antibody production.

An alternative to *in vivo* immunisation is *in vitro* immunisation of cultured spleenocytes (Borrebaeck, 1988). This procedure has several advantages over *in vivo* immunisations:

- It follows a reduced immunisation schedule leading to less time needed prior to fusion.
- It uses smaller amounts of the antigen of interest. This is particularly important as many substances are in extremely short supply due to expense or purification methods.
- It allows the production of antibodies to weakly immunogenic agents, to self-antigens and to toxic agents that cannot be immunised *in vivo*.
- It avoids the ethical arguments associated with *in vivo* immunisation (Borrebaeck, 1988).

The main disadvantage however, is that the primary immunological response is obtained, which means that IgM antibodies are produced rather than IgG antibodies that are usually produced by the *in vivo* immunisation method. This means that the antibodies produced have a greater chance of being polyreactive, (Bouvet and Dighiero, 2000). McMahon and O’Kennedy (2001) looked at a panel of nine hybridomas, secreting IgM anti-goat IgG, that had been produced from spleenocytes that had been immunised *in vitro* with the goat IgG. The affinity constants of the antibodies against six antigens were examined and the specific anti-IgG activities of the hybridoma supernatant and corresponding affinity purified IgM fraction were determined. Nine antibodies were tested, eight were found to be polyreactive. The degree of polyreactivity may be underestimated by screening techniques such as ELISA as discussed in the next section.

#### **4.1.3 Screening for antibodies of interest**

The successful fusion of the myeloma cells and spleenocytes produces a range of hybridomas that secrete antibodies. The spleenocytes are a heterogenous group of cells and so a different antibody is produced by each hybridoma. The mice used for the fusion would have been immunised for up to several months and kept in the laboratory animal facility and so would have been exposed to somewhat different environmental and endogenous factors along with the immunogen. This exposure means the immune

system is responding to other immunogens. There is also the issue that not all hybridomas will produce any antibody at all. The hybridoma cell when formed is tetraploid, because it is formed from two diploid cells. As the cells grow and divide, the extra chromosomes are lost. This means that some cells die, some stop producing the antibody and other successful ones go on to produce the antibody of interest. All of these factors mean that the screening procedure for the selection of hybridomas is of the utmost importance. The screening step is also the most time-consuming stage of hybridoma production and has to be performed alongside the continued cultures of the hybridomas. When the specific hybridoma cells are identified the cloning procedures must begin in order to isolate the single hybridoma that is secreting the required antibody. Screening must continue during this step as the hybridomas can be unstable and stop growing during the cloning or can continue to grow but cease to secrete antibody. The importance of screening in the production of antibodies to small haptens, such as drugs of abuse, is frequently discussed (Chappey *et al.*, 1992; Danilova, 1994). It is important to screen for antibodies that are reactive against the free hapten rather than the linker region between the hapten and protein or the protein. The common formats used for screening are ELISA, Western blots, and ELIspots. Danilova (1994) outlines the three main criteria that should be followed for the screening for antibodies against small haptens. They are as follows:

- The hapten-protein conjugate used as the immobilised conjugate in the ELISA format for screening should use a different protein compared to the hapten-protein used as the immunogen.
- Different methods of chemistry should be used to link the hapten and protein in the conjugates used for immunisations and screening.
- If possible, a different reactive group should be used for the conjugation of the screening conjugate.

Delcros *et al.* (1995) investigated the reactivity of an anti-spermine monoclonal antibody towards three different polyamines either free or covalently bound through EDC or glutaraldehyde to a solid surface by using equilibrium dialysis and ELISA. They found that the affinity of the antibody for putrescine, spermidine and spermine depends on whether it is free or bound. The reactivity of the antibody differs according to the nature of the link to the solid phase. This should be considered when the screening method uses immobilised antigen in the ELISA format. If this approach is

being used, an inhibition ELISA should be performed to determine the reactivity of the antibody to the free antigen.

Chappey *et al.* (1992) discusses the issue of controlled monoclonal polyspecificity towards haptens with the same core chemical skeleton, for example metabolites, versus uncontrolled polyspecificity involving the cross reactivity of monoclonal antibodies with compounds different from the native hapten. They propose that the solution to this problem is to produce a large number of monoclonal antibodies and then subsequent selection of the antibody with the appropriate specificity. McMahon and O'Kennedy (2001), suggest that the degree of polyreactivity from *in vitro* immunisation-based hybridomas can be determined to a greater extent by using an ELIspot method whereby washed cells could be resuspended in PBS. The binding of the antibodies to the culture components such as proteins, lipids, sugars, would be eliminated and a more accurate determination of the polyreactivity determined.

#### **4.1.4 Cloning techniques**

Immediately after the first round of screening the cells should be examined and the positive clones scaled up from 96 well plates, containing 0.1 ml medium to 48 well plates, containing 0.2mls medium. The cells are subsequently scaled up to 6 well plates, containing 1.6mls medium, and supernatants should be screened, ideally with each scale up. The cells should be cloned as early as possible, to ensure that a given culture contains only one cell type (Hurrell, 1983). If the cloning is not carried out at an early stage there is the likelihood that cells not producing specific antibody will increase and overgrow the specific clone of interest.

The main methods used for cloning cells are reviewed by McCullough and Spier (1990) and are as follows:

- Cloning by limiting dilution
- Cloning by isolation of colony by microscope and micromanipulation
- Cloning in semi-solid medium
- Cloning using a fluorescence-activated cell sorter

The easiest technique to master is probably cloning by limiting dilution. This method is based on diluting the cells and growing them at very low densities, starting at 5 cells/well to 1 cell/well. The main problem associated with seeding at 1 cell/well is

that the hybridomas have a tendency to die at such a low seeding density. This is why it is advisable to reseed several plates at a range of different densities. After the screening process, the positive wells are examined and only the cells that look to be of a single colony are expanded and re-cloned. This procedure is repeated for at least three cloning cycles. The cycle involves screening at each stage of growth, including a competitive screen for the antigen of interest, subsequent scale up of positives and the cloning out process is repeated again.

Monoclonal antibody production from hybridomas is a specialised technique that takes time to master. Like any specialist, the carer of a hybridoma gets to know the characteristics of the hybridoma, including the times suitable for screening, the growth stage suitable for cloning, the appropriate colour of the media and how indicative it is of growth and the general appearance of a clone and its stability.

#### ***4.1.5 Scale up process for production***

The application of a monoclonal antibody determines the quantities of antibody that need to be produced. Small amounts of less than 0.1gram are needed for most research purposes. Diagnostic kit reagents usually require medium scale quantities from 0.1 to 1.0gram. Larger amounts, over 1gram are used for routine diagnostic and therapeutic procedures. The production of large amounts of the antibody can be achieved through two means: *in vivo*, by intraperitoneal injection of a mouse to create ascites or, by *in vitro* tissue culture. The *in vivo* method was very common and has many advantages. The main advantage being that it is a method familiar to many labs, is relatively easy, and high concentrations of antibody can be produced. However, the major disadvantage is the use of mice and the associated ethical dilemma and veterinary considerations. *In vitro* tissue culture is the method that is being encouraged and is the primary method to be attempted before a licence to produce ascites is approved by the regulatory bodies. The general thinking is that *in vitro* methods can often provide an adequate means of generating most of the monoclonal antibodies needed by research. It is the responsibility of the researcher to be able to justify using mouse ascites as a method. Due to ethical and scientific pressure, the Committee on Methods of Producing Monoclonal Antibodies, Institute for Laboratory Animal Research, National Research Council (USA) conducted a study on the production of monoclonal antibodies. They issued a very comprehensive report that details their findings. In their executive



summary they issued the following recommendations (Committee on Methods of Producing Monoclonal Antibodies, 1999):

**Recommendation 1:** There is a need for the scientific community to avoid or minimise pain and suffering by animals. Therefore, over the next several years, as tissue-culture systems are further developed, tissue culture methods for the production of monoclonal antibodies should be adopted as the routine method unless there is a clear reason why they cannot be used or why their use would represent an unreasonable barrier to obtaining the product at a cost consistent with the realities of funding of biomedical research programs in government, academia, and industry. This could be accomplished by establishing tissue culture production facilities in institutions.

**Recommendation 2:** The mouse ascites method of producing antibodies should not be banned, because there is and will continue to be scientific necessity for this method.

**Recommendation 3:** When the mouse ascites method for producing monoclonal antibody is used, every reasonable effort should be made to minimize pain or distress, including frequent observation, limiting the number of taps, and prompt euthanasia if signs of distress appear.

**Recommendation 4:** Monoclonal antibody now being commercially produced by the mouse ascites method should continue to be so produced, but industry should continue to move toward the use of tissue culture methods.

The following section will concentrate on the *in vitro* methods for monoclonal antibody production.

#### **4.1.5.1 Batch tissue culture method**

This is the simplest method for producing batches of antibody. The current range of media and specially formulated hybridoma additives, e.g. BRIClone, Bio Research Ireland, Dublin City University, support the growth of hybridomas without the need for feeder cells. Fetal calf serum can sometimes be blamed for contamination, to avoid this the hybridomas can be adapted to grow in 1% FCS or in FCS-free media, (Federspiel, 1991). Due to the large volumes of medium involved in batch production, spinner flasks and roller bottles are used to increase the concentration of dissolved oxygen in the media. This increases cell viability and growth and so leads to an increase in antibody production (Reuveny, 1986). Another addition to the market is the gas permeable bag, i-MAB (Diagnostic Chemical Ltd., Canada), this allows for greater exchange of gases. The normal procedure is then for the cells to be grown for approximately 10 days and then the supernatant concentrated and purified for use. The disadvantage of this is that the overall quantity of antibody produced is quite low.

#### **4.1.5.2 Semipermeable membrane-based systems**

Semipermeable membrane-based devices can allow cells to grow at high densities. The basis of the technique is the separation of the cells and monoclonal antibody produced from a larger compartment that contains the media. Supplements can be added to the media to enhance the growth of the cells. The waste products diffuse across into the larger volume to equilibrium. This method can produce antibody concentrations comparable to those produced by the *in vivo* mouse ascites method. Two commercial systems are the mini-PERM (Unisyn Technologies, MA, USA), and the CELLine (Integra Bioscience, MD, USA).

Hollow fibre bioreactors are a variation of the semipermeable membrane system, and consist of three parts, the hollow fibre cartridge, a gas permeable tubing through which the media is oxygenated and the medium reservoir. The hollow fibre unit is composed of a bundle of semi-permeable fibres that run through a chamber that contains the hybridoma cells grown at a high density. The molecular weight cut-off of the membrane allows the cells to grow to a high density by not allowing them through, while it does allow the movement of nutrients and waste products. The hollow fibre bioreactor can produce large amounts of antibody.

#### **4.1.6 Recombinant antibodies**

Advances in molecular biology in the last ten to twenty years have transformed antibody production. The principles behind chimeric and humanised antibodies are discussed below. One very important technique crucial to the production of recombinant antibodies is PCR, the polymerase chain reaction, (Chaudhary *et al.*, 1990). The generation of different antibody fragments can be achieved through recombinant antibody display technology. The generation of antibody libraries and developments in display technologies have synergistically driven this field. One major feature distinguishing hybridomas from recombinant antibody technology is that hybridomas are confined to non-human antibodies whereas conceptually antibody libraries allow the generation of antibody fragments from any species including humans whose immunoglobulin genes are identified. A huge degree of antibody diversity can be created through production of recombinant antibody fragments and then the specific

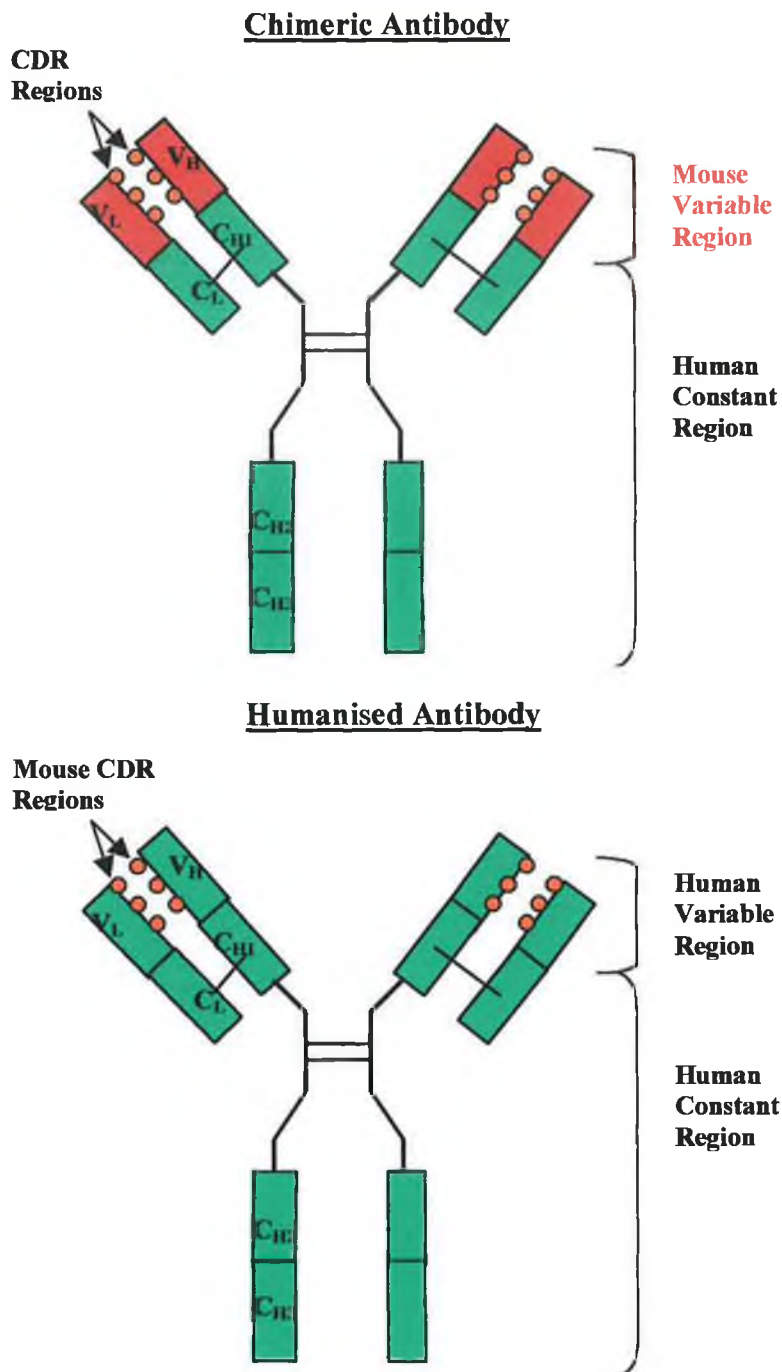
fragments of interest can be selected for by screening or panning. The variable chain genes, from naïve or immunised cell DNA are combined at random and cloned into a phage genome for fusion with a coat protein that is then subsequently expressed and displayed. (Hoogenboom *et al.*, 1998). The array of antibody fragments that can be created through this means is huge, and it allows for fusion of the fragments with other antibody fragments or other peptides or enzymes. The genomic DNA of the antibody fragments can be obtained from many sources as mentioned above including naïve or immunised cells (Hoogenboom, 1992), or mRNA can be extracted from hybridoma cells (Winter and Milstein, 1991; Krebber *et al.*, 1997).

#### **4.1.7 Chimeric and Humanized Antibodies**

The use of monoclonal antibodies has contributed to changes in the field of analytical science and diagnostics. However, in the field of therapeutics the use of monoclonal antibody has not provided the expected breakthroughs and we have only a limited number of antibody-based therapeutic agents. One major disadvantage of the hybridoma technology with respect to therapies is the inefficiency in immortalising human B cells. The inherent problem of using rodent antibodies as part of a treatment is that the antibodies will be detected by the host immune system. To overcome this, it is necessary to reduce the immunogenicity of the therapeutic antibodies. The many advances in engineering of antibodies and their fragments have led to major advancements in this field and, as a result we are closer to Paul Erlich's 'magic bullets'. However, while reduction of the immunogenicity of the antibody or antibody fragment can be achieved through genetic engineering, other key aspects of the antibody can be jeopardised, particularly in the case of humanised antibodies where the mouse complementarity determining regions (CDRs) are grafted into the human variable regions. These problem areas include antigen binding and recruitment of human effector cells.

Initially, the basis of antibody treatment for cancer was dependent on the antibodies' ability to elicit the patients' defence mechanisms to kill tumour cells. Successful techniques have been developed to reduce the antigenicity of murine antibodies for human therapeutic use. The chimeric antibody is created through the cloning of the heavy and light mRNAs of the murine hybrid myeloma line, and the fusion of the DNA of the mouse VH and VL domains to the human constant domains, C<sub>H1</sub>, hinge, C<sub>H2</sub>, and

C<sub>H3</sub> (Morrison *et al.*, 1984) This technology has progressed to producing humanised antibodies, where all of the antibody is of human origin with the exception of the CDR regions which are derived from a mouse as shown in Figure 4.3, (Jones *et al.*, 1986; Riechmann *et al.*, 1988; Sheets *et al.*, 1998). The theory is that the immunogenicity of such an antibody would be weakened as the number of epitopes recognised as foreign is decreased as compared to the traditional murine monoclonal antibodies. The specific antigen binding ability of the antibody can be approached through novel molecular biology tools such as molecular modeling, and cloning and sequencing of regions, (Nagahira *et al.*, 1999; Saldanha *et al.*, 1999). Developments in the field of humanised antibodies have led to antibodies that do exhibit potent anti-tumour cell activity against the target cells by antibody-dependent cell-mediated cytotoxicity (Ono *et al.*, 1999). Another exciting prospect in the field of development of human antibodies is the transgenic mouse. Cell Genesys Inc, California, USA (Green *et al.*, 1994; Jakobovits *et al.*, 1995), developed a strategy for producing human monoclonal antibodies in mice by the introduction of large segments of the human heavy and light chain immunoglobulin genes on yeast artificial chromosomes into the mouse germline. High levels of human antibodies are produced by these transgenic mice. This provides another avenue for production of human monoclonal antibodies and also provides a model for looking at the human antibody response. The possibilities of this technology makes one wonder if the transgenic mouse could be renamed the 'Mighty Mouse'.



**Figure 4.3:** The structure of the chimeric and humanised antibodies. The chimeric antibody is composed of the mouse variable region and the human constant region. The humanised antibody is composed of a human antibody, with the specific mouse CDR regions grafted into the variable region.

#### **4.1.8 Clinical applications of antibodies**

Antibodies play a key role in the clinical analysis of many biological constituents. The sensitivity and specificity of antibodies have been exploited to a large degree over the last decade for the development of diagnostic tests. These rapid, non-invasive antibody-based tests have dramatically improved screening and diagnosis of a wide range of clinical conditions from detection of drugs of abuse to cancer. There is an ever expanding range of approved kits available now that detect minute amounts of hormones, drugs, and specific disease markers. The approval of many of these tests for use at home, at the bedside, in the local physician's clinic and in the laboratory has accelerated the screening and monitoring of medical conditions (Fitzpatrick *et al.*, 2000).

##### **4.1.8.1 Detection of drugs of abuse**

The detection of drugs of abuse can range in complexity from immunoassays to gas chromatography/mass spectroscopy (GC/MS) (Braithwaite *et al.*, 1995). Quicker less invasive methods are being developed for use in screening for drugs of abuse in the work place, and rehabilitation clinics. The development of rapid immunoassays for the detection of illicit drug use that would be suitable for roadside testing would mean that screening could be performed in the same manner as current alcohol testing. The confirmatory test for these assays would be the existing gold standard, GC/MS. Currently, the most popular biological matrices for quantitative measurement of illicit drugs are plasma and urine. However, saliva is now becoming common because of the obvious advantage of the non-invasive nature of collection and the correlation between psychological impairment and the level of detection of the illicit drugs and metabolites. There are many different immunological test formats available now, as outlined in Chapter 1. In the development of the rapid roadside tests, the following considerations should be taken into account. The test must be specific and sensitive, with positive results correlating to the legal cut off level as determined by authorities such as the National Institute of Drugs of Abuse (NIDA). The recognition of metabolites of the

drugs of interest, and closely related designer drugs, such as the amphetamine derivatives, MDMA, or MDEA by the test antibodies should also be taken into account.

#### ***4.1.8.2 Detection of cancer***

The presence of specific protein markers on cancer cells is exploited for the detection of cancer using antibodies specifically directed to these markers. This is commonly used for detection and for the management of cancer patients. In order to detect cancers as early as possible, researchers are focusing on molecular methods including protein products of oncogenes and tumour suppressor genes as targets of detection in immunoassays. An example of one such test is the BTA test™, manufactured by Bion Diagnostic Sciences Inc. It allows recovering bladder cancer patients to monitor their risk for a recurrence of cancer. The BTA stat test™ is a lateral flow immunoassay that detects tumour antigens in urine. The solid phase monoclonal antibody reacts with human complement factor H-related protein (hCFHrp), which is secreted in urine by bladder cancer cells (Sarosdy *et al.*, 1997; Kinders *et al.*, 1998). The test is used as an adjunct to cystoscopy and based on the outcome of the test, the physician can decide the next investigative step.

The coupling of antibodies to radioactive isotopes can serve as contrast agents in diagnostic imaging products and are also used in the development of radioimmunotherapy. Firstly, for the purposes of detection, the antibodies that are specific to the disease marker are labelled with the radioactive isotope and travel to the disease site. This is then detected using sophisticated nuclear medical equipment. Antibody tracers are currently available for diagnosis of colorectal, lung, prostate and ovarian cancer. One such example is the Proscint™ test (Cytogen Corporation) used to determine if prostate cancer has remained local within the prostate or if it has spread to lymph nodes in the body (Proscint Package Insert). An example of radioimmunotherapy is the monoclonal antibody directed toward prostate-specific membrane antigen (PSMA). It is attached to a radioactive tracer, indium 111, and is injected into the patient. Expression of the antigen is higher in prostate adenocarcinoma cells than in non-malignant tissue and higher in metastatic lesions than in tumours (Sodee *et al.*, 1996). Lymph nodes that have been invaded by the prostate cancer cells appear as hot spots on the imaging detection system.

Goldenburg (2001) has reviewed the role of radiolabeled antibodies in the treatment of non-Hodgkin's lymphoma (NHL). Four radiolabeled antibodies are in clinical trials currently for NHL. These are being tested in combination with chemotherapy or after chemotherapy and at least two of these products show very promising results.

The collaborative efforts of biotechnology companies have resulted in antibody based systems for the analysis and treatment of disease. One example of this is the HercepTest™ (DAKO) and Herceptin® (Genentech) treatment used in women diagnosed with breast cancer associated with the overexpression of the HER2 protein (Pauletti *et al.*, 1996). The HercepTest™ is an immunohistochemical test used on biopsy samples from breast cancer patients. The Herceptin® treatment will be discussed in more detail below.

#### ***4.1.8.3 Antibodies as therapeutic agents***

Antibodies have been developed as therapeutic agents in recent years and there are now approved antibody treatments for cancers, auto-immune diseases and graft rejection. The inherent problems of using antibodies have been discussed previously, along with the relevant developments in recombinant humanised antibodies. Initially, the basis of antibody treatment for cancer was dependent on the antibodies' ability to elicit the patients' defence mechanisms to kill tumour cells. Immunotoxins provide a new method for killing tumour cells. The immunotoxins contain an antibody or antibody fragment conjugated to toxins, produced by bacteria or plants. This has resulted in a vast array of possible immunotoxins. The antibodies bind to the specific cell surface receptor that is targeted on the cancer cell, the molecule is then internalised by the cell and the toxin part of the conjugate exerts its cytotoxic effects. The immunotoxins currently under investigation are reviewed by Kreitman and Pastan (1998) and Trail and Bianchi (1999).



**Table 4.1:** Current Licenced Antibody Therapies in USA and Europe. Monoclonal antibodies account for over a quarter of the therapies currently being developed by biotechnology companies. Adapted from Breedveld (2000) and Fitzpatrick *et al.*, (2000).

Generic Name	Trade Name	Company	Indication
Moromonab	Orthoclone OKT3	Ortho Biotech	Graft rejection
Basiliximab	Simulect	Novartis	Prevention of renal graft rejection
Daclizumab	Zenapax	Roche	Prevention of renal graft rejection
Infliximab	Remicade	Centocor	Rheumatoid arthritis, Crohn's disease
Rituzimab	Rituxan	Genentech-Roche	Low grade and follicular non-Hodgkin lymphoma
Trastuzuamb	Herceptin	Genentech	Metastatic breast cancer
Abciximab	Reopro	Lilly	Prevention of cardiac ischemic complications
Palivizumab	Synagis	Abbott Laboratories	Respiratory syncytial virus

#### 4.1.8.4 Approved antibody therapies

The recent advances made in the field of antibody production and molecular biology in general, open up a world of possibilities, however the process, time and expenditure required to bring an idea from the lab bench to the pharmacy shelf is enormous. Table 4.1 shows the currently approved antibody therapies. Hopefully, we will see the rate of

therapies available increase faster in the future as currently there are many antibody based therapies in clinical trials. The anti-beta amyloid human antibody treatment, AN-1792, for Alzheimers disease being developed by Elan in association with American Home Products and Cambridge Antibody Technology (Thatte, 2001), is one classic example of how a dream 'magic bullet' can make it so far but then meet with unexpected results and its development currently suspended. At the time of suspension, 360 patients suffering from Alzheimers Disease had received AN-1792. The dosage was stopped when four patients in France developed clinical signs of inflammation of the central nervous system. The other side of this coin is the extreme success of the some of the approved treatments. Herceptin is an example of a humanised antibody. Abciximab, remicade and rituxan are examples of chimeric antibodies, while OKT3 is an example of a murine antibody. These are reviewed by Fitzpatrick et al, (2000), as an example Herceptin is described in the following paragraph.

Herceptin is a recombinant DNA-derived humanized monoclonal antibody that binds with high affinity to the extracellular domain of the human epidermal growth factor receptor 2 protein, HER2. The antibody is an IgG1 kappa that contains the human framework regions with the complementarity-determining regions of a murine antibody. By binding to the HER2 receptor it mediates the antibody-dependent cellular cytotoxicity (Herceptin Package Insert, 1998). The clinical trials with herceptin have resulted in the approval of the medication with an indication for the treatment of patients with metastatic breast cancer whose tumours overexpress the HER2 protein and who have received one or more chemotherapy regimens for their metastatic disease. Herceptin is also indicated in combination with paclitaxel for treatment of patients with metastatic breast cancer whose tumours overexpress the HER2 protein and who have not received chemotherapy for their disease.

#### ***4.1.9 Current advances – pharmacogenomics, proteomics***

The application of pharmacogenomics and proteomics to the field of drug discovery means that specific therapies can be developed for specific patient genotype populations within a patient population. Clinical pharmacogenomics is the use of genetic information from a patient population to predict the efficacy and safety of a therapy. Disease populations are not homegenous for a particular disease. An example of this is rheumatoid arthritis (RA). A monoclonal antibody therapy is approved for RA,

infliximab, however not all RA patients benefit from it. The concept behind pharmacogenomics is that candidate disease genes and polymorphisms can be identified and correlated to therapy and clinical outcome. An extension of this is the development of molecular genetic tests for detection of these sub-populations and the use of this information in deciding on the appropriate treatment strategy. It is becoming a common practice in drug development to explore pharmacogenetics and analyse clinical trial patient mRNA by microarray analysis. The powerful tools available such as micro array analysis, monoclonal antibody production, phage display antibody libraries, bioinformatic technologies and rapid biosensors, all contribute greatly to drug discovery research.

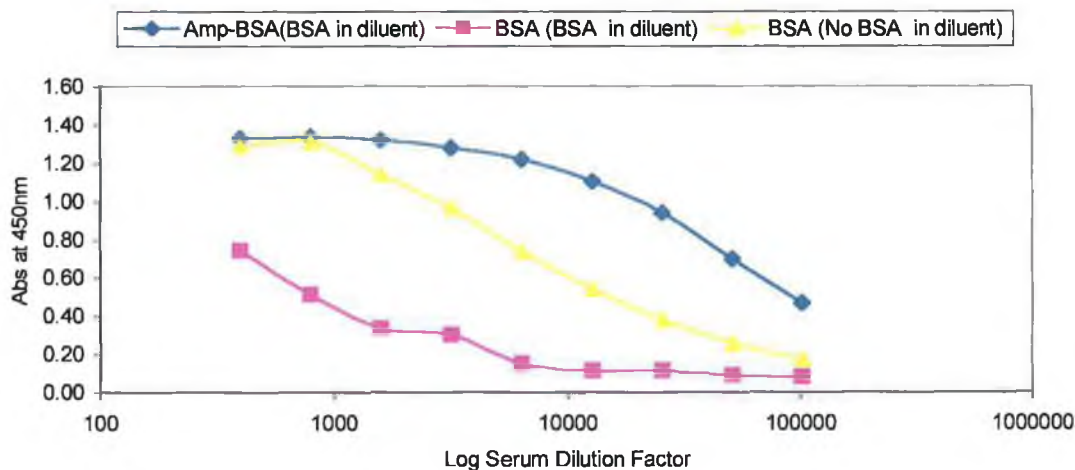
The following results detail the production, characterisation and application in ELISA of two different antibodies, i.e., the anti-amphetamine and the anti-methamphetamine monoclonal antibodies.

## ***4.2 Results***

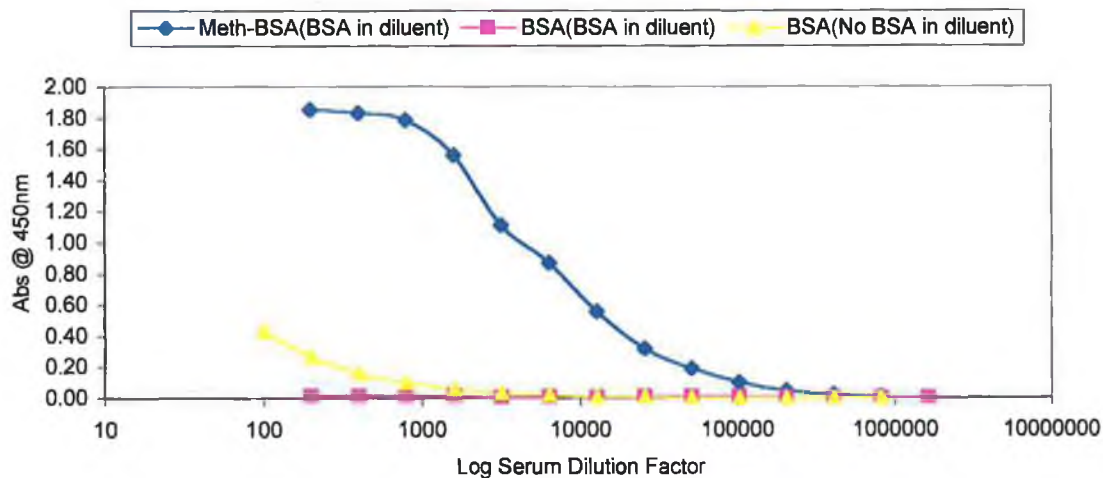
### ***4.2.1 Antibody Titre of mice used for monoclonal antibody production***

Two groups of mice were immunised with amphetamine-BSA (Group M1) and methamphetamine-BSA (Group M2) for the production of monoclonal antibodies against amphetamine and its derivatives. Mice were immunised using the schedule described in Section 2.6.1. Tail bleeds were performed on the mice 7 days post immunisation boost. The blood was collected and the serum separated and a conventional ELISA was performed as described in Section 2.6.4 and 2.9.2 to determine the titre of antibodies raised against the conjugate. The same conjugate was used for both immunisations and screening so an additional ELISA was performed using the BSA as the coating protein. Serum from pre-immunised mice was used as a control in an initial ELISA. Figure 4.4 shows the titre obtained for the amphetamine-BSA immunised mice (M1) and for the methamphetamine-BSA mice (M2) after ten months of immunisations.

### Titre of M1 Serum (Immunogen amphetamine-BSA)



### Titre of M2 Serum (Immunogen methamphetamine-BSA)



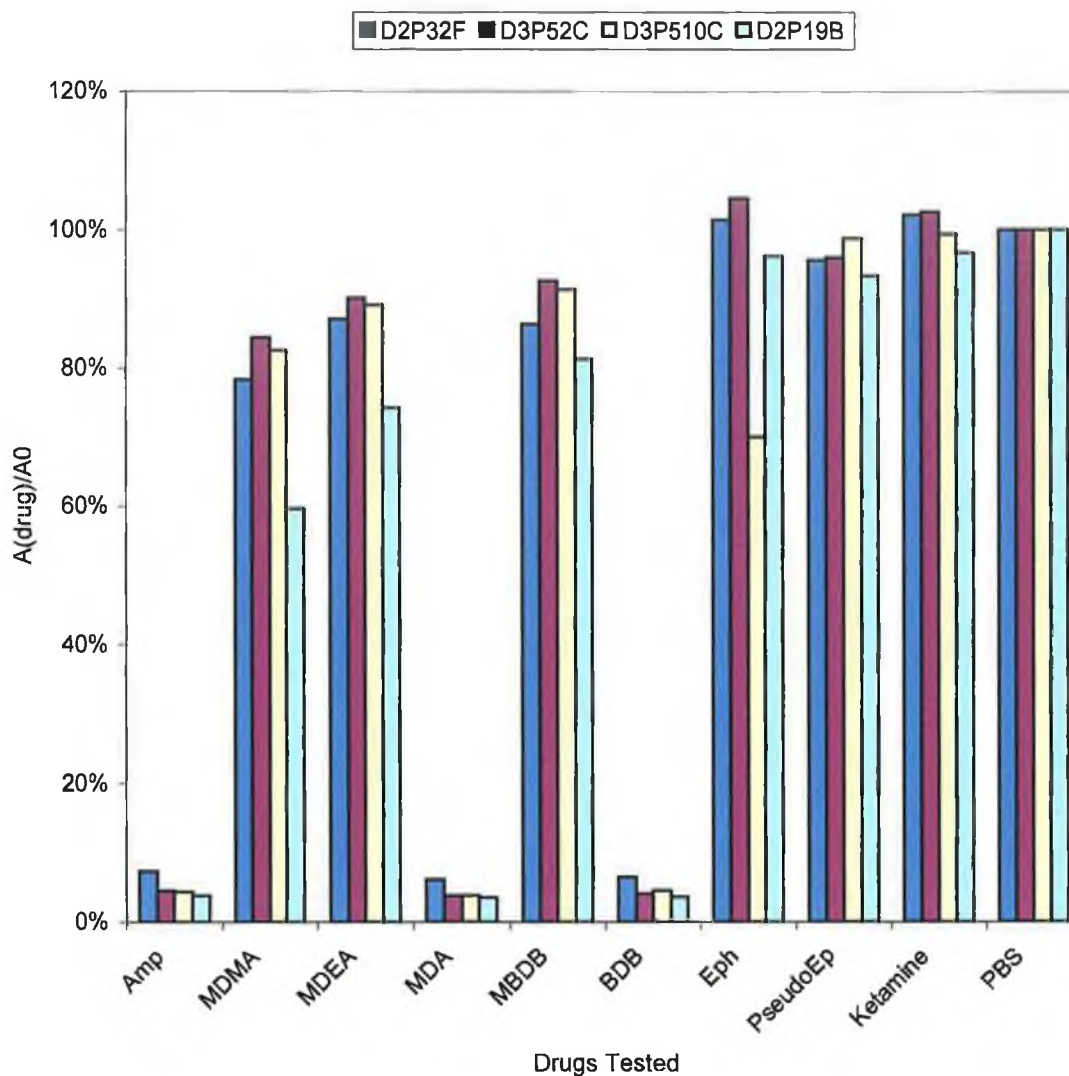
**Figure 4.4:** Final titre of serum from mice used for hybridoma production, M1 immunised with amphetamine-BSA (upper), and M2 (lower) immunised with methamphetamine-BSA. For M1, the coating conjugate used in the ELISA was amphetamine-BSA. The serum was also titred against BSA, with and without BSA in the diluent. For M2, the coating conjugate was methamphetamine-BSA. The serum was also titred against BSA, with and without BSA in the diluent. A sufficient titre was obtained for both mice and the spleens were extracted and used for the hybridoma production.

#### ***4.2.2. Screening of hybridoma supernatants from Group M1 fusion***

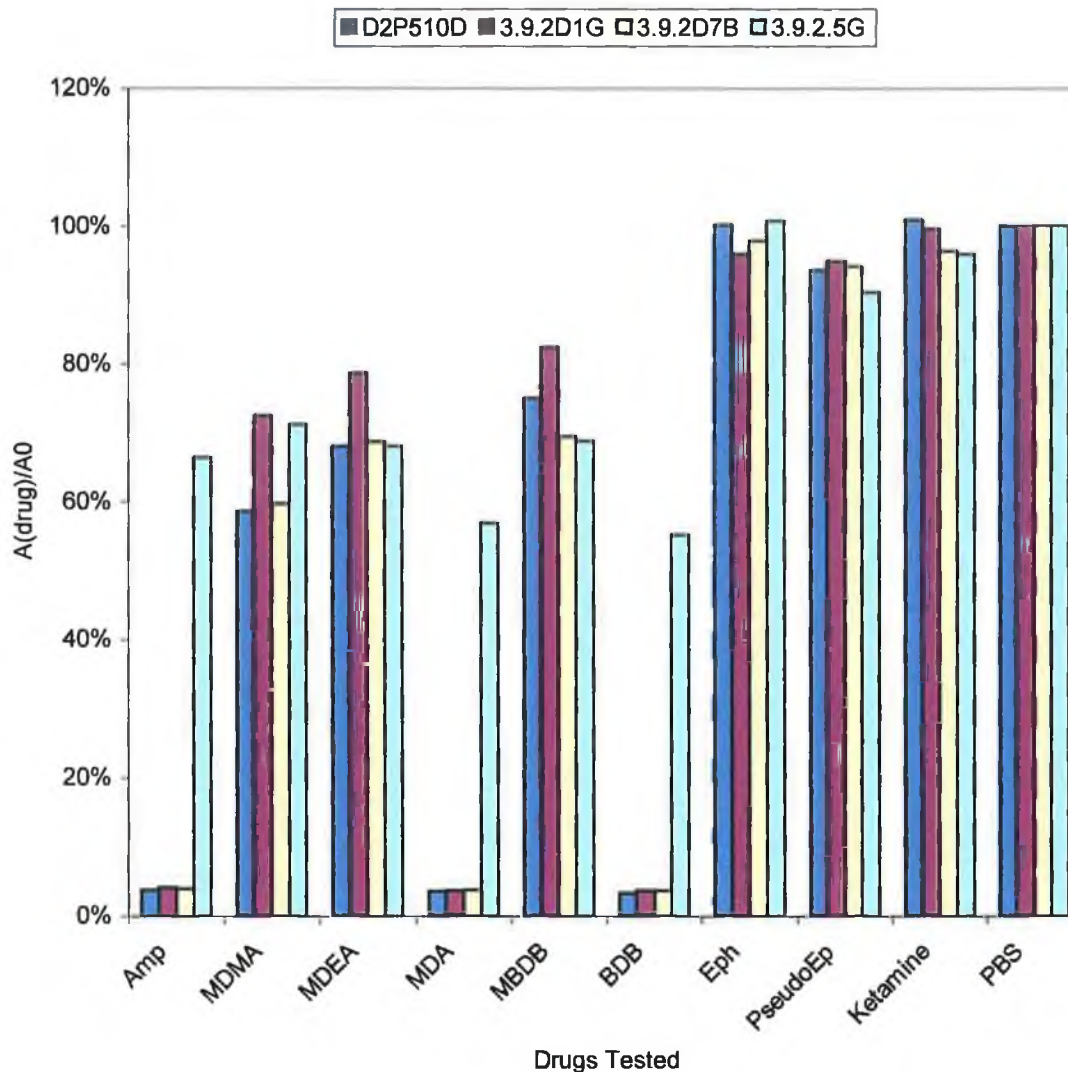
Once a sufficient titre had been obtained, approximately 8 months after the initial immunisation, the final immunisation boost was administered to the mice. Five days later, a final tail bleed was taken and the final titre determined. The mouse was sacrificed and the spleen removed. The spleen cells were used for the hybridoma production as discussed in Section 2.7.3. Eight days after the fusion, supernatants from the wells of the hybridoma cells were screened for reactivity towards amphetamine using conventional ELISA format by immobilising amphetamine-BSA onto the well surfaces. Supernatants were also screened against BSA to confirm the reactivity towards the amphetamine molecule. Positive wells were taken to 48 well plates with 0.2ml medium. Subsequent positives were scaled up to 24 well, 12 well, 6 well plates and to T-25 and T-75 flasks. As the volume of supernatants available increased, these supernatants were also screened using a competitive ELISA format with competition between the free drug (amphetamine and amphetamine derivatives at a concentration of 50µg/ml) and immobilised amphetamine-BSA conjugate. Cells were then cloned by limiting dilution. After the third step of cloning at 1-2 cells/well the cells were statistically monoclonal.

The concept behind the screening process for the anti-amphetamine antibody was to select a clone that would be specific for amphetamine but also recognise the amphetamine derivatives. Given the structures of the derivatives it was anticipated that the antibody would recognise the amphetamine molecule and derivatives to different degrees. In summary the results show that clones 10FP12F, 10FP13F, 4EP13C, 4EP18E, and 4EP18F secreted monoclonal antibodies that recognised amphetamine and BDB primarily (approx 90% displacement), but also recognise MDMA, MDEA, MDA, and MBDB (approx 50% displacement). These clones showed little reactivity towards ephedrine, pseudoephedrine or ketamine. Clones D2P510D, D2P32F, D2P32F, D3P52C, D3P510C, 392D1G, 392D7B, 3925G showed similar reactivity towards amphetamines but did not show strong reactivity towards the amphetamine derivatives. Clone 4EP18E was subsequently scaled up and the monoclonal antibody purified and characterised.

The following graphs (Figure 4.5 – 4.9) represent the results obtained using the competitive ELISA format for the range of different supernatants from the monoclonal cell lines.

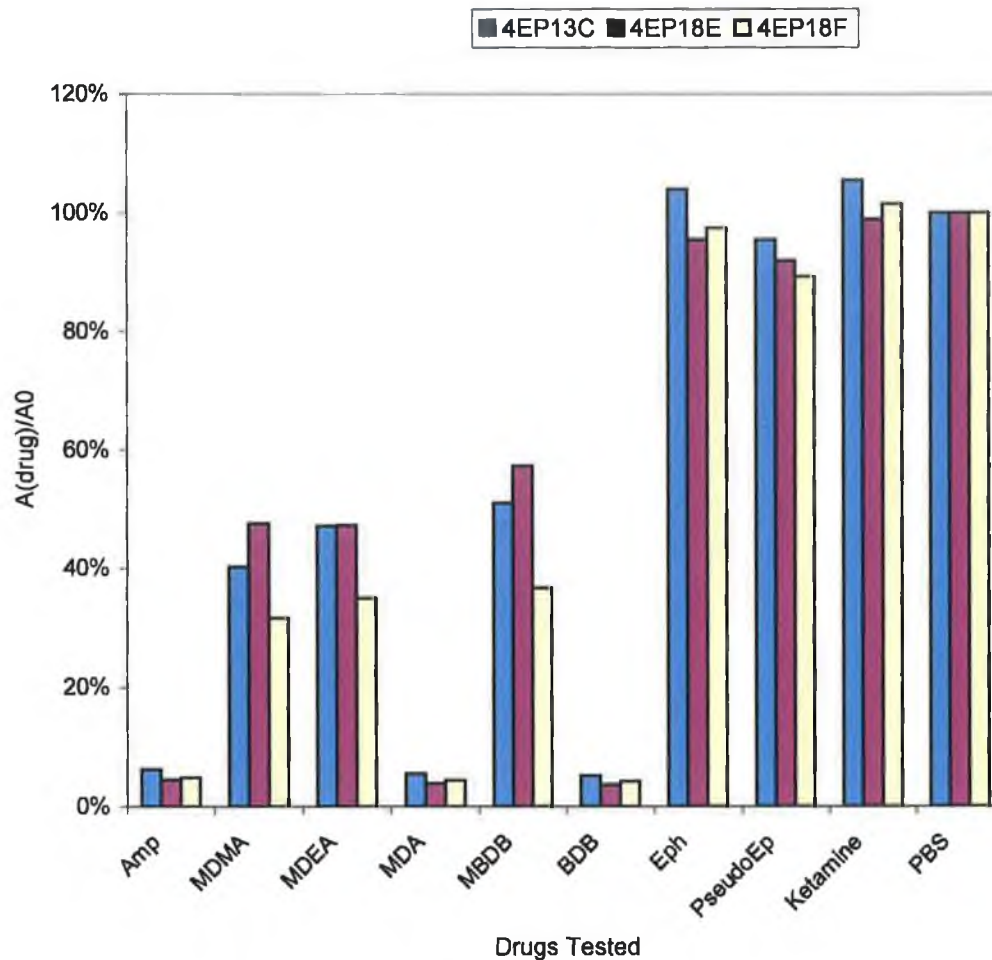


**Figure 4.5:** Reactivity of the different clones of monoclonal antibody supernatant (D2P32F, D3P52C, D3P510C, D2P19B) in the presence of amphetamine, MDMA, MDEA, MDA, MBDB, BDB, ephedrine, pseudoephedrine, ketamine and PBS (control). The reactivity of the antibody to immobilised amphetamine-BSA in the presence of the free drugs, A(drug), is expressed as a percentage of the reactivity of the antibody in the presence of PBS alone, A0.

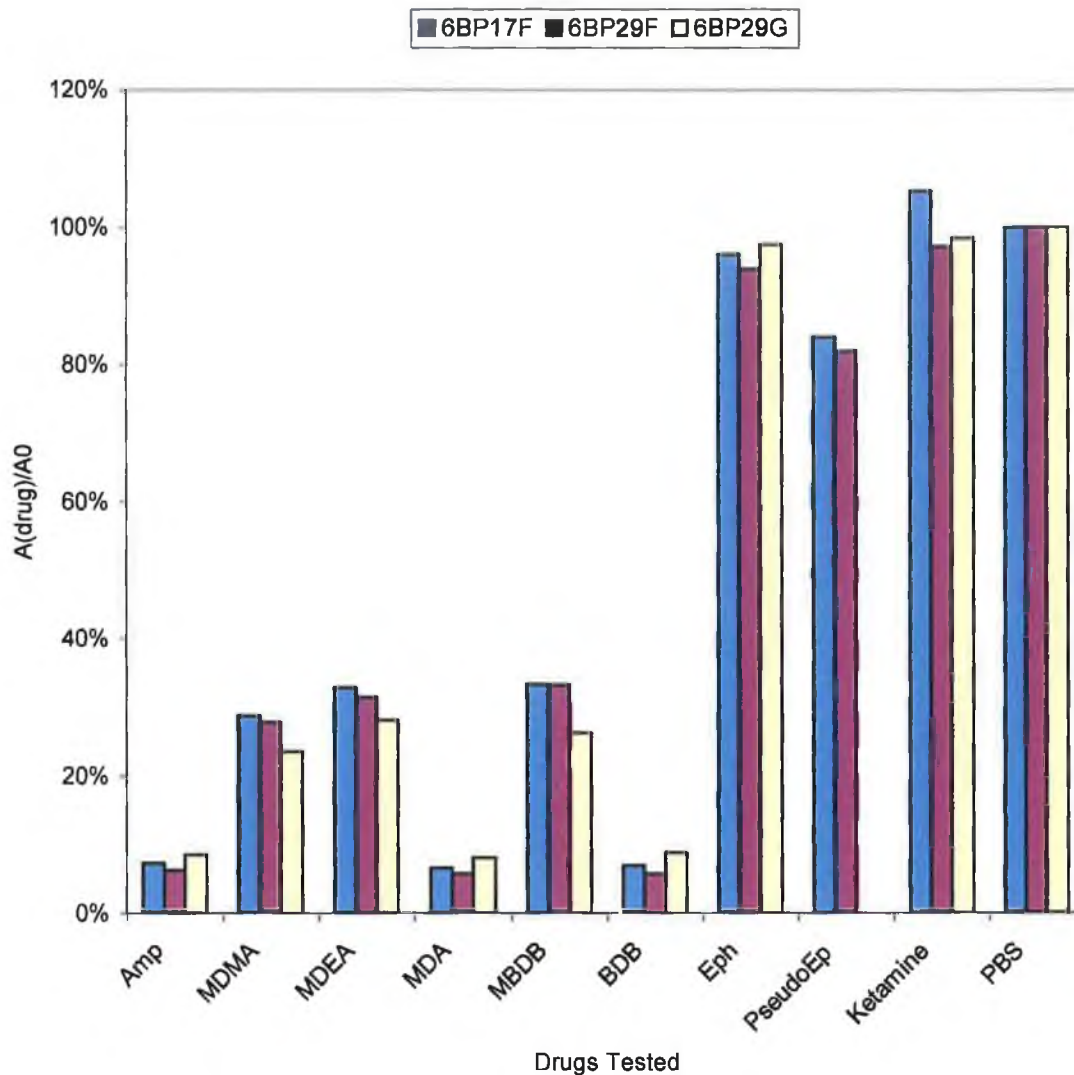


**Figure 4.6:** Reactivity of the different clones of monoclonal antibody supernatant (D2510D, 3.9.2D1G, 3.9D7B, 3.9.2.5G) in the presence of amphetamine, MDMA, MDEA, MDA, MBDB, BDB, ephedrine, pseudoephedrine, ketamine and PBS (control). The reactivity of the antibody to immobilised amphetamine-BSA in the presence of the free drugs, A(drug), is expressed as a percentage of the reactivity of the antibody in the presence of PBS alone, A0.

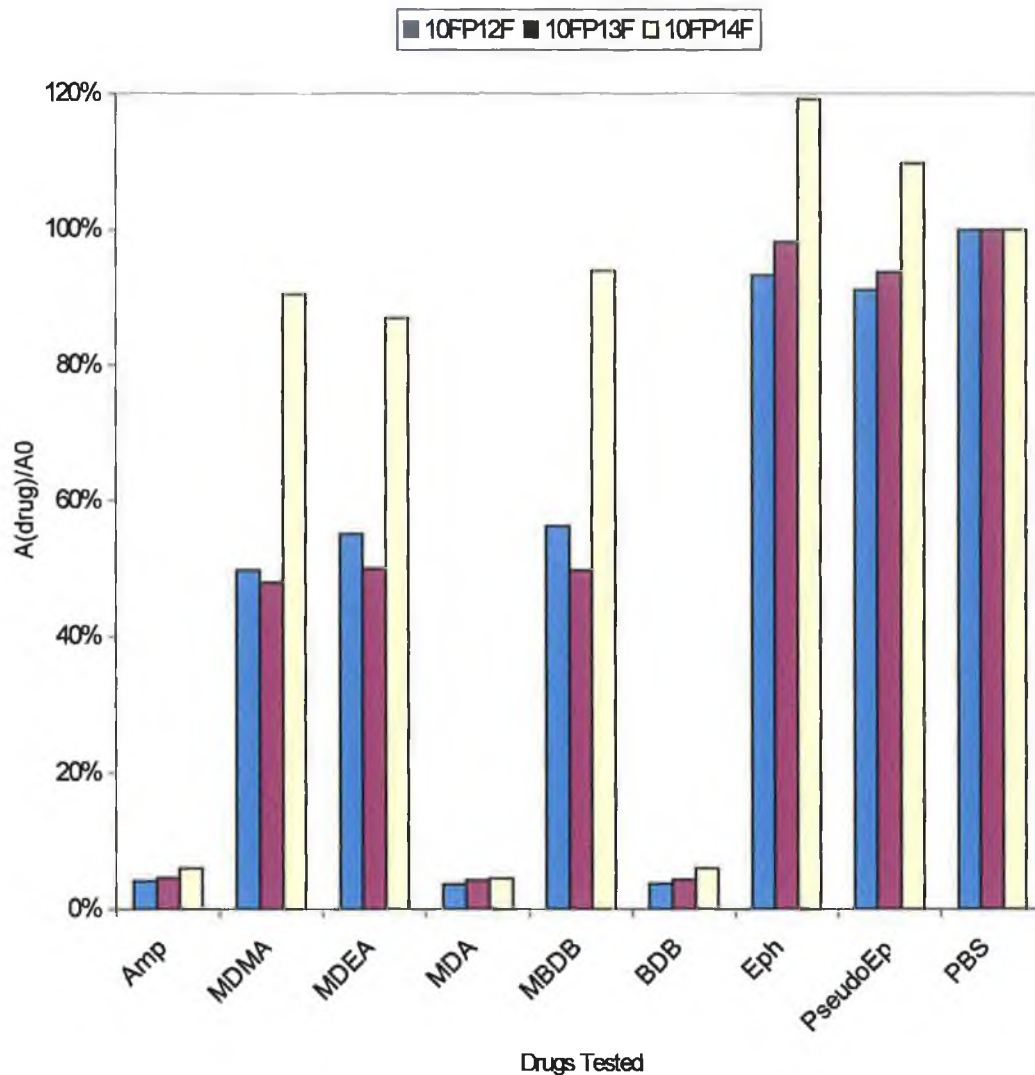




**Figure 4.7:** Reactivity of the different clones of monoclonal antibody supernatant (4EP13C, 4EP18E, 4EP18F) in the presence of amphetamine, MDMA, MDEA, MDA, MBDB, BDB, ephedrine, pseudoephedrine, ketamine and PBS (control). The reactivity of the antibody to immobilised amphetamine-BSA in the presence of the free drugs,  $A(\text{drug})$ , is expressed as a percentage of the reactivity of the antibody in the presence of PBS alone,  $A_0$ .



**Figure 4.8:** Reactivity of the different clones of monoclonal antibody supernatant (6BP17F, 6BP29F, 6BP29G) in the presence of amphetamine, MDMA, MDEA, MDA, MBDB, BDB, ephedrine, pseudoephedrine, ketamine and PBS (control). The reactivity of the antibody to immobilised amphetamine-BSA in the presence of the free drugs,  $A(\text{drug})/A_0$ , is expressed as a percentage of the reactivity of the antibody in the presence of PBS alone,  $A_0$ . (The test against pseudoephedrine was not done for Clone 6BP29G.)



**Figure 4.9:** Reactivity of the different clones of monoclonal antibody supernatant (10FP12F, 10FP13F, 10FP14F) in the presence of amphetamine, MDMA, MDEA, MDA, MBDB, BDB, ephedrine, pseudoephedrine, ketamine and PBS (control). The reactivity of the antibody to immobilised amphetamine-BSA in the presence of the free drugs, A(drug), is expressed as a percentage of the reactivity of the antibody in the presence of PBS alone, A0.

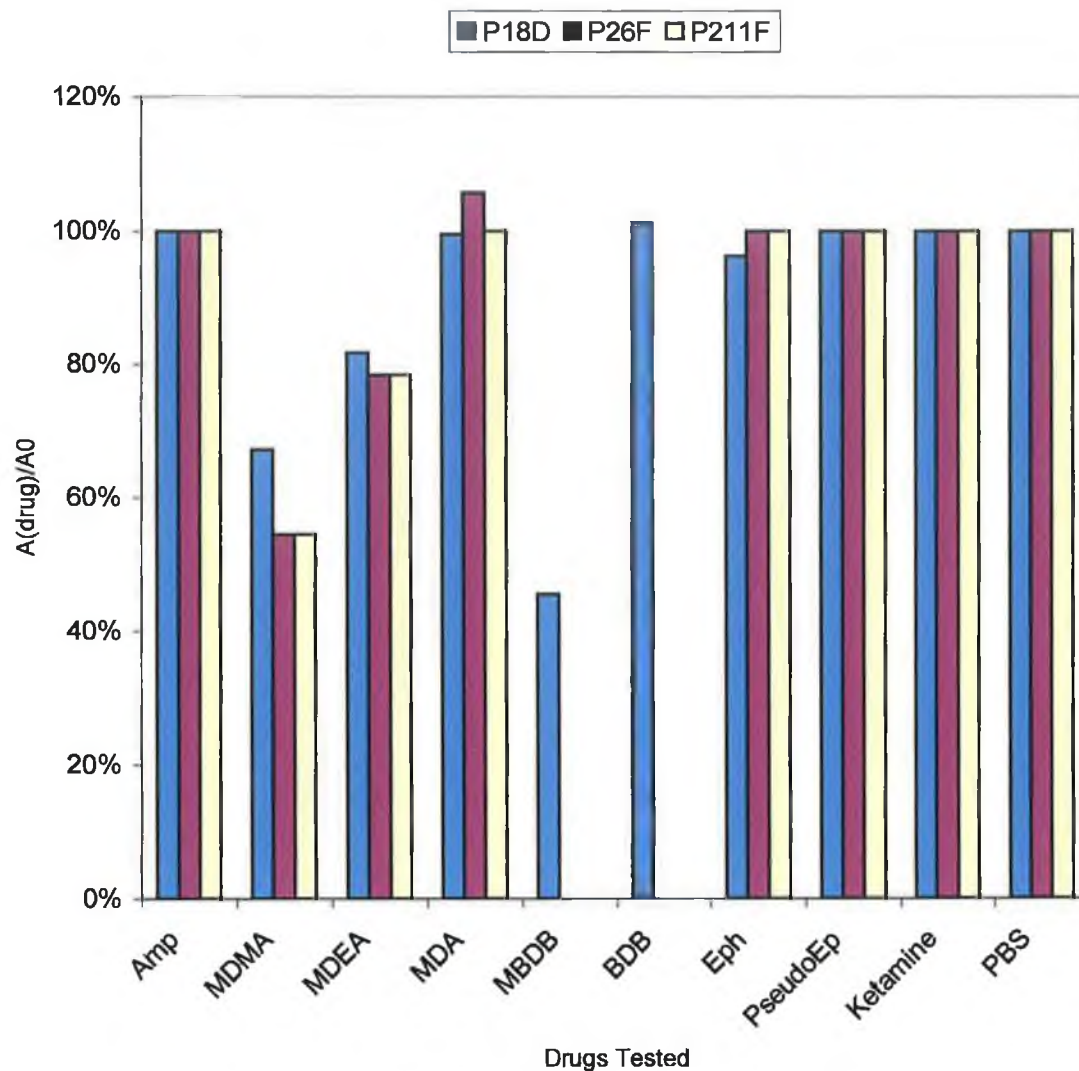
### *4.2.3 Screening of hybridoma supernatants from Group M2 fusion*

Supernatants from the wells of the hybridoma cells were screened for reactivity towards methamphetamine using conventional ELISA format by immobilising methamphetamine-BSA in the same format as the anti-amphetamine screening. Supernatants were also screened against BSA to confirm the reactivity towards the methamphetamine molecule. Positive wells were scaled up as described for the anti-amphetamine monoclonal production in Section 4.2.2. As the volume of supernatants available increased, the supernatants were also screened using a competitive ELISA format with competition between the free drug (methamphetamine and amphetamine derivatives at a concentration of 50µg/ml and 12.5µg/ml) and immobilised methamphetamine-BSA conjugate. This was to confirm that the clones were secreting antibodies that recognised free drug. Cells were then cloned by limiting dilution. By the third step of growing at 1-2 cells/well the cells were statistically monoclonal.

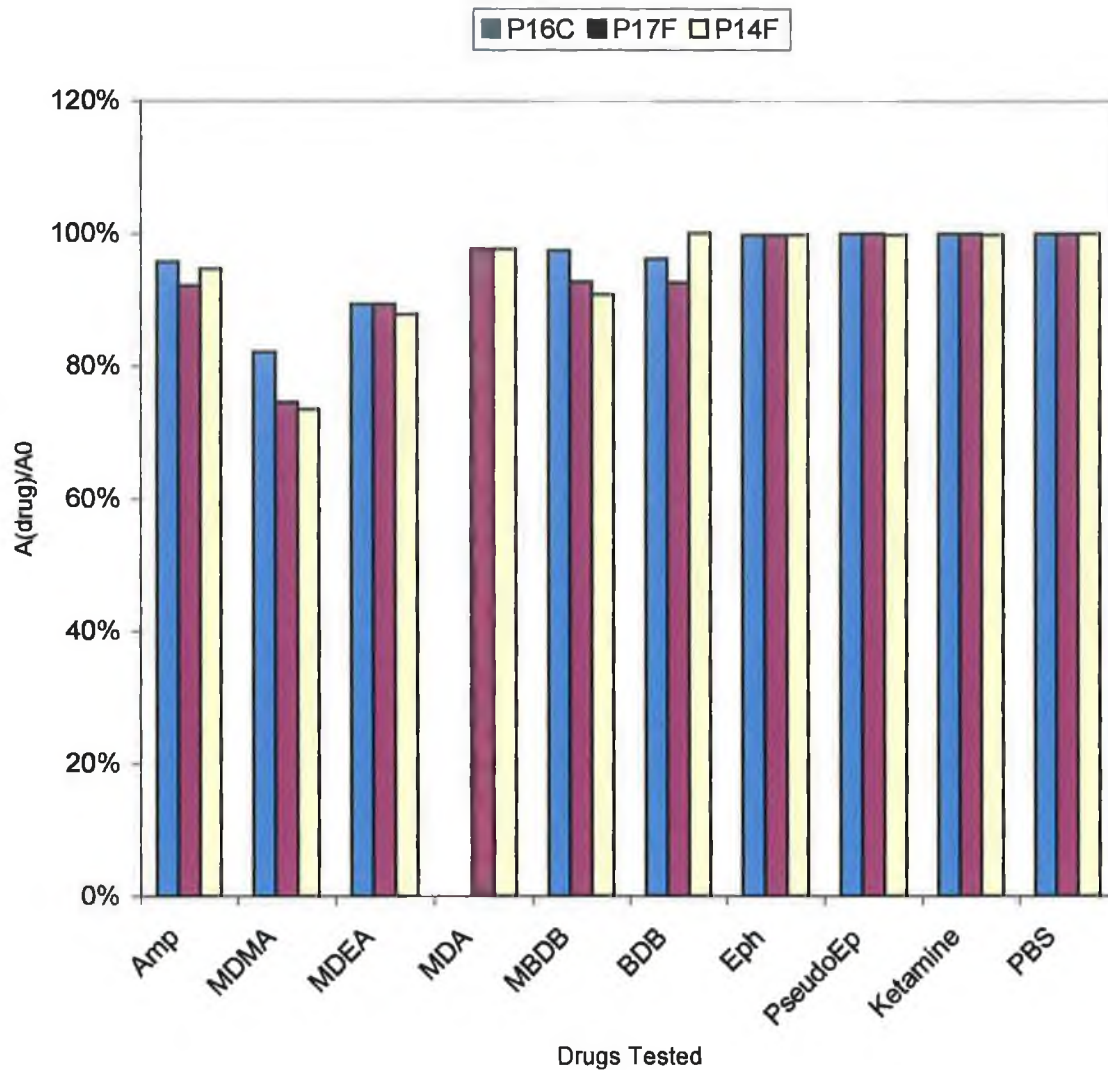
Graphs shown as Figure 4.10 – 4.12 represent the results obtained using the competitive ELISA format for the range of different supernatants from the monoclonal cell lines.

As discussed previously, the purpose behind the production of the anti-amphetamine and anti-methamphetamine antibody was to produce an antibody that would recognise the whole range of amphetamine and methamphetamine derivatives. Methamphetamine is a metabolite of many of the derivatives so it would be more likely that this antibody would recognise these designer amphetamine derivatives. In summary the results show that clones P18D, P26F and P211F secrete monoclonal antibodies that recognise MDMA, MDEA, MBDB. They were tested against methamphetamine later when the analytical standard was replenished. These clones showed no reactivity towards ephedrine, pseudoephedrine or ketamine. The Clones P15E and P15G which originated from a different clone were shown not to be as reactive against the derivatives and they were not characterised further. The same results were seen with P16C, P17F, and P14F, these were not as reactive as the P18D, P26F or P211F.

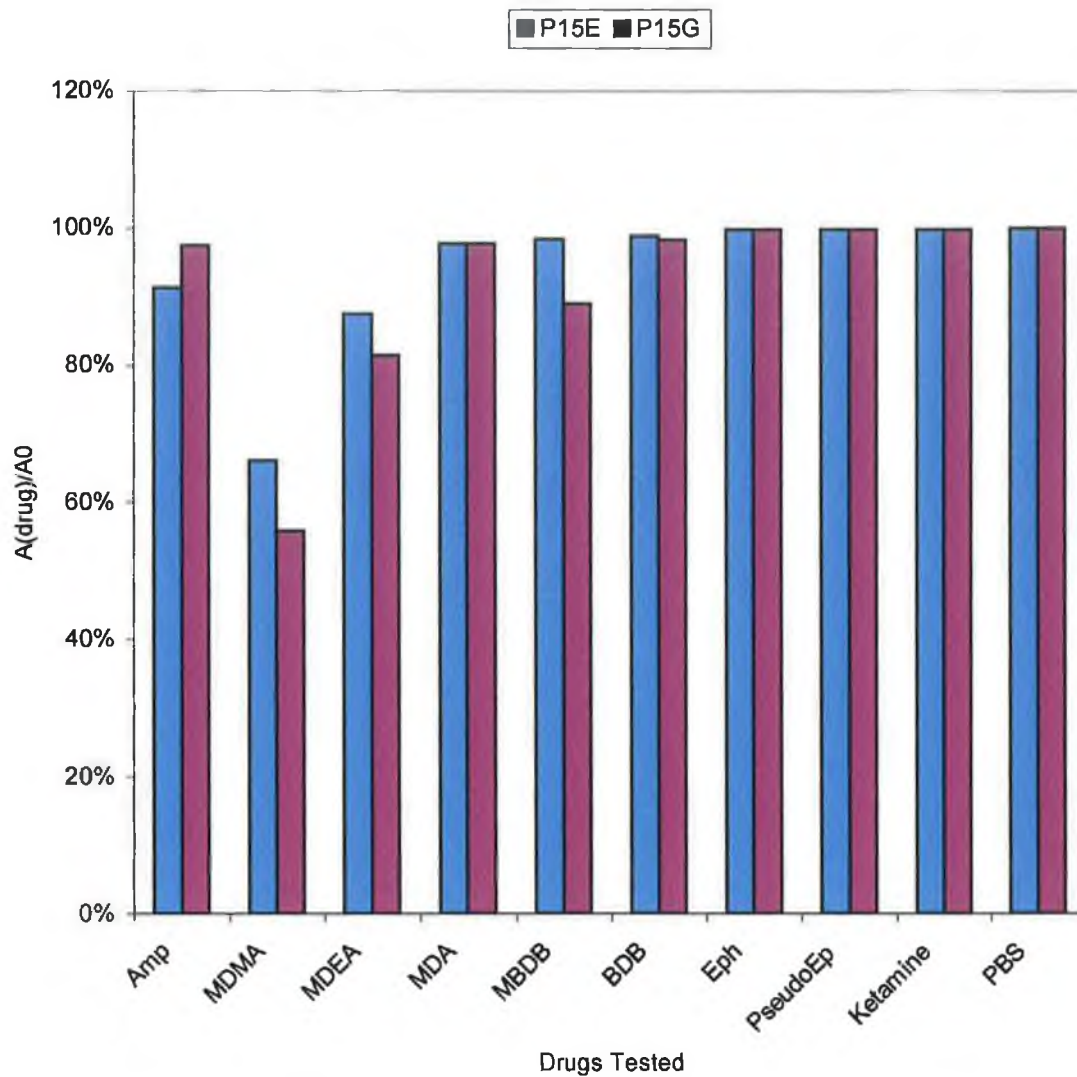
Clone P18D was subsequently scaled up as it was a robust cell line and showed good cross reactivity. The monoclonal antibody purified and characterised as described below. Stocks of the reactive clones were scaled up to 75cm<sup>3</sup> (T75) flasks.



**Figure 4.10:** Reactivity of the different clones of monoclonal antibody supernatant (P18D, P26F, and P211F) in the presence of amphetamine, MDMA, MDEA, MDA, MBDB, BDB, ephedrine, pseudoephedrine, ketamine and PBS (control). The reactivity of the antibody to immobilised methamphetamine-BSA in the presence of the free drugs (12500ng/ml MDMA, and 50000ng/ml for other drugs), A(drug), is expressed as a percentage of the reactivity of the antibody in the presence of PBS alone, A0. (The test against MBDB and BDB for P26F and P211F was not performed.)



**Figure 4.11:** Reactivity of the different clones of monoclonal antibody supernatant (P16C, P17F, and P14F) in the presence of amphetamine, MDMA, MDEA, MDA, MBDB, BDB, ephedrine, pseudoephedrine, ketamine and PBS (control). The reactivity of the antibody to immobilised methamphetamine-BSA in the presence of the free drugs (12500ng/ml MDMA, and 50000ng/ml for other drugs), A(drug), is expressed as a percentage of the reactivity of the antibody in the presence of PBS alone, A0. (The test against MDA for P16C was not performed.)



**Figure 4.12:** Reactivity of the different clones of monoclonal antibody supernatant (P15E and P15G) in the presence of amphetamine, MDMA, MDEA, MDA, MBDB, BDB, ephedrine, pseudoephedrine, ketamine and PBS (control). The reactivity of the antibody to immobilised methamphetamine-BSA in the presence of the free drugs (12500ng/ml MDMA, and 50000ng/ml for other drugs), A(drug), is expressed as a percentage of the reactivity of the antibody in the presence of PBS alone, A0.

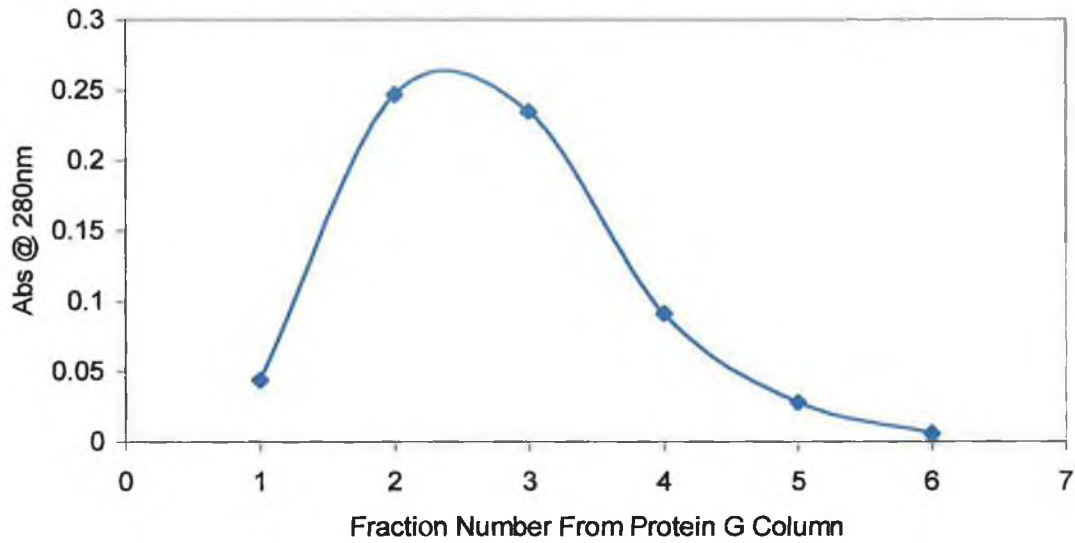
#### ***4.2.4 Purification of monoclonal antibody from hybridoma supernatant***

The 4EP18E clone and P18D clones were grown up in T75 flasks. The cells were grown and the spent media was removed after 1-2 weeks. The media was collected and stored at 4°C. 50mls of media was then concentrated to 5mls using the Amicon Filtration System. The monoclonal antibody was then purified by applying this concentrate to a Protein G immobilised Sepharose 3B column. The monoclonal antibodies were eluted from the column with 0.1M glycine, pH 2, as described in Section 2.8.3. The fractions collected were neutralised with 2M Tris, pH 8.6. The fractions were then read spectrophotometrically at 280 nm to determine the protein content. Figure 4.13 shows a typical elution profile. As an extra step to ensure the fractions contained the specific antibody of interest, an ELISA was performed. The fractions containing the antibody were pooled and dialysed in PBS overnight at 4°C with a two changes of PBS.

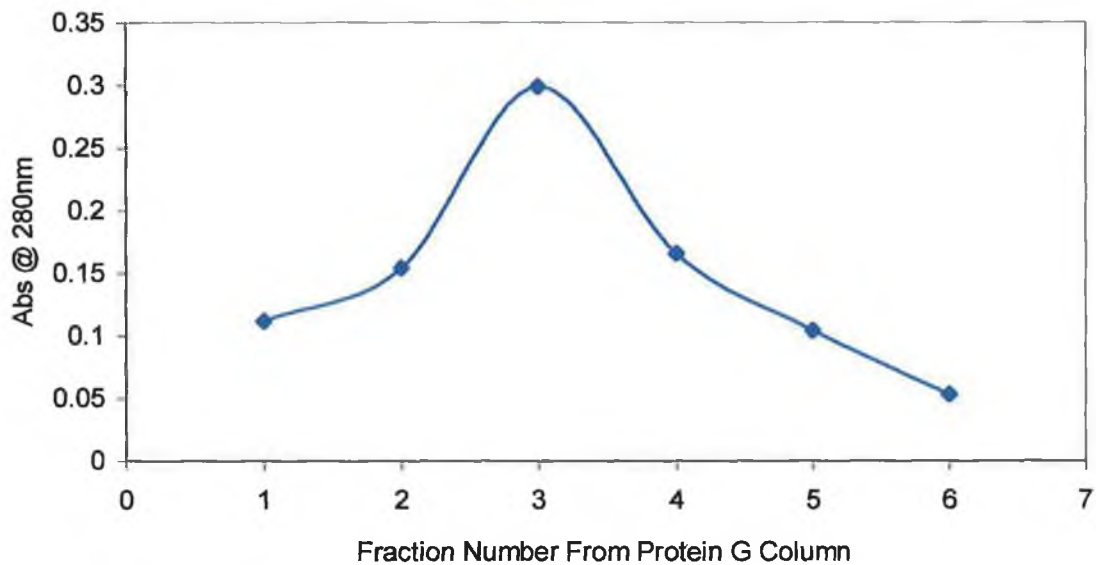
A sample of the purified antibody fractions were also run on an SDS-PAGE to determine purity, as shown in Figure 4.14 and 4.15.



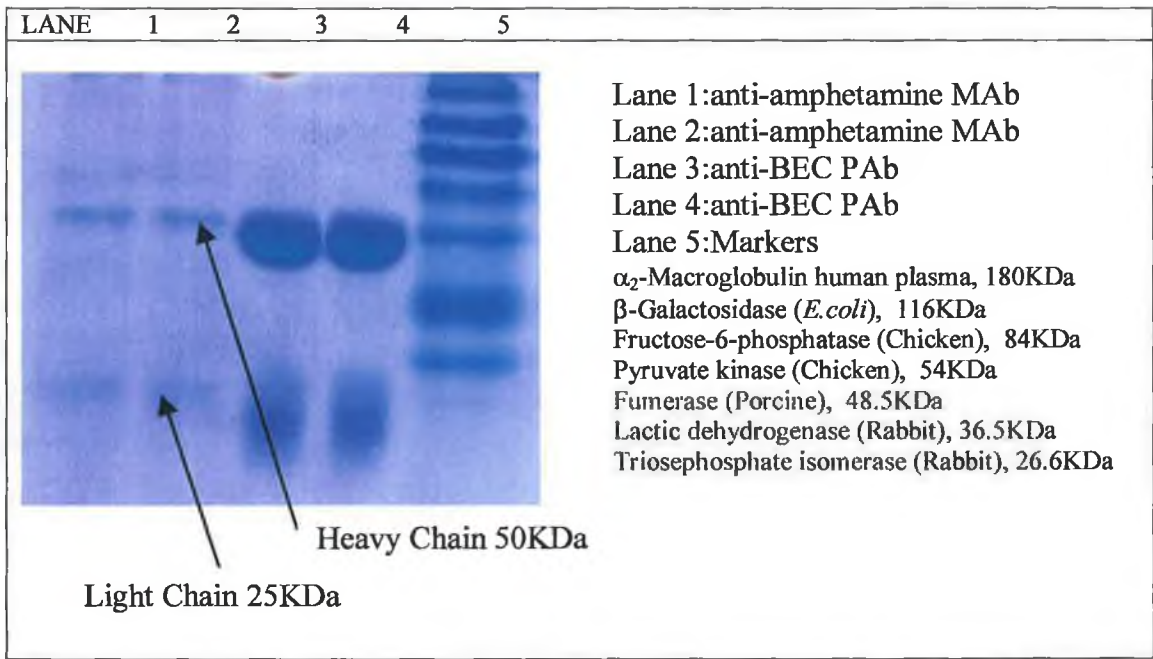
### Anti-Amphetamine Monoclonal Antibody



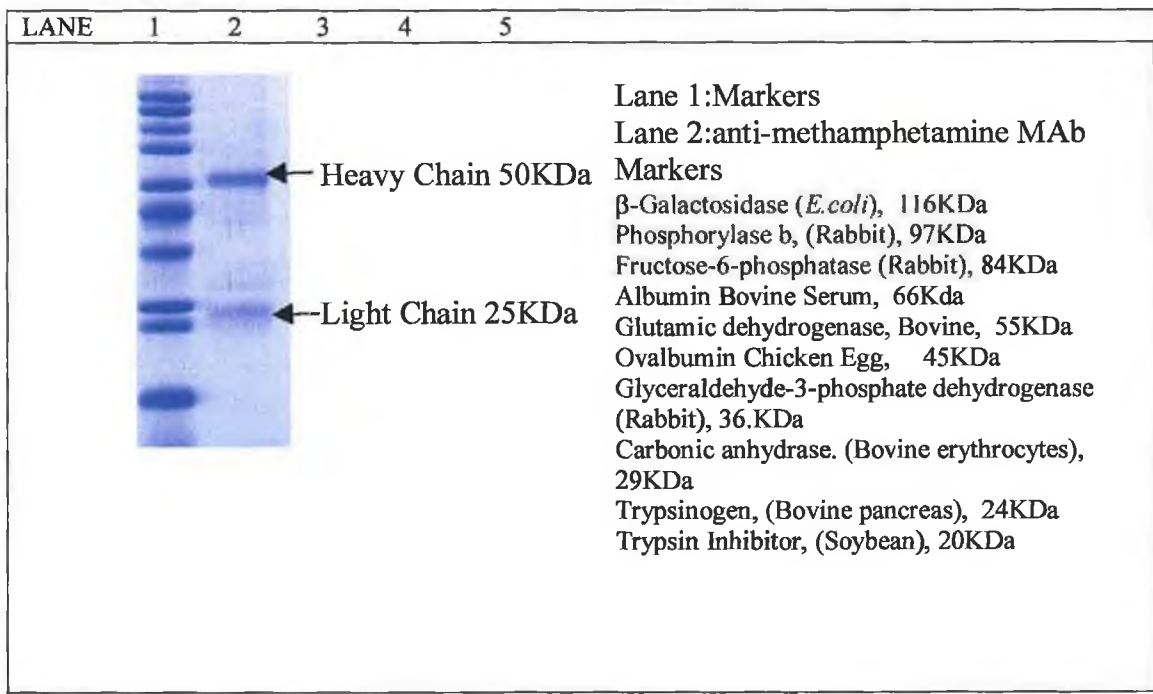
### Anti-Methamphetamine Monoclonal Antibody



**Figure 4.13:** Typical elution profile for the purification of anti-amphetamine and anti-methamphetamine monoclonal antibody. The concentrated hybridoma media was applied to a 2.5ml protein G-sepharose column. The column was washed and the bound antibody was eluted with 0.1M glycine/HCl, pH 2.5. 0.9ml fractions were collected in tubes containing 0.1ml 2M Tris, pH 8.6. This served to neutralise the glycine. The absorbance of each fraction, (1-6), was read at 280nm and the fractions containing protein were pooled and dialysed.



**Figure 4.14:** Characterisation by SDS-PAGE gel of the anti-amphetamine monoclonal antibody. Two bands can be seen, the top one at 50KDa representing the heavy chain and the fainter lower band at 25KDa representing the light chains.

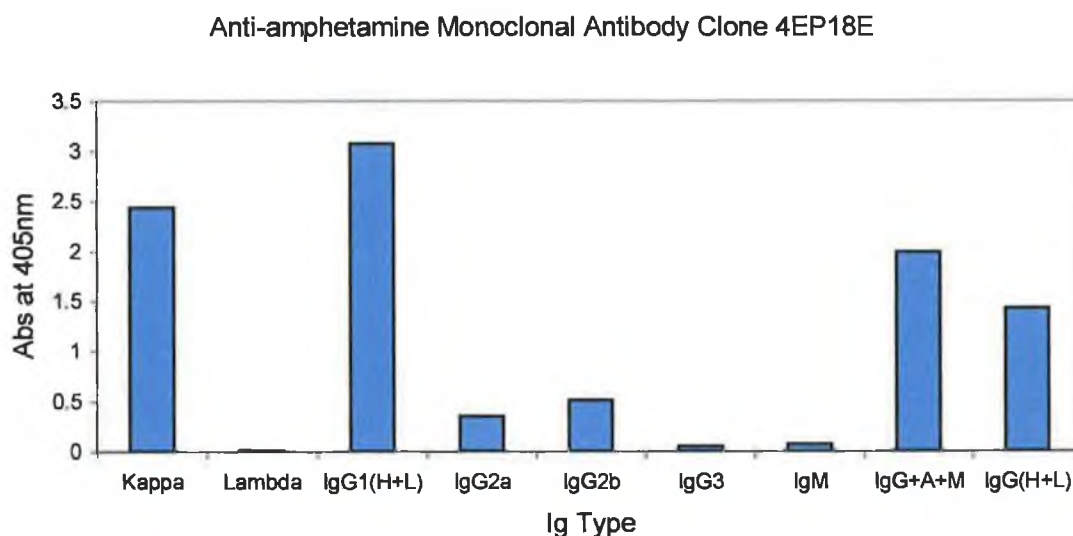


**Figure 4.15:** Characterisation by SDS-PAGE gel of the anti-methamphetamine monoclonal antibody. Two bands can be seen, the top one at 50KDa representing the heavy chain and the fainter lower band at 25KDa representing the light chains.

## 4.2.5 Antibody Isotyping

### 4.2.5.1 Antibody Isotyping – anti-amphetamine monoclonal antibody Clone 4EP18E

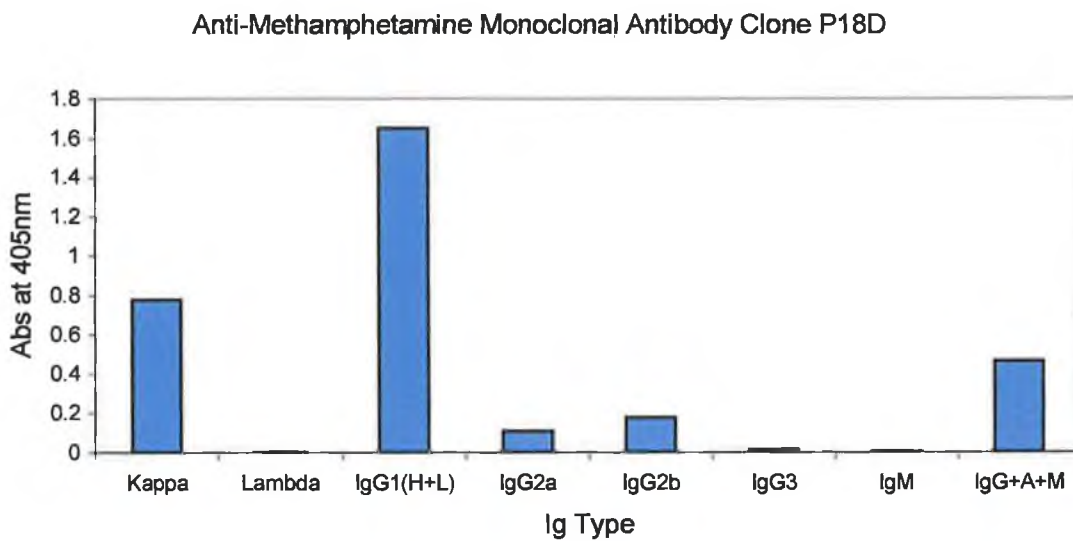
The isotype of the purified anti-amphetamine monoclonal antibody was determined by ELISA as described in Section 2.9.5. Figure 4.16 shows the relevant reactivity towards the different secondary antibodies. The results show that the antibody is an IgG1, with kappa light chains.



**Figure 4.16:** Isotype profile of purified anti-amphetamine monoclonal antibody, Clone 4EP18E. ELISA plates were coated and blocked with amphetamine-BSA and milk protein, respectively. Anti-amphetamine monoclonal antibody was added and incubated. After washing, alkaline phosphatase-labelled goat anti-mouse immunoglobulins were added to the wells and the ELISA developed. The absorbance indicates the monoclonal antibody isotype. This antibody is composed of IgG1 heavy chains and kappa light chains.

#### 4.2.5.2 Antibody Isotyping – anti-methamphetamine monoclonal antibody Clone P18D

The isotype of the purified anti-methamphetamine monoclonal antibody was determined by ELISA as described in Section 2.9.5. Figure 4.17 shows the relevant reactivity towards the different secondary antibodies. The results show that the antibody is an IgG1, with kappa light chains.



**Figure 4.17:** Isotype profile of purified anti-amphetamine monoclonal antibody, Clone 4EP18E. ELISA plates were coated and blocked with amphetamine-BSA and milk protein respectively. Anti-amphetamine monoclonal antibody was added and incubated. After washing, alkaline phosphatase-labelled goat anti-mouse immunoglobulins were added to the wells and the ELISA developed. The absorbance indicates the monoclonal antibody isotype. This antibody is composed of IgG1 heavy chains and kappa light chains.

#### ***4.2.6 Antibody Concentration Determination***

The protein content of the purified antibody was obtained from the BCA assay and an idea of the concentration can be obtained by reading at 280nm on a spectrophotometer. However, these techniques give the total protein content of the solution and not the antibody concentration. The ELISA using purified polyclonal goat anti-mouse immunoglobulin was performed as described in Section 2.9.7.

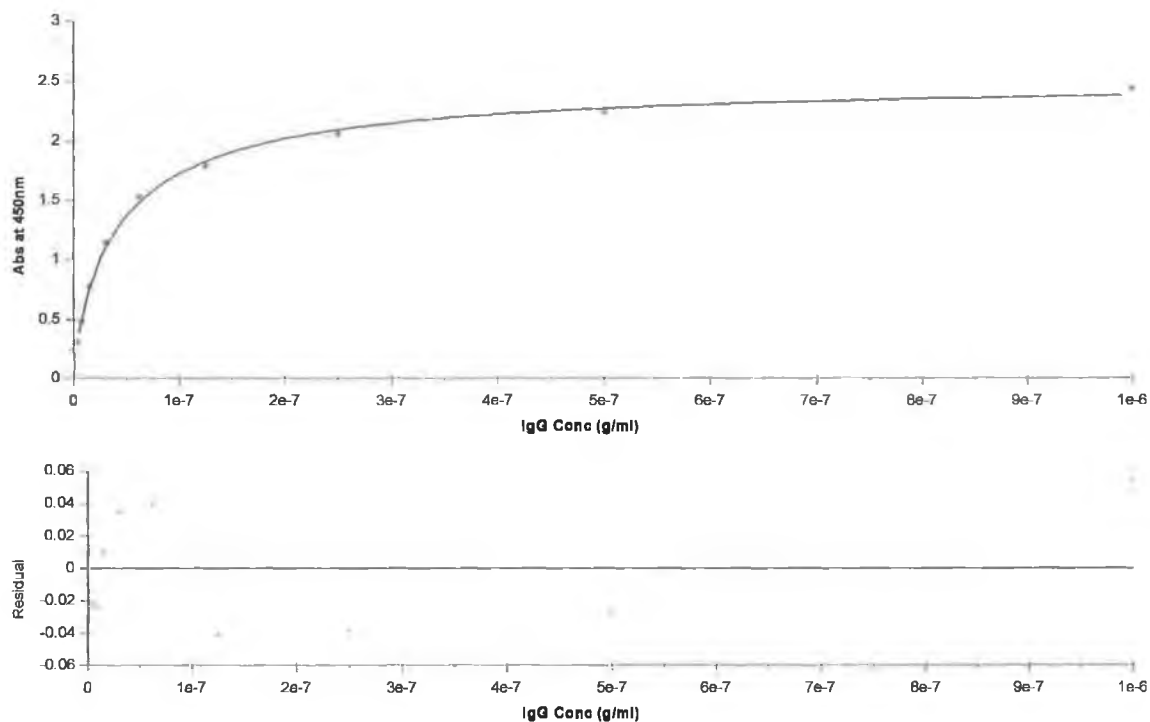
Goat anti-mouse immunoglobulin was used to coat the wells of an ELISA plate. Blocking was performed by adding 2% (w/v) milk. Serial dilutions of purified mouse immunoglobulin were prepared in dilutions containing from 1µg/ml to 76 pg/ml. The antibody dilutions were from 1/10 to 1/400.

##### ***4.2.6.1 Concentration of active anti-amphetamine monoclonal Clone 4EP18E***

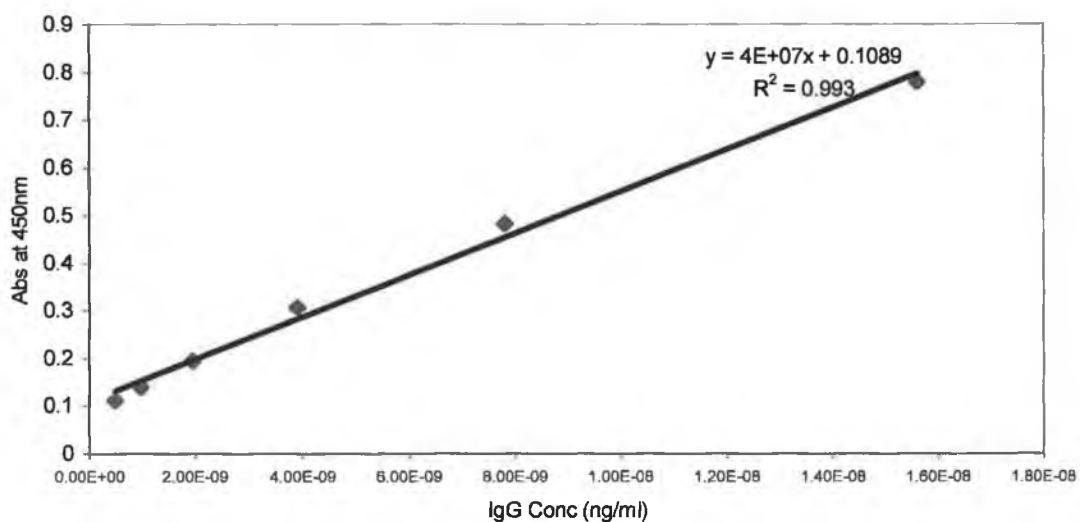
A 4-parameter equation was fitted to the data using the BIAevaluation software. This program calculated the concentration of the purified antibody solution to be  $5.31 \times 10^{-7}$  g/ml, (Figure 4.18). The data was also fitted to an Excel linear standard curve. The concentration of the antibody determined by the linear regression analysis was  $6.84 \times 10^{-7}$  g/ml (Figure 4.19).

##### ***4.2.6.2 Concentration of active anti-methamphetamine monoclonal Clone P18D***

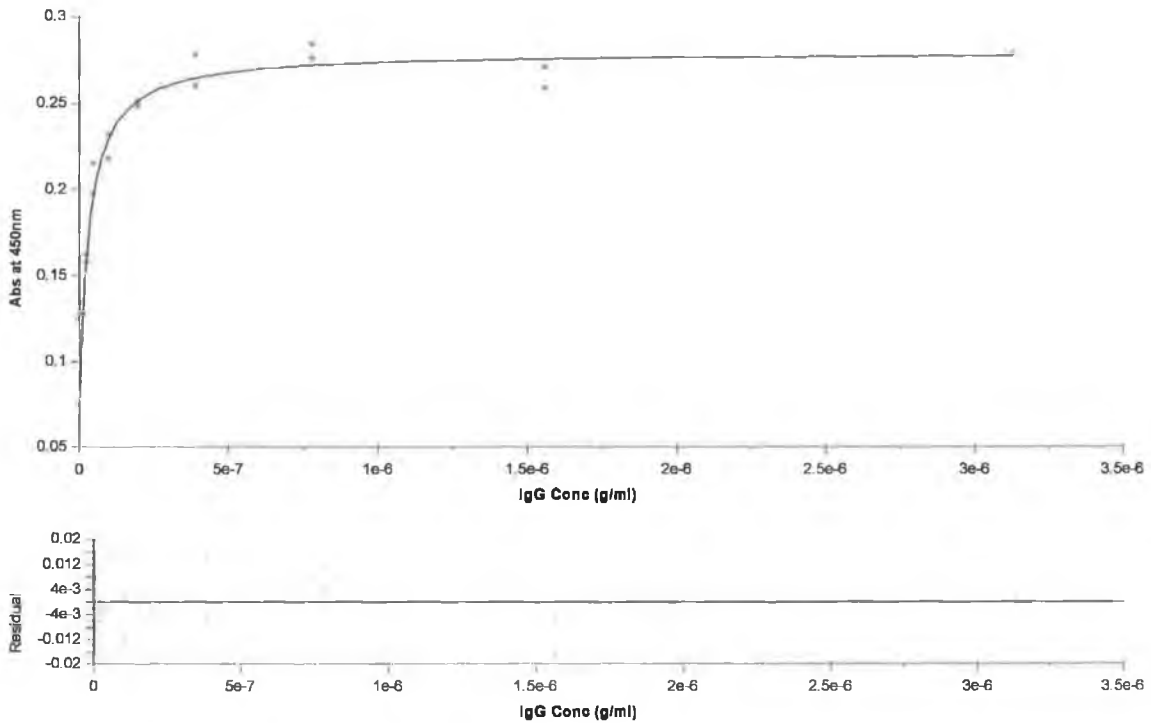
A 4-parameter equation was fitted to the data using the BIAevaluation software. This program calculated the concentration of the purified antibody solution to be  $1.60 \times 10^{-7}$  g/ml (Figure 4.20). The concentration of the antibody determined by the linear regression analysis was  $1.55 \times 10^{-7}$  g/ml (Figure 4.21).



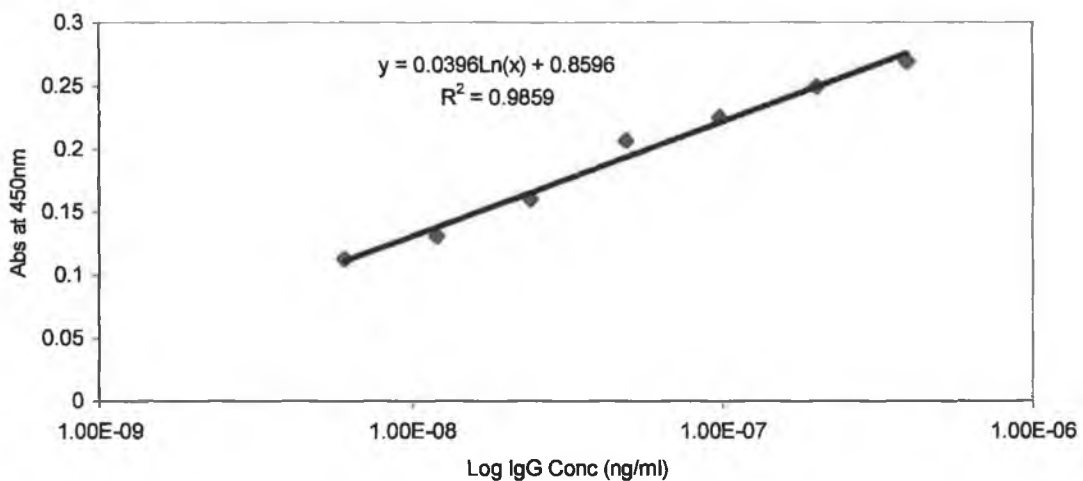
**Figure 4.18:** Mouse IgG calibration curve from BIAevaluation software. A 4-parameter fit is applied to the data allowing for the calculation of the mouse IgG amount in the purified anti-amphetamine monoclonal antibody batch, Clone 4EP18E. The concentration of the antibody determined by the 4-parameter fit was  $5.31 \times 10^{-7}$  g/ml.



**Figure 4.19:** Mouse IgG calibration curve from Excel software. A linear regression curve is applied to the data allowing for the calculation of the mouse IgG amount in the purified anti-amphetamine monoclonal antibody batch, Clone 4EP18E. The concentration of the antibody determined by the linear regression curve was  $6.84 \times 10^{-7}$  g/ml.



**Figure 4.20:** Mouse IgG calibration curve from BIAevaluation software. A 4-parameter fit is applied to the data allowing for the calculation of the mouse IgG amount in the purified anti-methamphetamine monoclonal antibody batch, Clone P18D. The concentration of the antibody determined by the 4-parameter fit was  $1.60 \times 10^{-7}$  g/ml.



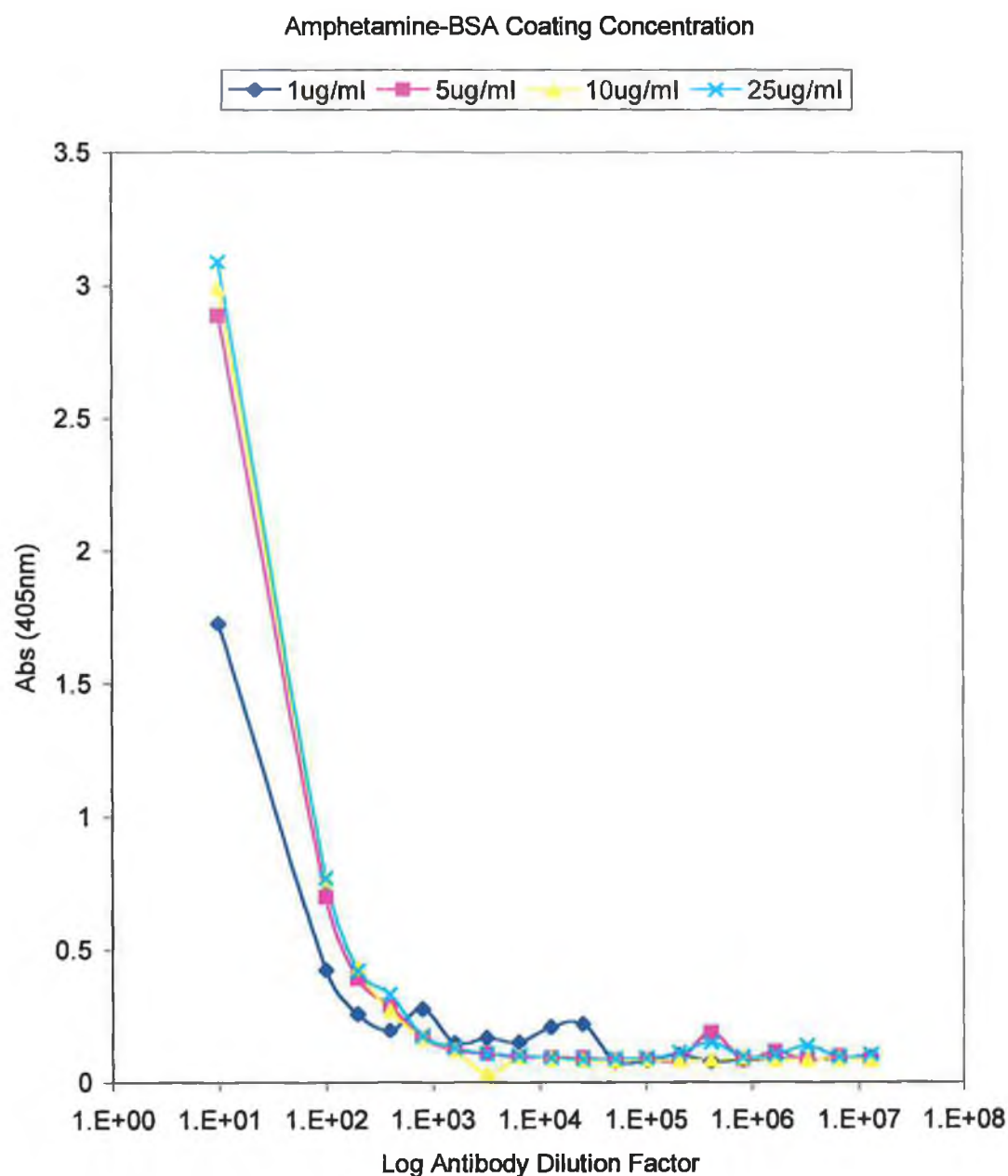
**Figure 4.21:** Mouse IgG calibration curve from Excel software. A linear regression curve is applied to the data allowing for the calculation of the mouse IgG amount in the purified anti-methamphetamine monoclonal antibody batch, Clone P18D. The concentration of the antibody determined by the linear regression curve was  $1.55 \times 10^{-7}$  g/ml.

#### 4.2.7 *Application of anti-amphetamine MAb in ELISA*

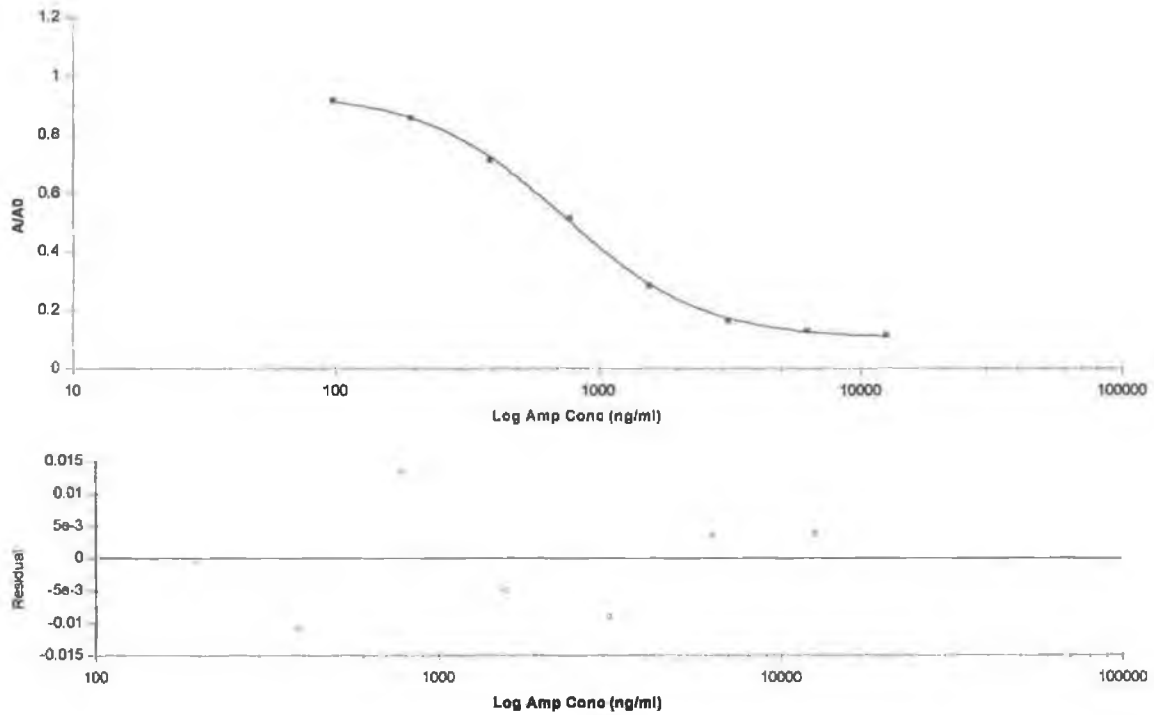
The optimal coating concentration of amphetamine-BSA and the optimal antibody dilution to use was determined by an indirect checkerboard ELISA. The results can be seen in Figure 4.22, the conjugate coating concentration ranged from 1 µg/ml to 10 µg/ml. BSA was also used to coat the plate at a concentration of 25 µg/ml. The antibody dilutions ranged from 1/10 to 1/13,107,200 in PBS/Tween containing 1% (v/v) milk powder. The coating concentration of the amphetamine-BSA analysed that gave the best sensitivity was 5 µg/ml and this was chosen as the concentration for the competitive ELISA. The optimum antibody dilution was 1 in 300.

The competitive assay was performed as described in Section 2.9.4. The standard amphetamine sample was prepared from a stock solution of 1mg/ml standard in ethanol. The intra-assay calibration is the results from three replicates performed on the same day (Table 4.2). The values from five assays were used to calculate the inter-assay mean, coefficients of variation and percentage recovery are shown in Table 4.3. The plot of the normalised absorbance and the concentration of free amphetamine for the inter-day assay has been shown in Figure 4.23. The range of detection of the assay is 97.7 to 6250 ng/ml. The percentage coefficients of variation for the intra-day assay and inter-day assay were very acceptable, being between 2.81 and 8.25%, and 7.71 and 24.29%, respectively. The degree of accuracy is determined by calculating the percent recovery of the known value for each concentration. This percent recovery is a quantitative measure of the closeness of the observed result to its theoretical true value, expressed as a percent of this theoretical value. The percent recoveries for the inter-day assay were 85.41 to 107.29%. These values indicate a very good accurate, reproducible assay for the detection of amphetamine.





**Figure 4.22:** Indirect checkerboard ELISA for determination of optimal concentration of coating conjugate concentration and antibody dilution. Amphetamine-BSA was coated at a range of concentrations between 1 and 25  $\mu\text{g/ml}$ . Dilutions from 1/ 10 to 1/13,107,200 of the anti-amphetamine monoclonal antibody were carried out.



**Figure 4.23:** Competitive ELISA for detection of free amphetamine. Anti-amphetamine monoclonal antibody was added to a range of amphetamine standards containing 0.19 to 50,000ng/ml. The data was fitted to a 4-parameter fit equation using BIAevaluation software. The residual plot show the difference between the experimental and fitted data. The results presented are the mean of 5 intra-day assays. The coefficient of variation, and mean back calculated value for each standard within the accepted range for the equation was determined and the degree of accuracy determined as shown in Table 4.2 and Table 4.3 for the intra-assay and inter-assay.

**Table 4.2:** Intra-assay variation (degree of precision) for the detection of amphetamine using the anti-amphetamine monoclonal antibody-based ELISA. The results are the mean of three replicates.

<b>Actual Amphetamine Conc (ng/ml)</b>	<b>Back-Calculated Amphetamine Conc. (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
97.7	88.8	4.57	90.96
195.3	193.8	2.81	99.24
390.6	403.2	8.25	103.22
781.3	757.7	8.22	96.99
1562.5	1639.4	5.14	104.92
3125.0	3099.4	3.36	99.18
6250.0	5376.5	3.30	86.02

**Table 4.3:** Inter-assay variation (degree of accuracy and reproducibility) for the detection of amphetamine using the anti-amphetamine monoclonal antibody-based ELISA. The results are the mean of five intra-day assays, each performed used three replicates.

<b>Actual Amphetamine Conc (ng/ml)</b>	<b>Back-Calculated Amphetamine Conc. (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
97.7	91.7	7.71	93.92
195.3	195.0	7.28	99.86
390.6	405.1	10.41	103.71
781.3	752.8	13.46	96.35
1562.5	1600.4	12.75	102.43
3125.0	3352.9	13.77	107.29
6250.0	5338.1	24.29	85.41

**4.2.8 Cross reactivity studies: anti-amphetamine monoclonal antibody, Clone 4EP18E**

The cross reactivity of the anti-amphetamine monoclonal antibody was determined against a range of amphetamine derivatives. From the previous section on screening the hybridomas, it was hoped that the anti-amphetamine clone would also recognise some of the derivatives to some degree. The structure of many of these popular designer amphetamines have a methylene dioxy group and substituted side chains. The structure of the MDA molecule is probably the closest to the amphetamine molecule. The degree of cross reactivity was determined with by a competitive ELISA described in Section 2.9.4. The amphetamine derivatives were obtained from a stock solution of 1mg/ml standard in ethanol. The degree of cross reactivity was determined as the concentration of cross reactant that gives a response of 50% or one-half of the observed maximum binding, (EC<sub>50</sub> – Cross Reactant) expressed a percentage of the specific analyte concentration that gives a 50% response(EC<sub>50</sub> – Specific Analyte).

$$\% \text{ Cross Reactivity} = \frac{\text{Concentration of Analyte (EC}_{50} - \text{SA)}}{\text{Concentration of Cross Reactant (EC}_{50} - \text{CR)}} \times 100\%$$

The degree of cross reactivity of the antiamphetamine antibody is expressed in Table 4.4. The point to note is that the assay does detect appreciable amounts of MDA and BDB.

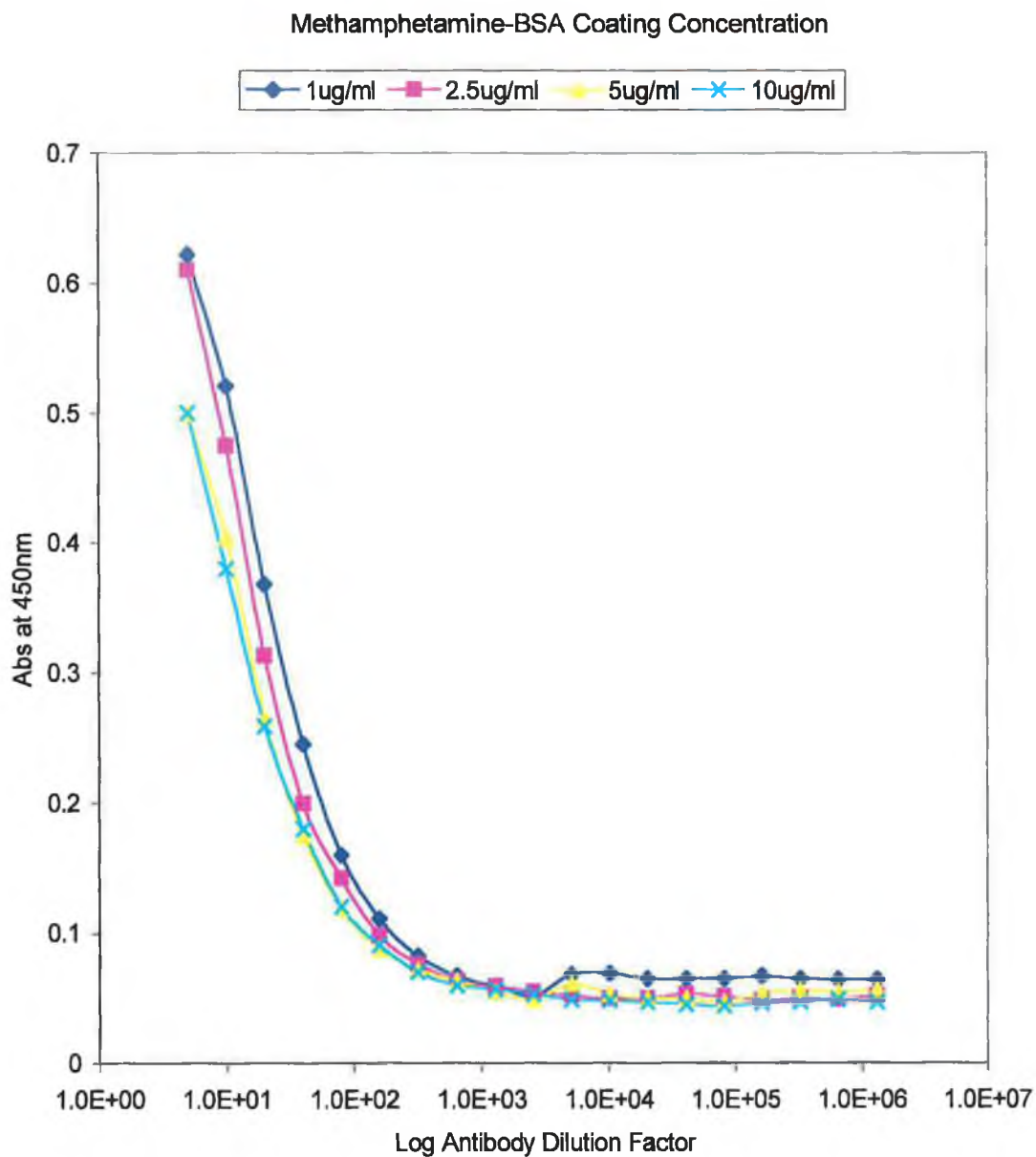
**Table 4.4:** Cross reactivity of anti-amphetamine monoclonal antibody. The results presented are the mean of three replicates.

<b>Drug</b>	<b>% Cross Reactivity</b>	<b>Range of Detection (ng/ml)</b>
Amphetamine	100%	97.7-6250.0
MDA	2%	3125.0-50000.0
BDB	1%	12500.0-50000.0
MDMA	0%	0
MBDB	0%	0
MDEA	0%	0
Ketamine	0%	0
Ephedrine	0%	0
Pseudoephedrine	0%	0

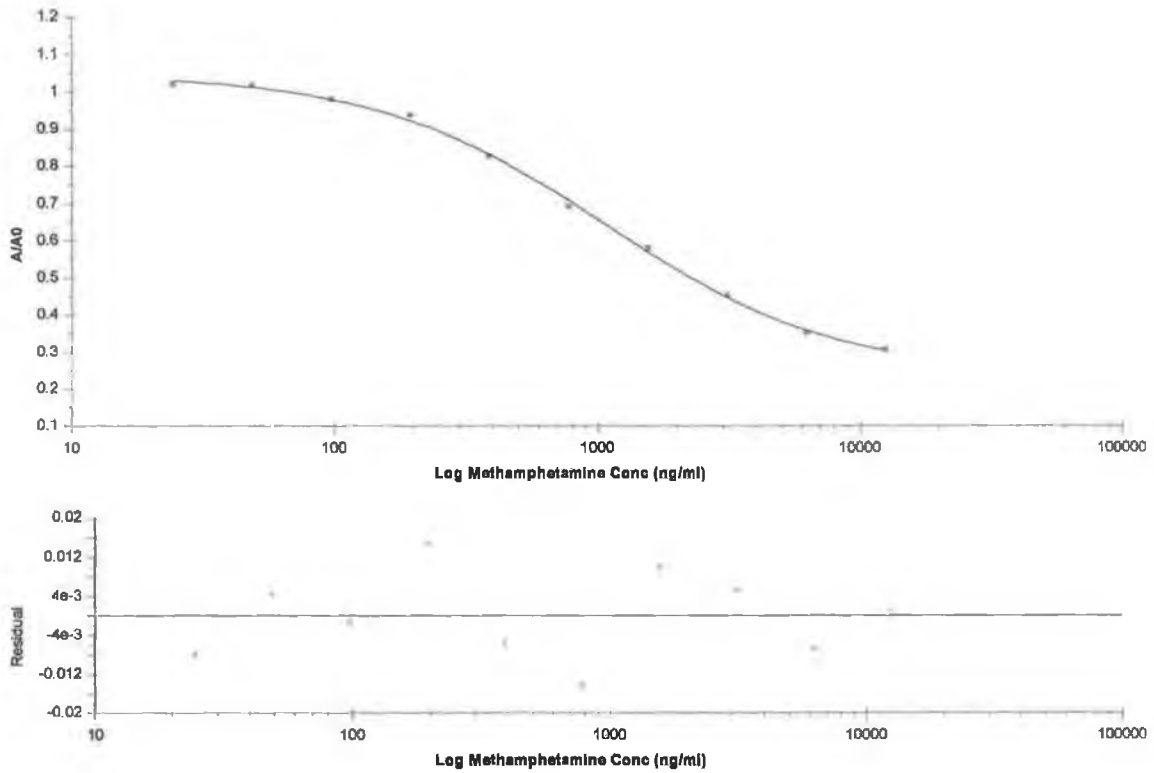
#### **4.2.9 Application of anti-methamphetamine MAb in ELISA**

The optimal coating concentration of methamphetamine-BSA and the optimal antibody dilution to use was determined by an indirect checkerboard ELISA. The results can be seen in Figure 4.24, the conjugate coating concentration ranged from 1 µg/ml to 10 µg/ml. There was no response to the BSA covered plate. The BSA was coated at a concentration of 25 µg/ml. The antibody dilutions ranged from 1/10 to 1/13,107,200 in PBS/Tween containing 1% (v/v) milk powder. The coating concentrations of the methamphetamine-BSA analysed all gave similar sensitivities, so 2.5 µg/ml was chosen as the concentration for the competitive ELISA. The optimum antibody dilution was 1 in 20.

The amphetamine derivative standards were prepared from a stock solution of 1mg/ml standard in ethanol. The intra-assay calibration is the results from three replicates performed on the same day, and these are shown in Table 4.5. The values from five assays were used to calculate the inter-assay mean, coefficients of variation and percentage recovery are shown in Table 4.6. The plot of the normalised absorbance and the concentration of free amphetamine for the inter-day assay is shown in Figure 4.25. The range of detection of the assay is 24.4 to 12500 ng/ml. The percentage coefficients of variation for the intra-day assay and inter-day assay are very acceptable, being between 0.94 and 5.68%, and 1.43 and 15.95%, respectively. The percent recoveries for the inter-day assay range from 87.23 to 145.42%. The highest value of 145.42% was obtained for the lowest value, 24.4ng/ml, of the range of detection. These values indicate a very good accurate, reproducible assay for the detection of amphetamine.



**Figure 4.24:** Indirect checkerboard ELISA for determination of optimal concentration of coating conjugate concentration and antibody dilution. Amphetamine-BSA was coated at a range of concentrations between 1 and 25  $\mu\text{g/ml}$ . Dilutions from 1/10 to 1/13,107,200 of the anti-amphetamine monoclonal antibody were carried out.



**Figure 4.25:** Competitive ELISA for detection of free methamphetamine. Anti-methamphetamine monoclonal antibody, Clone P18D, was added to a range of methamphetamine standards containing 0.04 to 12,500ng/ml. The data was fitted to a 4-parameter fit equation using BIAevaluation software. The results presented are the mean of 5 intra-day assays,  $\pm$  standard deviation. The coefficient of variation, and mean back calculated value for each standard within the accepted range for the equation was determined and the degree of accuracy determined .

**Table 4.5:** Intra-assay variation (degree of accuracy and reproducibility) for the detection of methamphetamine using the anti-methamphetamine monoclonal antibody based ELISA. The results presented are the mean of three replicates.

<b>Actual Methamphetamine Conc (ng/ml)</b>	<b>Back-Calculated Methamphetamine Conc. (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
24.4	30.1	5.68	123.14
48.8	60.8	1.99	124.59
97.7	83.4	5.16	85.44
195.3	158.7	1.11	81.24
390.6	438.2	1.31	112.19
781.3	847.2	2.95	108.45
1562.5	1340.6	1.55	85.80
3125.0	3191.4	1.31	102.13
6250.0	8275.7	3.43	132.41
12500.0	9819.5	0.94	78.56

**Table 4.6:** Inter-assay variation (degree of accuracy and reproducibility) for the detection of methamphetamine using the anti-methamphetamine monoclonal antibody based ELISA. The results presented are the mean of five intra-day assays, each performed using three replicates.

<b>Actual Methamphetamine Conc (ng/ml)</b>	<b>Back-Calculated Methamphetamine Conc. (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
24.4	35.5	1.43	145.42
48.8	42.6	2.35	87.23
97.7	99.7	4.23	102.06
195.3	168.3	5.47	86.18
390.6	405.2	7.59	103.72
781.3	839.2	10.20	107.42
1562.5	1483.1	12.71	94.92
3125.0	3017.9	11.78	96.57
6250.0	6681.1	10.59	106.90
12500.0	12302.8	15.95	98.42



#### **4.2.10 Cross reactivity studies: anti-methamphetamine monoclonal antibody, Clone P18D**

The cross reactivity of the anti-methamphetamine monoclonal antibody was determined against a range of amphetamine derivatives. As discussed previously, the planning behind the immunisations with methamphetamine-BSA was that the anti-methamphetamine clone would also recognise some of the derivatives to a better degree than the monoclonal produced with the amphetamine-BSA immunisations. The structure of many of the designer amphetamines have a methylene dioxy group and substituted side chains. The structure of the MDMA molecule is probably the closest to the methamphetamine molecule. The degree of cross reactivity was determined by competitive ELISA, as described in Section 2.9.4. Basically, a typical ELISA was performed, substituting the methamphetamine for the drug of interest. The amphetamine derivatives were obtained from a stock solution of 1mg/ml standard in ethanol. The degree of cross reactivity was determined as described above in Section 4.2.8.

The degree of cross reactivity of the antiamphetamine antibody is expressed in Table 4.7. The point to note is that the assay does detect appreciable amounts of MDMA, MBDB, and MDEA.

**Table 4.7:** Cross reactivity of anti-amphetamine monoclonal antibody. The results presented are the mean of three replicates.

<b>Drug</b>	<b>% Cross Reactivity</b>	<b>Range of Detection (ng/ml)</b>
Methamphetamine	100%	24.4-12500.0
MDA	0%	0
BDB	0%	0
MDMA	146%	48.8-390.6
MBDB	47%	390.6-12500
MDEA	18%	390.6-12500
Ketamine	0%	0
Ephedrine	0%	0
Pseudoephedrine	0%	0
Phenylpropanolamine	0%	0

**4.2.11 Application of anti-amphetamine monoclonal antibody in ELISA for detection of amphetamine in saliva samples**

The competitive assay as described above, was repeated using saliva samples spiked with amphetamine. The saliva caused very little interference with the assay and similar results were obtained with regard to the level of detection. Each assay had three replicates for each standard concentration. The values from five assays were used to calculate the inter-assay mean, coefficients of variation and percentage recovery are shown in Table 4.8. The coefficient of variations were a little higher than the values obtained in the PBS model ELISA. This can be accounted for by variations in saliva itself that may be causing slight interference.

**Table 4.8:** Inter-assay variation (degree of accuracy and reproducibility) for the detection of amphetamine using the anti-amphetamine monoclonal antibody based ELISA. The results presented are the mean of five intra-day assays, each performed using three replicates.

<b>Actual Amphetamine Conc (ng/ml)</b>	<b>Back-Calculated Amphetamine Conc. (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
97.7	78.4	8.83	80.23
195.3	226.6	5.05	116.02
390.6	386.9	10.99	99.03
781.3	737.7	19.43	94.43
1562.5	1663.2	21.54	106.45
3125.0	3025.6	20.53	96.82

**4.2.12 Application of anti-methamphetamine monoclonal antibody in ELISA for detection of methamphetamine in saliva samples**

The competitive assay, as described above, was repeated using saliva samples spiked with methamphetamine. Each assay had three replicates for each standard concentration. The values from five assays were used to calculate the inter-assay mean, coefficients of variation and percentage recovery are shown in Table 4.9. Similar results were obtained with regard to the level of detection. However, the percentage recovery, which is a measure of the accuracy was lower for the lower values of the detection limit, as shown in Table 4.9 below. This can be accounted for by variations in saliva itself that may be causing slight interference. So in this case, the lower limit of 24.4 ng/ml should not be included in the acceptable range of detection.

**Table 4.9:** Inter-assay variation for the detection of methamphetamine using the anti-methamphetamine monoclonal antibody based ELISA. The results presented are the mean of five intra-day assays, each performed using three replicates.

<b>Actual Methamphetamine Conc (ng/ml)</b>	<b>Back-Calculated Methamphetamine Conc. (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
24.4	10.5	1.07	42.93
48.8	70.6	3.96	144.65
97.7	133.2	4.10	136.43
195.3	150.7	4.07	77.17
390.6	412.5	6.03	105.60
781.3	724.0	5.82	92.68
1562.5	1447.9	8.68	92.67
3125.0	3707.0	5.10	118.62
6250.0	6924.1	8.70	110.79
12500.0	10404.4	13.21	83.24

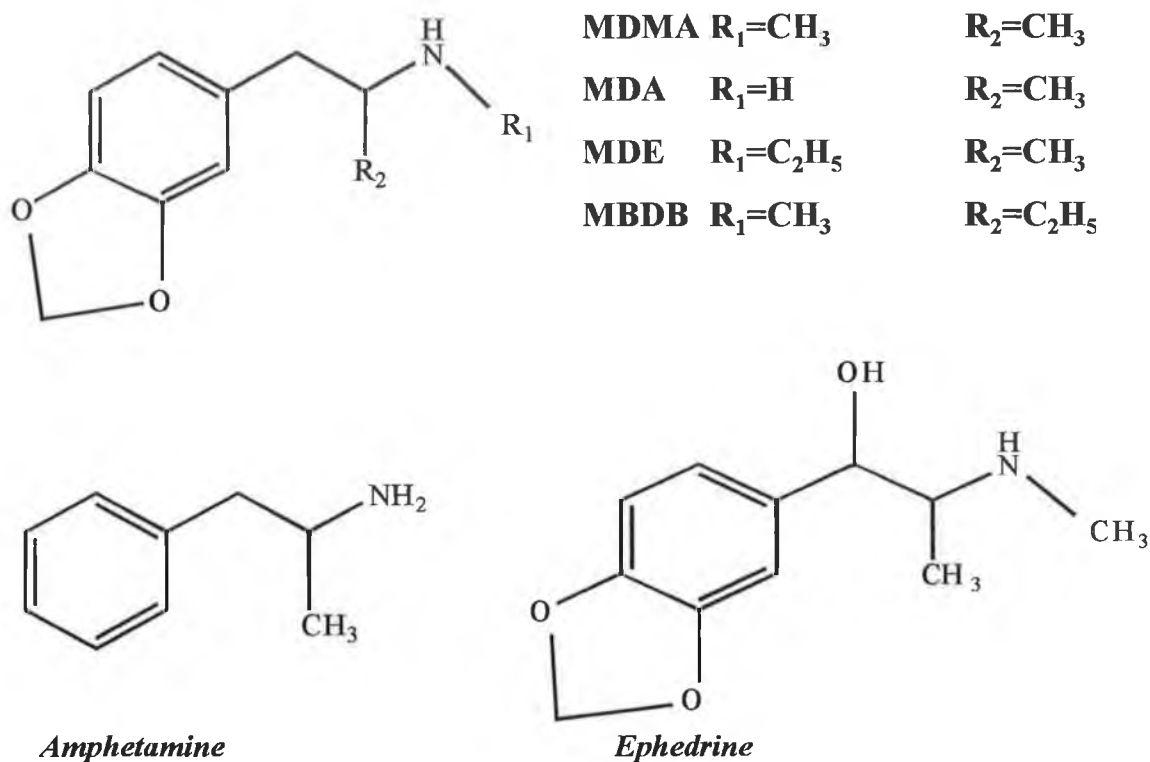
### 4.3 Discussion

Monoclonal antibody production was described in this chapter. The aim of the work was to prepare antibodies that would recognise amphetamine and amphetamine derivatives. This was at the outset a challenging task as the range of designer amphetamine derivatives made by clandestine laboratories is continually increasing, correlating with the increased use of these so called 'recreational' drugs. The other major consideration that had to be taken into account was the other 'amphetamine-like' derivatives that are used in common over the counter cold and flu medications, for example ephedrine, pseudoephedrine, and phenylpropanolamine, (Figure 4.26).

The original application for these antibodies is in the screening of saliva samples from drivers being tested for drugs of abuse. A key aspect of such a test is that the amount of false positives be kept to the absolute minimum. Otherwise the screening would not be advantageous, or contributing to, different screening methods, for example, psychometric testing by specialised police personnel. Therefore, the aim of the project presented two challenges, i.e. (a) to produce antibodies that would be specific for the amphetamine designer drugs, and (b) that would not cross react with structurally-related legal medications.

The immunogens chosen for the *in vivo* immunisations were amphetamine-BSA and methamphetamine-BSA. The only structural difference between amphetamine and methamphetamine is as the name suggests, the presence of a methyl group on the carbon side chain. Amphetamine was chosen as it is the main parent structure of these drugs. Methamphetamine was chosen as it is abused in this form, and the designer amphetamine derivatives have substituted amphetamine side chains, and so this structure would be more closely related to these. There is very little work published concerning the form of these amphetamine derivatives that are present in saliva after ingestion. From discussions, with our collaborators on the SMT project, the emphasis with regard to detection was placed on amphetamine, methamphetamine, MDMA, MDA and MDEA. It is the *d*-isomer of amphetamine that has potent psychological effects and so it was this form that was examined in all assays.

Due to the large amounts of amphetamine and methamphetamine that are needed to derivatise these drugs for conjugation to proteins, and the time needed, it was decided to use commercially available conjugates as they could be acquired without a need for an import licence.



**Figure 4.26:** Structure of amphetamine and the main amphetamine derivatives

The screening procedure for the antibodies was complicated by the fact the same conjugate used for immunisation had to be used for the screening. This goes against all the recommendations, mentioned in the introduction, that the screening conjugate should be a different conjugate, in terms of the protein used, and the chemistry used. In order, to compensate for this, it was decided that extensive screening needed to be performed. This screening took the format of an inhibition ELISA. If a supernatant was positive on the amphetamine-BSA (M1 Fusion) or methamphetamine-BSA (M2 Fusion), it was then screened in the inhibition ELISA against all the derivatives. This required a substantial amount of time to perform but the end result was very successful.

The successful clones were grown up in T75 flasks. Sufficient quantities were produced by this method for the purposes of characterisation and testing in an ELISA format.

A competitive ELISA was developed for the detection of amphetamine with the anti-amphetamine monoclonal antibody, and a 4 parameter fit was applied to the data. This antibody showed a range of detection between 97.7 to 6250 ng/ml amphetamine. The degree of cross reactivity at 50% was 2% for MDA and 1% for BDB. Although, a 2% cross reactivity may seem low, perhaps a better idea of cross reactivity can be seen from looking at the range of detection of MDA by the anti-amphetamine antibody, which is 3125.0 to 50000.0ng/ml. This means that the presence of 3125ng/ml MDA will cause an appreciable decrease in the absorbance of the assay and this is taken as a positive result. It is interesting that of all the other amphetamine derivatives, the only one that could be detected was BDB. This recognition of MDA can be explained by its structure as it contains the amphetamine structure substituted with the methylene dioxy group on the phenyl ring. BDB does not have the methylene dioxy group at the phenyl ring, but is substituted on the carbon side chain. The important issue with regard to cross reactivity was that it did not recognise ephedrine or pseudoephedrine. The assay also showed very good precision as determined by the intra-assay coefficients of variation (2.81% to 8.25%), and very good reproducibility as determined by the inter-assay coefficients of variations (7.71 – 24.29%). The high percentage CV of 24.29% was obtained for the value of 6250 ng/ml amphetamine. This large CV could have been a result of the fact that it is at the high end of the range of detection, and so there is more variability as a result. The degree of accuracy was also determined by a calculation of the percentage recovery. As described by Findlay (2000) this is a concept that expresses the closeness of agreement between a measured test result and its theoretical true value. The percentage recoveries for the inter-assay were very good, between 85.41% and 107.29%. Overall, the anti-amphetamine monoclonal antibody, Clone 4EP18E, competitive ELISA for the detection of amphetamine was a very good, accurate, reproducible assay.

A competitive ELISA was developed for the detection of methamphetamine with the anti-methamphetamine monoclonal antibody, and a 4 parameter fit was applied to the data. This antibody showed a range of detection between 24.4 to 12500 ng/ml

methamphetamine. The degree of cross reactivity at 50% was 146% for MDMA, 47% for MBDB, and 18% for MDEA. The 146% cross reactivity for MDMA, can be explained by the fact that during the screening procedures, the lab supply of methamphetamine was exhausted and this necessitated using MDMA as the primary drug of interest, as it most closely related to methamphetamine. The range of detection of MDMA by the anti-amphetamine antibody, is 48.8 to 390.6ng/ml. This means that the presence of MDMA will cause an appreciable decrease in the absorbance of the assay and this is taken as a positive result. This recognition of MDMA can be explained by its structure as it contains the methamphetamine structure substituted with the methylene dioxy group on the phenyl ring. MBDB and MDEA have additional butyl and ethyl groups on the carbon side chain. Again, with this antibody, specific clones were chosen that did not recognise ephedrine, pseudoephedrine or phenylpropanolamine.

The assay also showed very good precision as determined by the intra-assay coefficients of variation (0.94 to 5.68%), and very good reproducibility as determined by the inter-assay coefficients of variations (1.43 to 15.95%). The high percentage CV of 15.95% was obtained for the value of 12500 ng/ml methamphetamine. This larger CV value could have been a result of the fact that it is at the high end of the range of detection, and so there is more variability. The degree of accuracy was also determined by a calculation of the percentage recovery as discovered for the anti-amphetamine antibody. The percentage recoveries for the inter-assay were very good, between 87.27 and 145.42%. This high CV of 145.42% was obtained for the low methamphetamine concentration of 24.4ng/ml. For an accurate assay, this value should be removed from the range of detection, changing the range to 48.8 to 12500ng/ml. All other CVs were within an acceptable range of 87.27% to 106.9%. Overall, the anti-methamphetamine monoclonal antibody, Clone P18D, competitive ELISA for the detection of amphetamine is a very good, accurate, reproducible assay. It also has the added benefit of detecting appreciable amounts of MDMA, MBDB and MDEA, without interacting with ephedrine, pseudoephedrine or phenylpropanolamine.

The assay format was then applied to saliva samples spiked with amphetamine and methamphetamine. There was a very good correlation between the model assay in PBS and the assay in saliva. The competitive assay for saliva samples spiked with

amphetamine, showed similar results were obtained with regard to the level of detection. The coefficient of variations were a little higher than the values obtained in the PBS model ELISA. This can be accounted for by variations in saliva itself that may be causing slight interference. These could involve proteins or extraneous components. The protein content of saliva is composed of the same constituents as blood, in that it contains lipoproteins and digestive enzymes. However, the total protein concentration in saliva is significantly less (under 1%) than that in plasma. There are also additional constituents in saliva such as dead epithelial cells and usually some food debris also, (Samyn *et al.*, 1999).

The competitive assay for saliva samples spiked with methamphetamine, again, showed similar results with regard to the level of detection, however the percentage recovery, which is a measure of the accuracy was lower for the lower concentrations of the detection limit. Again, this can probably be accounted for by variations in saliva itself that may be causing slight interference.

The production of the two monoclonal antibodies was a success. The key to selecting the specific antibodies was the intensive screening process, in which not only reactivity towards the drugs of interest was investigated but also the reactivity towards the structurally close relatives that it was hoped would not be detected. The reactivity of the antibodies in saliva mirrored that seen in the PBS assay and so these assays can be directly applied to testing saliva samples for amphetamine, MDA, methamphetamine, MDMA, MBDB, and MDEA.

The next chapter, continues the characterisation of these monoclonal antibodies and their application in a BIAcore competitive assay for the detection of amphetamines.



## *Chapter 5*

### *Characterisation and Application of Anti-Amphetamine and Anti-Methamphetamine Monoclonal Antibodies*

## **5.1 Introduction**

### **5.1.1 Biosensors**

The basis of a sensor is the ability to detect a change in an environment, and then to process this information and display it to the user. A biosensor consists of three main components, a biosensing material, a transducing mechanism and a processing unit. One definition of a biosensor is a sensor that uses a biological component, for example an antibody or enzyme (Figure 5.1A). McCormack *et al.* (1998), have reviewed the different biomaterials that can be used for biosensors. These include enzymes, antibodies, modified by labeling and also by the production of hybrid immunoglobulin molecules that have been changed to either enhance the specificity or to incorporate a signal generating component. Other biomaterials include cells, used in place of purified enzymes, microorganisms, which are commonly used as indicators of toxicity, mammalian tissue slices, plant tissue, DNA and RNA, and cell receptors. There are many different types of biosensors and other chemical sensors that are not specifically biosensors but that are used in analysis of biological systems. An example of one would be ion-selective electrodes. These are chemical sensors in that they do not possess a biological component but can be used for direct *in vivo* measurements in mammalian muscle cells (Fanning, 1995).

The ideal properties of a sensor include the following (Diamond, 1998):

- Rapid measurement
- Sensitive
- Selective
- Sensor signal should return to baseline after measurement
- Noise to signal ratio must be low as this determines the limit of detection
- Signal output must bear mathematical relationship to amount of analyte in sample.

### **5.1.2 Surface Plasmon Resonance**

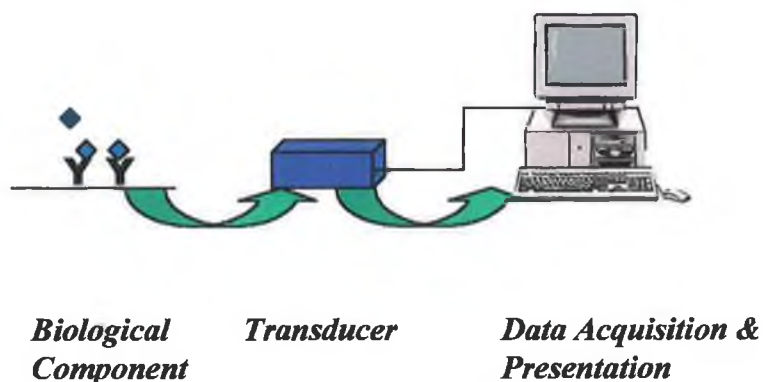
Surface plasmon resonance is a method for measuring biomolecular interactions. BIAcore is an analytical system based on surface plasmon resonance. It detects and measures binding interactions in 'real-time'. The detection system depends on changes in concentration at the sensor surface. The sensor chip is a glass slide coated on one side with a gold film to which a matrix of carboxymethylated dextran is covalently

attached. Surface plasmon resonance is a property of thin metal layers. When a wedge of light is focused on the rear side of the sensor chip, total internal reflection occurs above a certain angle of incidence, and a photodiode array detector detects the intensity of the reflected light. At an angle of incidence to the gold layer, total internal reflectance occurs, the photons from the light resonates with the free electrons (plasmons) in the gold layer creating an evanescent wave. The angle at which the surface plasmon resonance occurs is dependent on the refractive index of the other side of the gold film. A change in mass bound to the gold layer is proportional to a change in refractive index. When binding occurs between the immobilised molecules in the dextran layer and the molecules from the solution that is continuously passed over the chip, changes in mass bound occur causing changes in refractive index and subsequent changes in the SPR angle (Figure 5.1B). The output of the data is in the form of a sensorgram (Figure 5.2). Kinetic data on the interaction between the antigen and ligand can be obtained by this 'real-time' measurement.

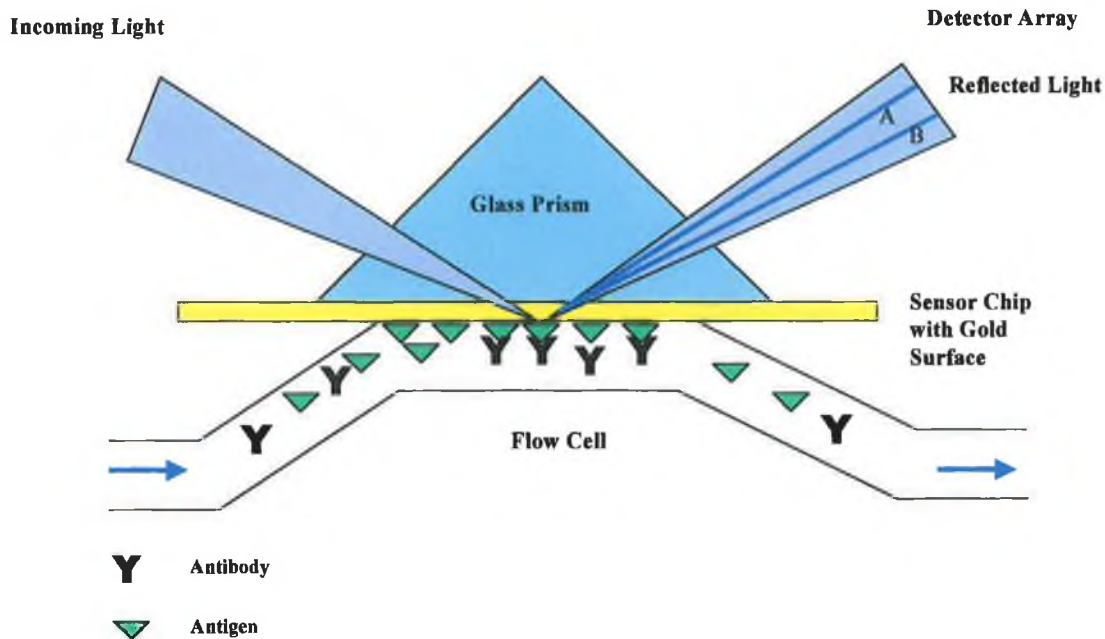
SPR-based biosensors have considerable advantages over other technologies, including the following:

- There are no labeling requirements, so the mechanisms of the interactions are not effected by a modification. This also involves less preparatory work.
- The reactions are monitored in 'real-time', so providing rapid, quantitative data.
- The stability of the sensor surface can be monitored by the sensorgram readings.
- The systems are fully automated and can handle large amounts of samples.
- The assays developed require little or no sample preparatory steps. Reports include assay development in blood (Quinn *et al.*, 1997), cell culture supernatant (Fanning, unpublished data), urine (Dillon, 2001), saliva (as detailed in Section 5.2 and Chapter 6), and bile (Fitzpatrick *et al.*, unpublished data).
- Kinetic and affinity data over a wide range of constants can be determined, ( $k_a$ :  $10^3$ - $10^8$   $M^{-1}Sec^{-1}$ ,  $k_d$ :  $10^{-6}$ - $10^{-1}$   $Sec^{-1}$ ,  $K_D = 10^{-3} - 10^{-13}$ ) (Myszka and Rich, 2000).
- The feature of multi-channel analysis, means that the interactions can be monitored over different immobilised ligand sensor surfaces, and provide direct comparison.
- The amount of materials required for immobilisation of ligands and regeneration solutions are minimal.

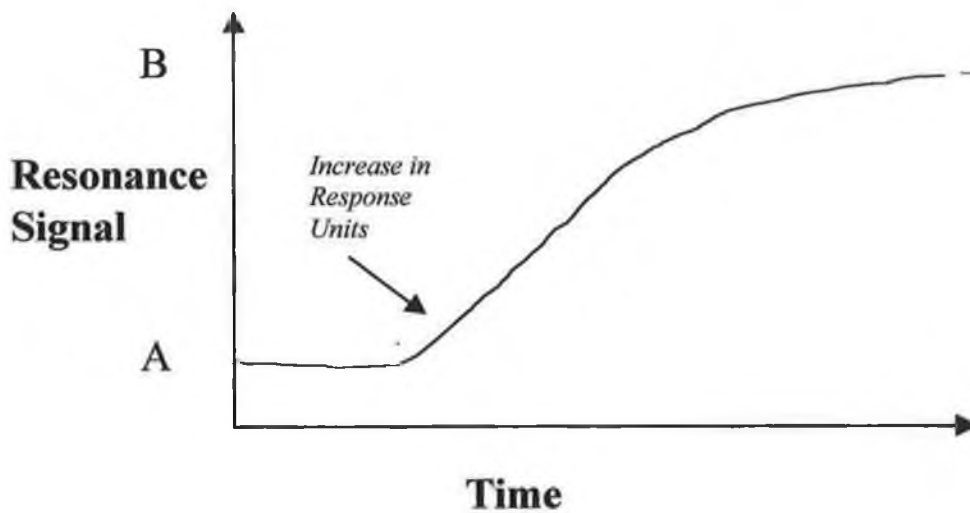
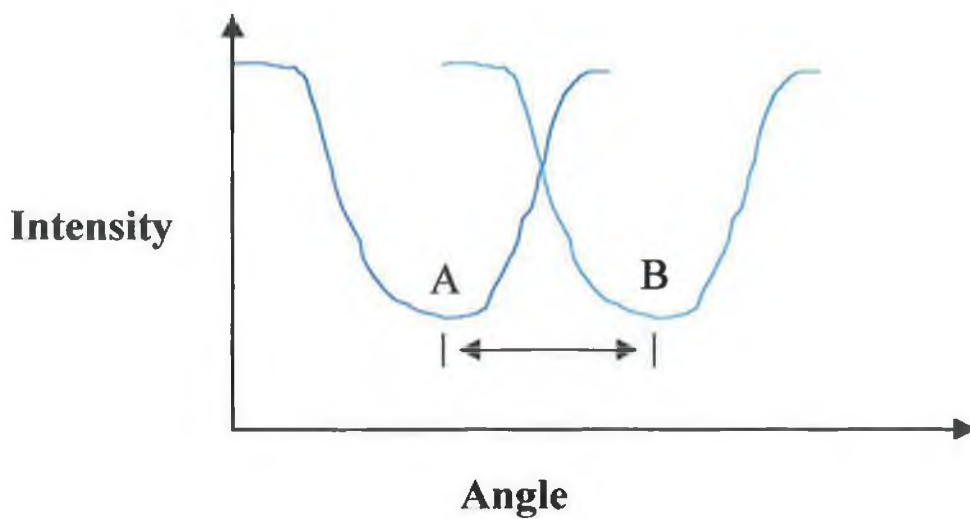
Pharmacia were the first company to introduce a commercial SPR biosensor, BIAcore, onto the market in 1990. In recent years, other optical biosensors have been developed and are also available. These include systems by Affinity Sensors, (IASys), IBIS Technologies BV, (IBIS), BioTul AG, (Kinomics), Nippon Laser and Electronics Lab, (SPR 670), and Texas Instruments, (TI-SPR), Artificial Sensing Instruments, (OWLS), Aviv, (PWR-400), and Quantech Ltd., (FasTraQ), (Rich and Myszka, 2000; McDonnell, 2001).



**Figure 5.1A:** Basic diagram of a biological sensor. It is composed of three main elements, the biosensor where the biological interaction occurs, a transducer component and, a data acquisition and presentation part. Figure 5.1B shows a schematic of the surface plasmon resonance, which is the basis of the BIAcore biosensor.



**Figure 5.1B:** Schematic of the basis of surface plasmon resonance. In this example the antigen is immobilised on the sensor chip surface. Light from a high intensity emitting diode is focused onto the gold sensor chip surface by means of a glass prism under conditions of total internal reflection. The reflected light is measured using a two-dimensional photo-diode array. Under conditions of total internal reflection at the gold surface, an evanescent wave propagates into the medium of lower refractive index. The angle at which this occurs is known as the resonant angle. This angle is sensitive to changes in the refractive index of the layer adjacent to the gold surface. This can be used to monitor the interaction occurring at the surface in 'real-time'. In the above schematic the following is represented: the binding of the antibody to the immobilised antigen causes in a change in the refractive index at the sensor surface which resulted in a change in the angle of incidence of the reflected light, from A to B.



**Figure 5.2:** The change in the angle of incidence as described in Figure 5.1B is translated by the BIAcore software to display the SPR response curve. The interaction between the immobilised antigen and antibody as the antibody solution was passed over the surface, caused a mass change and an increase in the SPR angle. The result is the increase in response unit seen in the sensorgram.

### 5.1.3 Applications of BIAcore Technology

BIAcore was introduced onto the market in 1990, as the first SPR biosensor. Since then, extensive investigations have been performed in many different areas. This section will focus on BIAcore biosensors as it is the most publicised, however, it is possible that other SPR sensors could also be used in similar applications. The underlying characteristic of BIAcore is its ability to monitor biomolecular interactions. This opens up a plethora of potential uses for the BIAcore as this is an elemental step in basic research, analytical assays and drug discovery. Briefly, these applications include quantitative concentration determination of an analyte, measurement of binding constants to see how fast a complex forms and dissociates, measurement of the affinity of an antigen:antibody complex, and epitope mapping of monoclonal antibodies. These applications have been well documented over the last decade. (Malmqvist, 1993; Fägerstam & O'Shannessy, 1993; Johne, *et al.*, 1993; Daly *et al.*, 2000; Quinn & O'Kennedy, 2001).

BIAcore introduced the new BIAcore 3000 in 2001, this offers increased sensitivity over existing sensors and an 'on-line' reference curve subtraction. BIAcore could potentially be utilised for screening during the production of antibodies. Chapter 4 described the screening process for the monoclonal antibodies using ELISA. This is a very labour and time-intensive process. It is possible that the supernatant samples, which at the initial stages could number over 1000, could be screened using the BIAcore. The regeneration profile of the assay would have to be successful for this high number of samples. In the production of an antibody against a hapten, it is ideal that the antigen of interest would be immobilised onto the chip surface directly, without the need for a protein conjugate. If, however, the hapten-protein conjugate is immobilised, the on-line reference curve subtraction can be used to detect the non-specific binding of the supernatant components to the immobilised protein compared to the binding to the hapten-protein immobilised surface. The reason behind the preference for direct immobilisation of the hapten are two-fold. Firstly, the supernatant that interacted with the hapten would have a higher possibility of being specifically bound by it, as opposed to the case of a antigen-protein conjugate being immobilised, whereby, the antibody may recognise the linker region or protein part of the conjugate. Secondly, previous studies have shown, (Fitzpatrick, 2001) that a directly immobilised small hapten gives better reproducibility and stability leading to increased number of

regeneration cycles, compared to the hapten-protein conjugate. The direct immobilisation of a hapten is dependent on the presence of suitable reactive groups and the ease of derivatisation of the molecule if such reactive groups are not already present. The ability of BIAcore biosensors to collect eluted samples after binding to the immobilised surface on the sensor chip expands the potential applications. Malmberg & Borrebaeck (1995) panned a phage display library for the selection of phage-displayed antibody fragments. The specific bound phage particles were eluted from the immobilised surface and collected.

BIAcore have introduced a range of alternative sensor chip surfaces that provide different immobilisation chemistries to couple ligands to the sensor surface so that the interaction can be studied in the most appropriate manner. The basic chip has a layer of carboxymethylated dextran and this is suitable for most applications. Other chips include the B1, which has less carboxyl groups available, and the F1 that has a thinner dextran layer. This is suitable for analysis of solutions with large particles that may be hindered in the longer dextran matrix. The NTA chip is able to bind histidine-tagged molecules. This complements other laboratory techniques that involve His-tags, such as chelation chromatography. On the C1 chip the carboxyl groups are directly attached to the sensor surface. This chip without the dextran layer could be useful in work involving large particles such as cells.

Recent developments in the field of SPR include coupling the technique to mass spectroscopy. Common techniques for protein characterisation include the laborious two dimensional gel separation, and subsequent sequencing. Mass spectroscopy has many advantages including specificity and sensitivity. The coupling of SPR biosensors to mass spectroscopy mean that the SPR technology can detect and capture specific proteins and then they can be analysed by MS. The combination of the technologies could result in a powerful tool for microarray analysis in proteomics, (Williams and Addona, 2000; Natsume *et al.*, 2001). Another application for SPR in proteomics is in the area of orphan ligand screening in drug development. The SPR biosensor can detect such ligands from a variety of sources of fluids and media (Myszka & Rich, 2000). Another area where SPR is becoming popular is in the analysis of biological interactions with biomaterials (Green *et al.*, 2000). The SPR sensor flow cell can be modified with a range of different surfaces by coating of thin layers above the gold surface. These surfaces can include dextran, lipid bilayers, adsorbed copolymers or self- assembled monolayers.



#### 5.1.4 Antibody Affinity

The affinity of an antibody describes the strength of the bond between the antibody and its specific antigen. It is not just a theoretical value as it is important in determining a key physiological property of the antibody and this in turn determines its potential usefulness. It is important to include the affinity of an antibody in the characterisation process. Determination of affinity can be done by many different methods. However, all are based on a system that allows the antibody and antigen to come to equilibrium. The interaction of the antibody and antigen at equilibrium can be described by:



- [A] = Free Antigen Concentration
- [B] = Free Antibody Concentration
- [AB] = Antigen:Antibody Complex
- $k_a$  = Association Rate Constant
- $k_d$  = Dissociation Rate Constant

By applying the Law of Mass Action the affinity constant can be determined:

$$k_a / k_d = K_A = [AB] / [A] [B] \quad \text{Equation 5.2}$$

Where  $K_A$  = Equilibrium association rate constant

$$K_A = 1/K_D$$

$K_D$  = Equilibrium dissociation rate constant

The common perception is that antibodies with high affinities for their specific antigen are better than those with lower affinity, given that the affinity is a measure of the strength and stability of the antibody-antigen complex. This is true in the case of many biological processes. However, in the case of an antibody-based detection assay an antibody of high affinity can cause problems. An example of this is an assay developed on the BIAcore system. The antigen of interest is immobilised onto the dextran layer of a gold sensor chip surface. Antibody is then injected over the immobilised surface and the binding causes a change in mass bound to the sensor chip surface. The change in

the refractive index at the surface causes a shift in the resonant angle of reflected light and this shift is proportional to the change in mass of antibody bound. This immobilised surface has to be used many times in some cases up to a couple of hundred for the assay to be considered efficient in terms of time and expense. The surface needs to be regenerated after each pulse of antibody:antigen solution, for example, in the case of an inhibition assay for the detection of an analyte, as discussed in Section 5.3.4. If the antibody is of a high affinity and is bound strongly to the immobilised antigen, a harsh regeneration solution is required to break the interaction. This has negative implications, as in some cases this could affect the immobilised antigen and cause degradation of the surface. It could also effect the dextran layer of the surface and destroy the immobilised surface. So, in this case an antibody of medium affinity is sufficient and more desirable.

#### **5.1.5 Affinity Measurement by ELISA**

Classical methods for the determination of affinity constants include equilibrium dialysis, immunoprecipitation, and fluorescence assays based on the changes in the fluorescent properties of the antibody or antigen occurring by the binding interaction. Friguet *et al.* (1985) described an ELISA-based method for the determination of the equilibrium dissociation constant, that compares favourably with results from immunoprecipitation and fluorescence transfer assays. A series of antigen standards are prepared and incubated with a constant nominal concentration of antibody. The mixtures are allowed to reach equilibrium overnight. Another set of dilutions of the antibody are set up and also left overnight. The solutions are then applied to ELISA plate wells that have been coated with the antigen of interest, and the ELISA is developed as described in Section 2.9.6. These antibody standards are used to construct a curve of nominal antibody concentration versus absorbance. A linear relationship can be seen from the amount of antibody added to the wells and the absorbance. If the antibody at a total concentration of  $i_0$  is incubated with antigen at a particular concentration, then  $i$ , the free antibody concentration, will be equal to the absorbance, divided by the absorbance of the antibody in the absence of antigen, as follows:

$$i/i_0 = A/A_0$$

**Equation 5.3**

According to the mass conservation equations, the concentrations of bound antibody,  $x$ , and free antigen,  $a$ , at equilibrium can be calculated as follows:

$$x = i_0 - i \quad \text{and} \quad a = a_0 - x$$

where:

$a_0$  = total concentration of antigen

$a_0$ ,  $x$ , and  $i_0$  are related to  $K_D$  by the Klotz equation:

$$1/x = 1/i_0 - (K_D/a \times i_0) \quad \text{Equation 5.4}$$

This can be related to the absorbance readings as follows:

$$x = i_0 \times (A_0 - A/A_0) \quad \text{Equation 5.5}$$

and

$$a = a_0 - i_0 \times (A_0 - A/A_0) \quad \text{Equation 5.6}$$

And so the equation 5.4 can be rewritten as follows:

$$A_0/A_0 - A = 1 + K_D/(a_0 - i_0) (A_0 - A/A_0) \quad \text{Equation 5.7}$$

As one of the prerequisites of the Friguet assay is that the antigen concentration is at least 10 times molar excess of the antibody, then  $a$  can be approximated by  $a_0$ , and the equation becomes the following:

$$A_0/A_0 - A = 1 + K_D/a_0 \quad \text{Equation 5.8}$$

The fraction of total antibody, bound by the antigen,  $(A_0 - A/A_0)$ , abbreviated to  $(V)$ , was calculated for each antigen concentration. The slope of the plot of  $1/V$  versus  $1/[\text{Antigen Concentration}]$ , known as the Klotz Plot, defined the equilibrium dissociation constant.

### 5.1.6 Affinity Measurement by BIAcore

The BIAcore can be used to determine the affinity of an antibody based on the assumption that the antibody-antigen interaction follows pseudo-first order reaction kinetics. Measurement of affinity in solution with the BIAcore programs is based on the determination of the free concentration of one of the interactants in equilibrium mixtures. A standard curve of known concentrations has to be constructed, as well as the equilibrium mixtures for determination of the affinity. In the case of an antibody-antigen affinity, the antigen is immobilised on the sensor chip surface. A range of antibody dilutions of known molar concentrations are passed over the surface and a standard curve constructed. A constant concentration of the antibody is mixed with a series of known concentrations of the antigen and allowed to reach equilibrium. The mixtures are then passed over the sensor chip surface. The remaining free concentration of the unbound antibody is determined by reference to the standard curve. The affinity constant is determined by applying the solution affinity fit to the data using the BIAevaluation software. The equation used is:

$$[B]_{\text{free}} = (B - A - K_D) / 2 + \sqrt{((A + B + K_D) / 4 - (A)(B))} \quad \text{Equation 5.9}$$

$B_{\text{free}}$  = free concentration of component B (antibody)

A = molar concentration of A

B = molar concentration of B

A basic factor determining the kinetics of antibody and antigen interactions is the mass transport of analyte across a stationary layer of solution at the surface of the sensor chip flow cell. The mass transfer coefficient is dependent on the flow cell geometry and the flow rates. If the mass transfer is limiting, the mass transfer determines the rate of binding of the antigen to the immobilised ligand. For the correct determination of kinetic constants using BIAcore technology, the mass transfer limitation must be eliminated, so the rate of binding is only determined by the kinetic constants. This can be achieved by using a high flow rate to remove the mass transfer limitation.

The development of BIAcore based immunoassays for the detection of amphetamine and derivatives is investigated in the following results sections, and applied to saliva samples. The anti-amphetamine and anti-methamphetamine monoclonal antibodies are used for this application. The affinities of the antibodies for amphetamine derivatives are also investigated using ELISA and BIAcore-based assays.

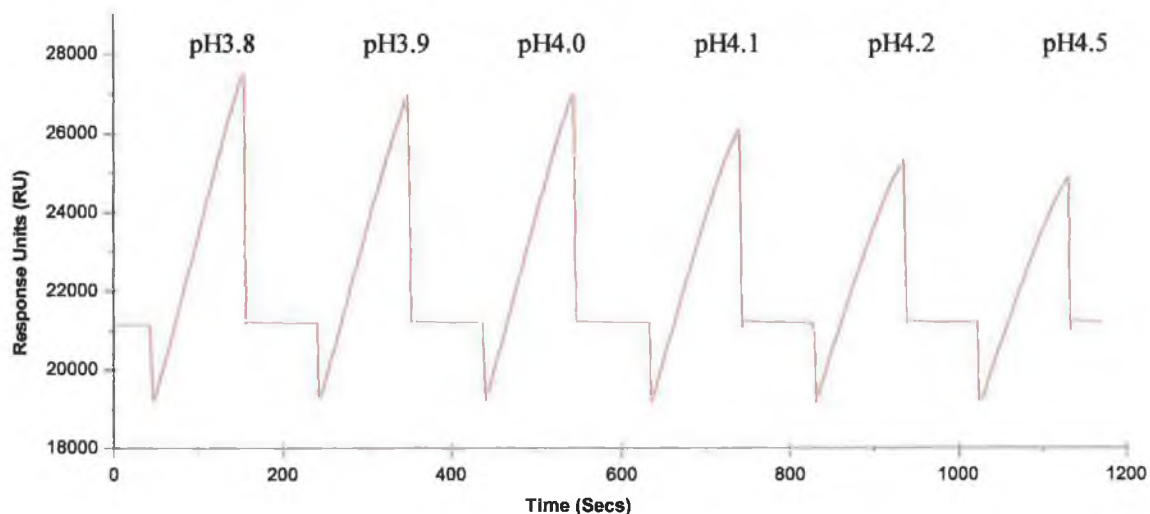
## **5.2 Results**

### **5.2.1 Development of BIAcore-based competitive immunoassay for the detection of amphetamine using anti-amphetamine monoclonal antibody, Clone 4EP18E**

#### **5.2.1.1 Preconcentration studies**

It is necessary to run a preconcentration step as a preliminary to the immobilisation of a drug-protein conjugate to the carboxymethylated dextran layer of the sensor chip. This step ensures that the immobilisation process is maximised.

Solutions of amphetamine-BSA were prepared in 10mM sodium acetate, with a range of pH from 3.9 to 5.5. The pH of the sodium acetate was adjusted with 10% (v/v) acetic acid. Each protein solution was sequentially passed over an underivatised sensor flow cell for 2 minutes at a flow rate of 5 $\mu$ l per minute. The injection pulse of each solution, is followed by a pulse of Hepes buffered saline that is sufficient to dissociate the electrostatic attraction between the drug-protein conjugate and the carboxymethylated surface. The results of the preconcentration step are shown in Figure 5.3. The optimal pH determined for immobilisation of amphetamine-BSA is pH 4.0, as the dextran layer is unable to tolerate lower pHs, because protonation of the carboxy groups on the dextran occurs leading to collapse of this layer. All immobilisations were carried out at pH 4.0. Although this pH contributes to the immobilisation process, it is necessary to chemically modify the carboxymethylated dextran to achieve immobilisation of the amphetamine-BSA conjugate.

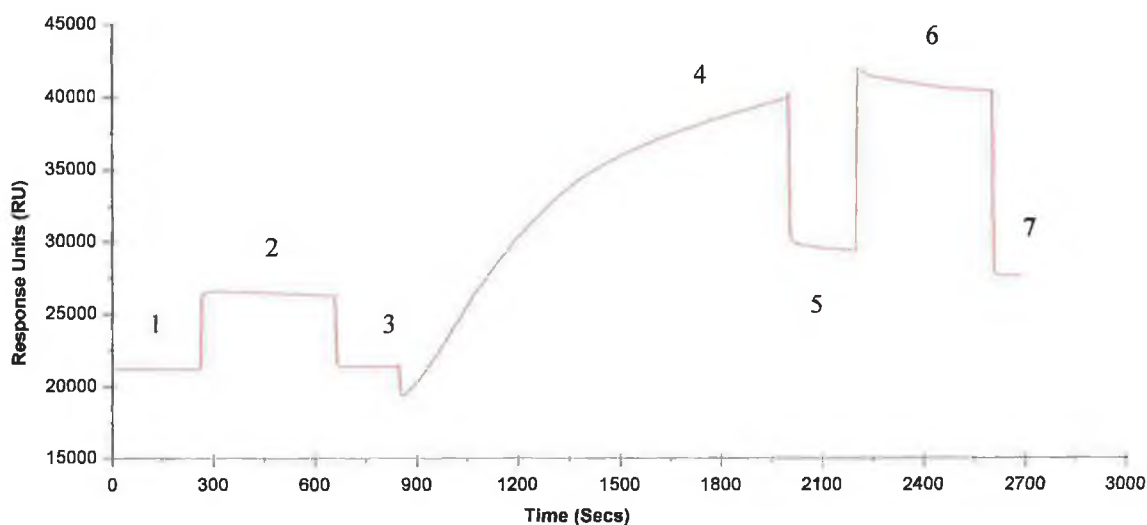


**Figure 5.3:** Preconcentration study of amphetamine-BSA in sodium acetate at various pH values onto the carboxymethylated dextran surface of the CM5 sensor flow cell. Solutions containing 50 $\mu$ g/ml of amphetamine-BSA were passed over the surface for 2 mins at a flow rate of 5 $\mu$ l/min. The response units for each solution is a measure of the electrostatic attraction between the negatively charged dextran and the positively charged protein conjugate. The ionic strength of the HEPES buffered saline is sufficient to dissociate the protein conjugate from the dextran layer. The optimal pH was determined to be pH 4.0 as shown on the figure. It is not advisable to use a pH lower than 4.0 on the dextran surface as this results in protonation of the carboxyl groups causing a collapse of the gel matrix.

### ***5.2.1.2 Immobilisation of amphetamine-BSA***

A solution of EDC/NHS was passed over the chip surface. The carboxyl groups on the dextran layer of the sensor chip were converted into active ester functional groups by the EDC, and stabilized by the NHS. The amphetamine-BSA conjugate in 10mM sodium acetate, pH 4.0, was passed over the chip. The NHS esters then react with the available amine groups on the amphetamine conjugate. Figure 5.4 shows a typical immobilisation profile.





**Figure 5.4:** Sensorgram of a typical immobilisation of amphetamine-BSA onto a CM5 dextran chip surface.

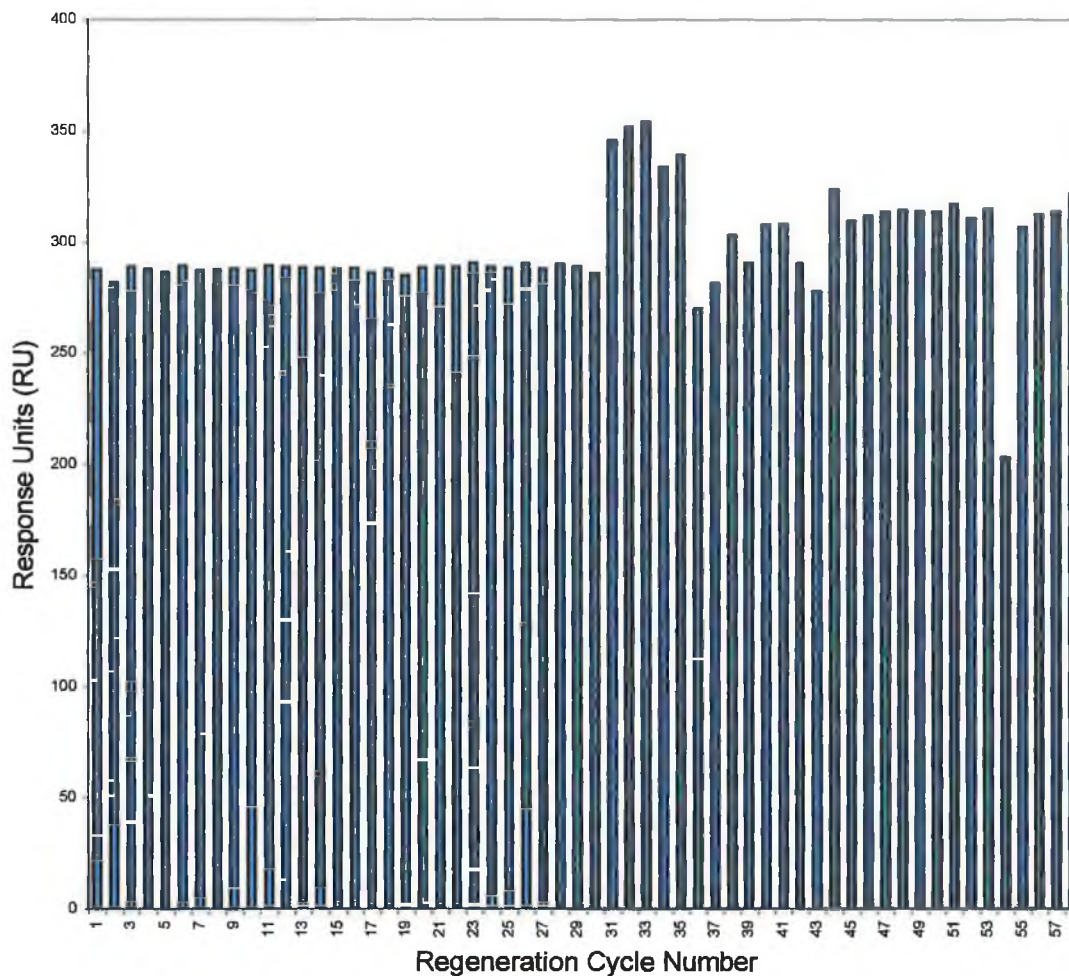
1. HBS buffer was passed over the surface and baseline measurement recorded.
2. A solution of EDC and NHS, final molarity 0.2M and 0.05M, respectively, was passed over the surface for 7 minutes at a flow rate of 5 $\mu$ l/min to activate the carboxymethylated groups.
3. After the pulse of EDC/NHS, the HBS buffer was run over the surface again. The activation of the surface was seen by the small change (approx 120-200) in response units.
4. A solution of 50 $\mu$ g/ml of amphetamine-BSA in 10mM sodium acetate, pH 4.0, was passed over the surface for 20 minutes at a flow rate of 5 $\mu$ l/min.
5. The HBS buffer was run over the surface and the excess conjugate eluted. The amount of bound conjugate was recorded as the change in response units from baseline.
6. The surface NHS-esters were deactivated by a pulse of 1M ethanolamine hydrochloride, pH 8.5. This also removed any excess non-covalently bound conjugate.
7. The HBS running buffer resumed flow over the surface and the amount of bound amphetamine-BSA can be seen from the change in the response units. Approximately 6,000RU's of amphetamine-BSA were bound on the chip surface.

### ***5.2.1.3 Regeneration Conditions***

The previous sections dealt with the immobilisation of the amphetamine-BSA conjugate on to the sensor chip. The regeneration conditions must also be optimised. It is preferable to be able to run multiple samples usually greater than 40 on a single cell of a sensor chip. To do this, the regeneration conditions must be examined and chosen carefully and a regeneration cycle set up to determine the effects of the regeneration solutions on the surface and on the binding of the antibody to the surface.

A 1/10 final dilution of anti-amphetamine monoclonal antibody was found to give a binding response of approximately 250 response units. A range of different molarities of HCl and NaOH were tried. The optimum combination found to give reproducible results was a 15 second pulse of 10 $\mu$ l of 20mM HCl and a 60 second pulse of 5 $\mu$ l 7.5mM NaOH. The surface was found to be reproducible for over 60 cycles of antibody binding and regeneration with this protocol. Figure 5.5 shows the response units for each cycle. An increase in response units was seen for cycles 31-34. This may have been due to extraneous substances at the chip surface. The decreased response units for cycle 54 may also be an anomaly, for example, an air bubble in the system.

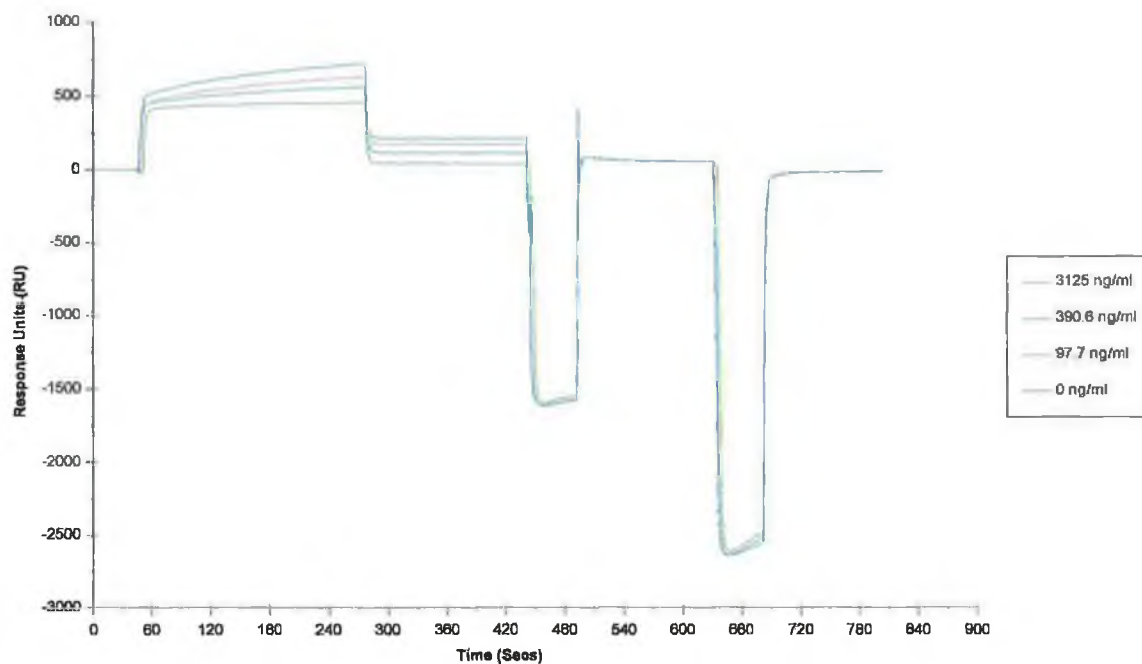
The interaction between the BSA portion of the conjugate and the antibody was examined by immobilising 50 $\mu$ g/ml BSA in 10mM sodium acetate, pH 4.0. The response of the antibody to this surface was zero RU. This response to dextran was also examined and found to be zero also.



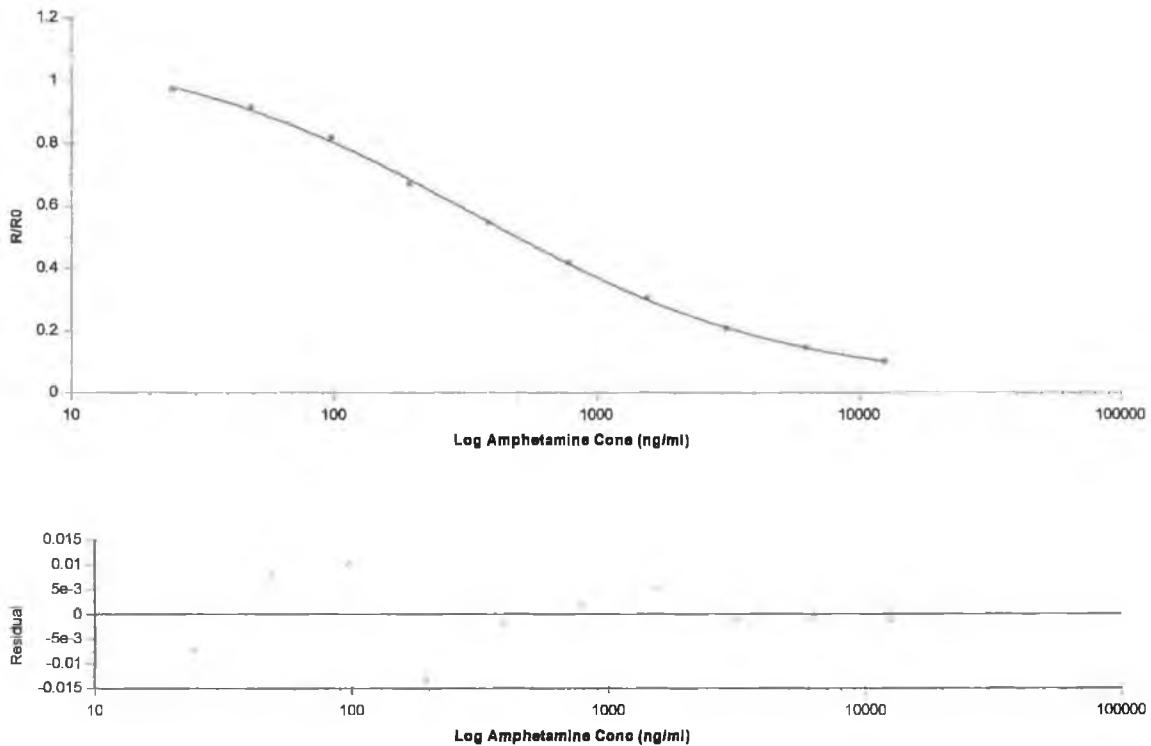
**Figure 5.5:** Regeneration profile of the anti-amphetamine monoclonal antibody binding to the amphetamine-BSA immobilised surface. A 1/10 dilution of antibody was passed over the surface for 4 minutes. The surface was regenerated with a 60 second pulse of 5 $\mu$ l of 20mM HCl and a 60 second pulse of 5 $\mu$ l of 7.5mM NaOH. An increase in response units was seen for cycles 31-34. This may have been due to an artefact. The decreased response units for cycle 54 may also be an anomaly, for example, an air bubble in the system.

#### *5.2.1.4 Determination of range of detection of amphetamine in the BIAcore competitive assay*

To determine the working range for detection of amphetamine with this assay, a number of standard amphetamine concentrations were prepared in HBS buffer, ranging from 0.09 to 25,000 ng/ml. The anti-amphetamine monoclonal antibody, diluted in HBS, was mixed with equal volumes of each standard and allowed to come to equilibrium for 15 minutes on the bench before being placed in the BIAcore for the assay run. The samples were passed over the amphetamine-BSA immobilised surface in random order. An example of the different sensorgrams are shown in the overlapped sensorgram diagram in Figure 5.6. Each cycle was followed by the regeneration cycle. Each drug-antibody solution was run over the surface three times in random order. This, therefore, eliminated any possible bias that could have been incorporated into the assay. Each value was normalised for that intra-assay by dividing the RU obtained by the RU for the positive control that only contained antibody and no amphetamine. An example of the intra-assay variability is shown in Table 5.1. The inter-assay calibration is the combination of three different assays run on three different days, (Table 5.2). The calibration curve for the inter-assay is plotted in Figure 5.7. The range of detection of the assay is 24.4 to 12,500ng/ml. The back-calculated values as determined by the four-parameter fit of the calibration curve for the amphetamine standards show the assay to very accurate for these values. The percentage recoveries ranged from 93.78 to 108.41%. The degree of accuracy can be determined by calculating the percent recovery of the known value for different concentrations. This percent recovery is a quantitative measure of the closeness of the observed result (back-calculated result) to its theoretical true value, expressed as a percent of the nominal, theoretical concentration. The percentage coefficient of variation (CV) for the range of detection is very acceptable, being between 1.66 and 6.90%, for the inter-assay. The degree of precision of the assay is expressed in the percent coefficient of variation of the intra assay variation as shown in Table 5.1.



**Figure 5.6:** Overlay plot showing examples of typical binding curves in the Biacore inhibition assay. This figure shows the binding response obtained when samples containing 0, 97.7, 390.6 and 3125 ng/ml amphetamine were incubated with anti-amphetamine monoclonal antibody and allowed to reach equilibrium. The samples were then passed over the amphetamine-BSA-coated sensor chip surface and the binding response measured. The samples were passed over the surface in triplicate and the assay was repeated over three days. The results were normalised and were used to construct the inter-day calibration curve, as shown in Figure 5.7.



**Figure 5.7:** Inter-day curve for the detection of amphetamine using the anti-amphetamine monoclonal antibody on an amphetamine-BSA immobilised surface. The data was correlated to a four-parameter model fit and the plot constructed using BIAevaluation 3.1 software. Each point on the graph is the average of three results obtained on three different days from a set of three replicates. Each value was normalised for that intra-assay by dividing the RU obtained by the RU for the positive control that only contained antibody and no amphetamine. The coefficient of variation, back-calculated amphetamine concentration and the percentage recovery are shown in Table 5.2. The range of detection of the assay is 24.4 – 12500ng/ml.

**Table 5.1:** Intra-assay variation (degree of precision) for the detection of amphetamine in the BIAcore-based competitive assay using the anti-amphetamine monoclonal antibody. The results are the mean of three replicates.

<b>Actual Amphetamine Conc (ng/ml)</b>	<b>Back-Calculated Amphetamine Conc (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
24.4	23.8	5.41	97.61
48.8	49.3	4.70	100.96
97.7	105.3	10.50	107.87
195.3	171.1	10.09	87.61
390.6	425.3	7.40	108.88
781.2	782.1	3.36	100.11
1562.5	1517.8	7.85	97.14
3125.0	3154.2	9.24	100.93
6250.0	6241.6	6.14	99.87
12500.0	12528.0	8.46	100.22

**Table 5.2:** Inter-assay variation (degree of accuracy and reproducibility) for the detection of amphetamine in the BIAcore-based competitive assay using the anti-amphetamine antibody. The range of detection of the assay is 24.4 – 12500 ng/ml. The results presented are the means obtained from three intra-day assays, each performed on three replicates.

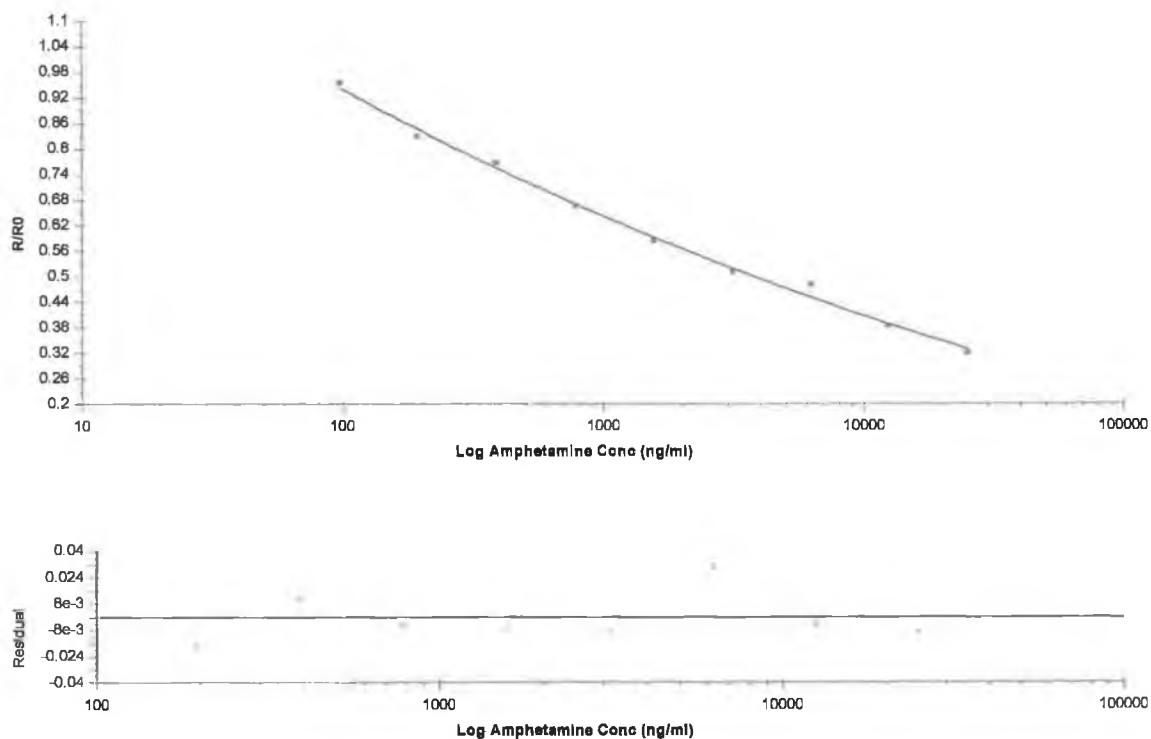
<b>Actual Amphetamine Conc (ng/ml)</b>	<b>Back-Calculated Amphetamine Conc. (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
24.4	26.5	6.81	108.41
48.8	45.8	3.34	93.78
97.7	91.8	2.97	93.98
195.3	209.4	6.90	107.20
390.6	393.8	4.81	100.81
781.2	773.3	2.70	98.98
1562.5	1508.2	2.83	96.53
3125.0	3159.0	1.66	101.09
6250.0	6295.7	3.23	100.73
12500.0	12771.1	5.54	102.17



### ***5.2.2 Development of a BIAcore-based competitive immunoassay for the detection of amphetamine in spiked saliva samples***

Saliva was applied to the BIAcore-based inhibition assay for the determination of amphetamine. Negative control saliva samples were initially applied to the assay to determine the characteristics of the saliva with regard to the assay format. There was no interference noted between the saliva and the immobilised amphetamine-BSA. The inhibition assay was then established for the saliva samples containing amphetamine as described for the model assay.

An example of the intra-assay variability is shown in Table 5.3. The inter-assay calibration is the combination of three different assays run on three different days. The calibration curve for the inter-assay is plotted in Figure 5.8. The range of detection of the assay is 97.7 to 25,000ng/ml. The back-calculated values as determined by the four-parameter fit of the calibration curve for the amphetamine standards show the assay to very accurate for these values, ranging from 72.55 to 114.16%. The degree of accuracy can be determined by calculating the percent recovery of the known value for different concentrations. The range of the percentage coefficient of variations are higher for the saliva assay, (2.65 to 29.36%) as compared to the model assay in buffer. The range of detection is also different in buffer compared to saliva, 24.4 to 12500ng/ml in the model buffer assay, compared to 97.7 to 25000 ng/ml in the saliva-based assay.



**Figure 5.8:** Inter-day curve for the detection of amphetamine in saliva samples using the anti-amphetamine monoclonal antibody on an amphetamine-BSA immobilised surface. The data was correlated to a four-parameter model fit and the plot constructed using BIAevaluation 3.1 software. Each point on the graph is the average of three results obtained on three different days from a set of three replicates. Each value was normalised for that intra-assay by dividing the RU obtained by the RU for the positive control that only contained antibody and no amphetamine. The coefficient of variation, back-calculated amphetamine concentration and the percentage recovery are shown in Table 5.3 (Intra-assay) and Table 5.4 (Inter-assay).

**Table 5.3:** Intra-assay variation (degree of precision) for the detection of amphetamine in saliva samples in the BIAcore – based inhibition assay using the anti-amphetamine monoclonal antibody. The results presented are the mean obtained from three replicates.

<b>Actual Amphetamine Conc (ng/ml)</b>	<b>Back-Calculated Amphetamine Conc. (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
97.7	101.8	2.93	104.23
195.3	191.1	1.51	97.82
390.6	374.5	3.96	95.87
781.2	830.9	0.99	106.37
1562.5	1481.4	9.70	94.81
3125.0	3239.2	2.46	103.65
6250.0	6366.3	4.05	101.86
12500.0	11390.2	11.63	91.12
25000.0	27153.4	14.40	108.61

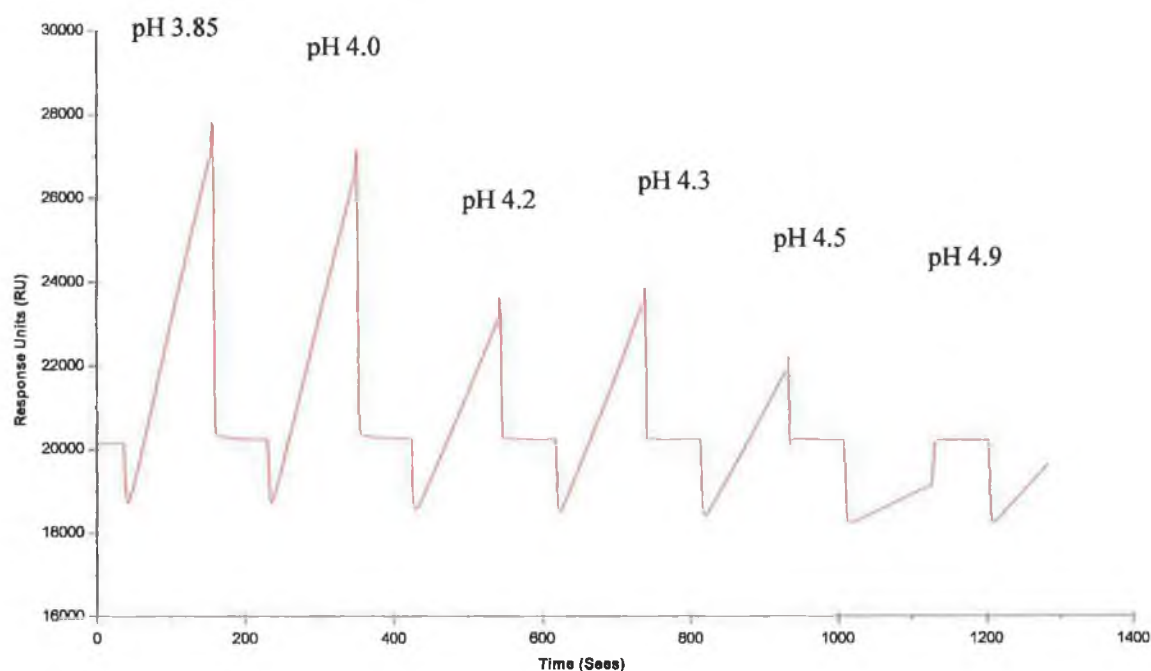
**Table 5.4:** Inter-assay variation (degree of accuracy and reproducibility) for the detection of amphetamine in saliva samples in the BIAcore – based inhibition assay using the anti-amphetamine monoclonal antibody. The range of detection of the assay was 97.7 to 25,000ng/ml. The results presented are the means obtained from three intra-day assays, each performed on three replicates.

<b>Actual Amphetamine Conc (ng/ml)</b>	<b>Back-Calculated Amphetamine Conc. (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
97.7	91.2	7.40	93.39
195.3	222.9	21.62	114.16
390.6	357.3	20.62	91.46
781.2	814.6	18.58	104.27
1562.5	1664.8	14.73	106.55
3125.0	3404.2	2.65	108.94
6250.0	4534.5	25.74	72.55
12500.0	13156.3	22.10	105.25
25000.0	27960.7	29.36	111.84

### 5.2.3 Development of BIAcore-based competitive immunoassay for the detection of methamphetamine using anti-methamphetamine monoclonal antibody, Clone P18D

#### 5.2.3.1 Preconcentration studies

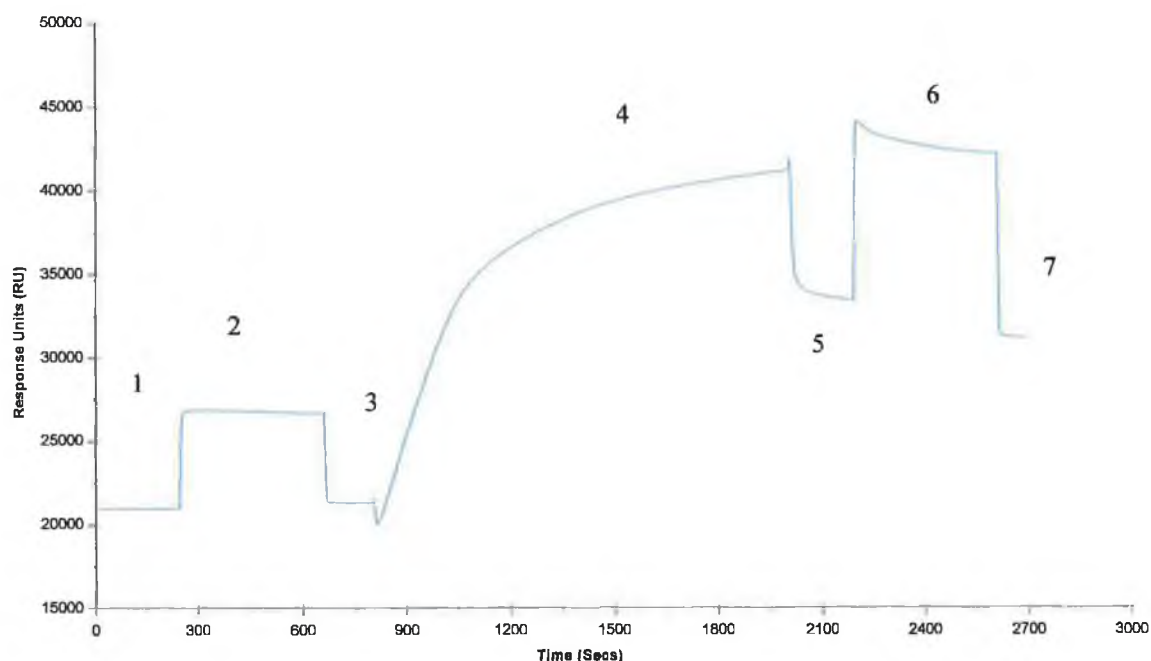
A range of solutions of methamphetamine-BSA were prepared in 10mM sodium acetate of various pH, from 3.8 to 4.9. The pH of the sodium acetate was adjusted with 10% (v/v) acetic acid. Each protein solution was sequentially passed over an underderivatised sensor as described previously. The results of the preconcentration step are shown in Figure 5.9. The optimal pH determined for immobilisation of methamphetamine-BSA is pH 4.0. All immobilisations were carried out at this pH.



**Figure 5.9:** Preconcentration study of methamphetamine-BSA in sodium acetate at a various pH values onto the carboxymethylated dextran surface of the flow cell. The solutions containing 50µg/ml of methamphetamine-BSA were passed over the surface for 2 minutes at a flow rate of 5µl/min. The response units for each solution is a measure of the electrostatic attraction between the negatively charged dextran and the positively charged protein conjugate. The ionic strength of the Hepes buffered saline is sufficient to dissociate the protein conjugate from the dextran layer. The optimal pH was determined to be pH 4.0 as shown on the figure.

### ***5.2.3.2 Immobilisation of methamphetamine-BSA***

The immobilisation of the methamphetamine-BSA was performed as described previously. A solution of EDC/NHS was passed over the chip. The carboxyl groups on the dextran layer of the sensor chip were converted into active ester functional groups by the EDC, and stabilized by the NHS. The methamphetamine-BSA conjugate in 10mM sodium acetate, pH 4.0, was passed over the chip. The NHS esters then react with the available amine groups on the methamphetamine conjugate. Figure 5.10 shows a typical immobilisation profile.



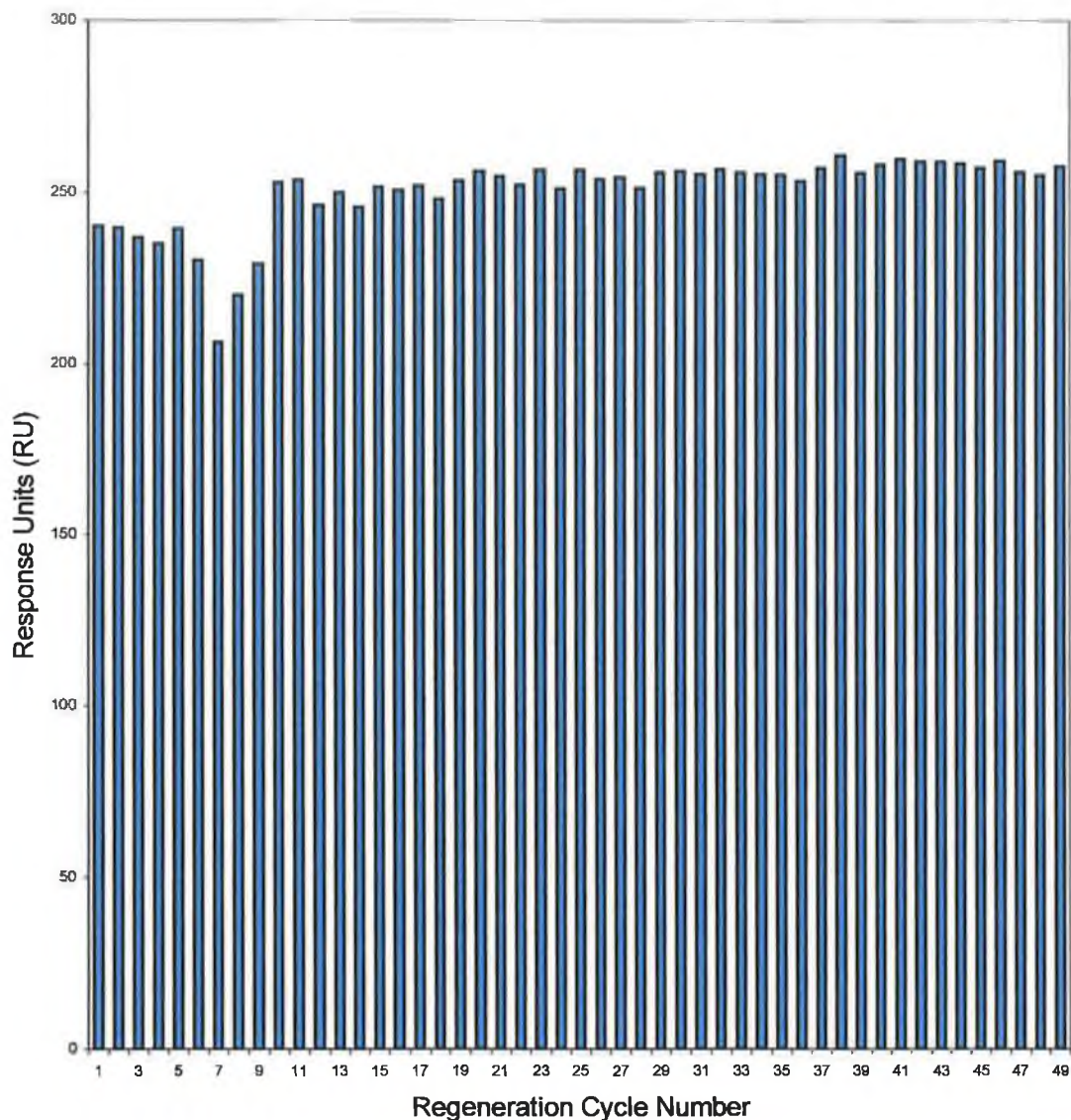
**Figure 5.10:** Sensorgram of a typical immobilisation of methamphetamine-BSA onto a CM5 dextran chip surface.

1. HBS buffer was passed over the surface and baseline measurement recorded.
2. A solution of EDC and NHS, final molarity 0.2M and 0.05M respectively, was passed over the surface for 7 minutes at a flow rate of 5 $\mu$ l/min to activate the carboxymethylated groups.
3. After the pulse of EDC/NHS, the HBS buffer was run over the surface again. The activation of the surface was seen by the small change (approx 120-200) in response units.
4. A solution of 50 $\mu$ g/ml of methamphetamine-BSA in 10mM sodium acetate, pH 4.0, was passed over the surface for 20 minutes at a flow rate of 5 $\mu$ l/min.
5. The HBS buffer was run over the surface and the excess conjugate eluted. The amount of bound conjugate was recorded as the change in response units from baseline.
6. The surface NHS-esters were deactivated by a pulse of 1M ethanolamine hydrochloride, pH 8.5. This also removed any excess non-covalently bound conjugate.
7. The HBS running buffer resumed flow over the surface and the amount of bound amphetamine-BSA can be seen from the change in the response units. Approximately 10,000RU's of methamphetamine-BSA were bound on the chip surface.

### ***5.2.3.3 Regeneration Conditions***

As discussed in the previous sections, the regeneration profile for the antibody and surface have to be established.

A 1/5 final dilution of anti-methamphetamine monoclonal antibody was found to give a binding response of approximately 200 response units. A range of different molarities of HCl and NaOH were tried and the optimum combination found to give reproducible results was a 60 second pulse of 5 $\mu$ l of 10mM HCl. The surface was found to be reproducible for over 50 cycles of antibody binding and regeneration with this protocol. Figure 5.11 shows the response units for each cycle and it can be seen that the first 8 cycles gave variable results. For this reason, for all assays, a preliminary run of 10 cycles of antibody binding and subsequent regeneration using 5 $\mu$ l 10mM HCl were run in order to optimise the system and eliminate the variability. The interaction between the BSA part of the conjugate and the antibody was examined by immobilising 50 $\mu$ g/ml BSA in 10mM sodium acetate, pH 4.0. The response of the antibody to this surface was zero RU. This response to dextran was also examined and found to be zero also.

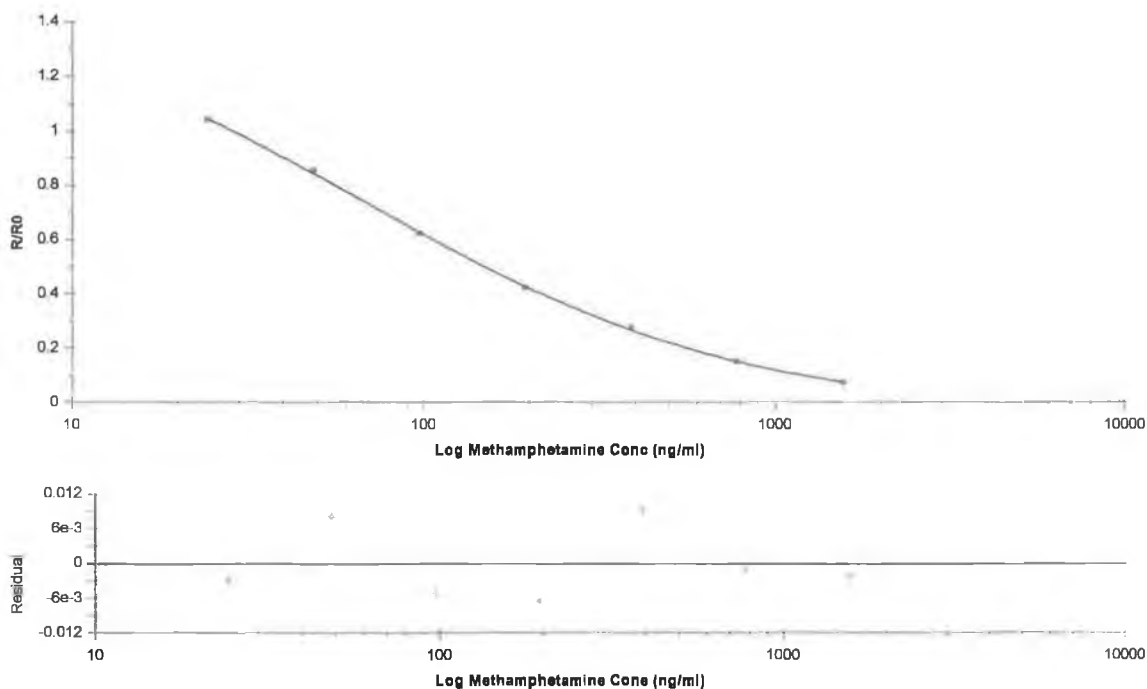


**Figure 5.11:** Regeneration profile of the anti-methamphetamine monoclonal antibody binding to the methamphetamine-BSA immobilised surface. A 1/10 dilution of antibody was passed over the surface for 4 minutes. The surface was regenerated with a 60 second pulse of 5  $\mu$ l of 10mM HCl. The first 8 cycles gave variable results. Hence, for all assays, a preliminary run of 10 cycles of antibody binding and subsequent regeneration using 5  $\mu$ l 10mM HCl were run in order to optimise the system and eliminate the variability.



#### ***5.2.3.4 Determination of range of detection of methamphetamine in the BIAcore inhibition assay***

To determine the working range of detection of amphetamine on this assay, a number of standard methamphetamine concentrations were prepared in HBS buffer, ranging from 0.09 to 25,000 ng/ml. The inhibition assay was established as described in Section 5.2.1.3 for the anti-amphetamine assay. The values obtained were normalised as described previously. An example of the intra-assay variability is shown in Table 5.5. The inter-assay calibration is the combination of three different assays run on three different days, (Table 5.6). The calibration curve for the inter-assay is plotted in Figure 5.12. The range of detection of the assay is 48.8 to 1562.5ng/ml. The back-calculated values as determined by the four-parameter fit of the calibration curve for the amphetamine standards show the assay to very accurate for these values, between 95.49 and 102.52%. The high degree of precision of the assay is expressed in the percent coefficient of variation of the intra assay variation as shown in Table 5.5 and is acceptable.



**Figure 5.12:** Inter-day curve for the detection of methamphetamine using the anti-methamphetamine monoclonal antibody on a methamphetamine-BSA immobilised surface. The data was correlated to a four-parameter model fit and the plot constructed using BIAevaluation 3.1 software. Each point on the graph is the average of three results obtained on three different days from a set of three replicates. Each value was normalised for that intra-assay by dividing the RU obtained by the RU for the positive control that only contained antibody and no methamphetamine. The coefficient of variation, back-calculated amphetamine concentration and the percentage recovery are shown in Table 5.6. The range of detection of the methamphetamine in the assay is 48.8 to 1562.5ng/ml.

**Table 5.5:** Intra-assay variation (degree of precision) for the detection of methamphetamine in the BIAcore-based competitive assay using the anti-methamphetamine monoclonal antibody. The results presented are the mean values obtained from three replicates.

<b>Actual Methamphetamine Conc (ng/ml)</b>	<b>Back-Calculated Methamphetamine Conc (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
48.8	48.8	15.21	100.02
97.7	100.9	13.98	103.57
195.3	181.7	7.77	93.19
390.6	399.0	5.64	102.32
781.2	817.6	8.49	104.82
1562.5	1510.2	18.45	96.75

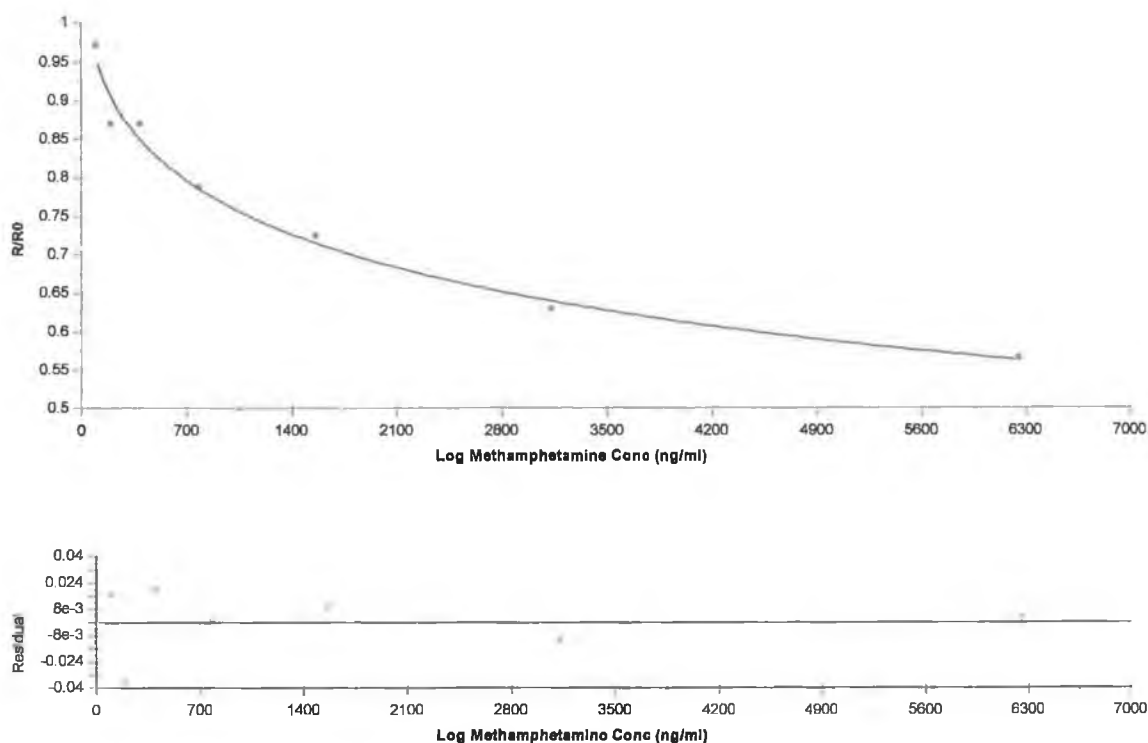
**Table 5.6:** Inter-assay variation (degree of accuracy and reproducibility) for the detection of methamphetamine in the BIAcore-based inhibition assay using the anti-methamphetamine monoclonal antibody. The range of detection was 48.8 to 1562.5ng/ml. The results presented are the mean results obtained from three intra-day assays, each assay had three replicates.

<b>Actual Methamphetamine Conc (ng/ml)</b>	<b>Back-Calculated Methamphetamine Conc. (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
48.8	47.5	17.60	97.37
97.7	99.2	17.83	101.70
195.3	199.9	22.02	102.49
390.6	372.4	17.52	95.49
781.3	784.2	12.70	100.53
1562.5	1600.3	23.79	102.52

#### ***5.2.4 Development of a BIAcore-based inhibition immunoassay for the detection of methamphetamine in saliva samples***

Saliva was applied to the BIAcore-based competitive assay for the determination of methamphetamine, as described for the anti-amphetamine antibody. A successful assay was established as shown in the calibration curve in Figure 5.13. The range of detection of the assay is 97.7 to 6250ng/ml. The percentage coefficients of variation are acceptable and are shown in Table 5.7 and Table 5.8 for the intra-day and inter-day assays, respectively. The percentage recoveries for the standards are also displayed in these tables and show a very accurate assay, 89.41 to 108.0%.

There is a different range of detection in saliva as compared to the assay in buffer, i.e. 97.7-6250 ng/ml compared to 48.8-1562.5 ng/ml. This follows the same trend as the anti-amphetamine antibody assay in the saliva-based assay.



**Figure 5.13:** Inter-day curve for the detection of methamphetamine in saliva samples using the anti-methamphetamine monoclonal antibody on a methamphetamine-BSA immobilised surface. The data was correlated to a four-parameter model fit and the plot constructed using BIAevaluation 3.1 software. Each point on the graph is the average of three results obtained on three different days from a set of three replicates. Each value was normalised for that intra-assay by dividing the RU obtained by the RU for the positive control that only contained antibody and no methamphetamine. The coefficient of variation, back-calculated methamphetamine concentration and the percentage recovery are shown in Table 5.8.

**Table 5.7:** Intra-assay variation (degree of precision) for the detection of methamphetamine in saliva samples in the BIAcore – based inhibition assay using the anti-methamphetamine monoclonal antibody. The results presented are the mean values obtained from three replicates.

<b>Actual Methamphetamine Conc (ng/ml)</b>	<b>Back-Calculated Methamphetamine Conc. (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
97.7	132.9	10.03	136.15
195.3	132.9	8.03	68.07
390.6	423.1	6.69	108.32
781.3	790.2	5.01	101.14
1562.5	1604.5	9.95	102.69
3125.0	2715.7	6.87	86.90
6250.0	7562.6	10.53	121.00

**Table 5.8:** Inter-assay variation (degree of accuracy and reproducibility) for the detection of methamphetamine in saliva samples in the BIAcore – based inhibition assay using the anti-methamphetamine monoclonal antibody. The range of detection of the assay was 97.65 to 6250 ng/ml. The results presented are the mean values obtained from three intra-day assays, each intra-assay had three replicates.

<b>Actual Methamphetamine Conc (ng/ml)</b>	<b>Back-Calculated Methamphetamine Conc. (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
97.7	105.5	10.20	108.00
195.3	198.3	22.47	101.53
390.6	349.3	12.76	89.41
781.3	840.2	14.89	107.55
1562.5	1535.9	5.13	98.30
3125.0	3096.1	11.99	99.07
6250.0	6301.6	22.08	100.83

### **5.2.5 Determination of affinity constant**

Many different techniques are used for the determination of affinity constants, as discussed in Section 5.1.5 and Section 5.1.6. The two techniques used in this study are the Friguet method, (Friguet *et al.*, 1985) and solution phase affinity determination using 'real-time' biomolecular interaction BIAcore technology.

#### **5.2.5.1 Determination of anti-amphetamine antibody and anti-methamphetamine antibody affinity constant by ELISA**

The Friguet method is based on the calculation of the dissociation constant of antibody-antigen mixtures at equilibrium in solution. A 96-well plate is coated with amphetamine-BSA. A series of antigen concentrations were incubated with a constant nominal amount of antibody. The antigen-antibody mixtures were allowed to reach equilibrium overnight. From a stock solution of antibody, serial doubling dilutions were made. These were used to construct a standard curve of nominal antibody concentration versus absorbance at 450nm. Following overnight incubation, the antibody standards and antigen-antibody mixtures were applied to the ELISA plate and the ELISA procedure completed. Absorbance readings at 450nm were related to the nominal concentration values, by reference to the constructed linear standard curve of nominal antibody concentration versus absorbance at 450nm. The fraction of total antibody that is bound by the antigen is represented by (V), and was calculated for each antigen concentration. The slope of the plot of  $1/V$  versus  $1/[\text{Antigen Concentration}]$ , known as the Klotz Plot, gives a straight line. The slope of the line defines the overall equilibrium dissociation constant for the antibody-antigen interaction at equilibrium.

Some conditions must be obeyed to satisfy the conditions of the Friguet assay. The first stipulation is that there must be a correlation between free antibody concentration and enzymatic activity, in the form of a nominal antibody concentration versus absorbance calibration curve. This curve is then used to calculate the concentration of antibody bound at equilibrium in the mixtures. The other prerequisite is that there is no readjustment of the antibody-antigen equilibrium mixture during the incubation period. This can be verified by incubating serial dilutions of the antibody for a time period, then transferring the solution to another series of similarly coated wells for the same time period. The absorbances obtained for the two sets of incubations should not differ

significantly. This would then imply that the amount of antibody bound by the solid phase immobilised antigen is negligible compared to the amount of antibody in solution, and, therefore, there is no significant displacement of the antibody-antigen mixture at equilibrium. The fraction of antibody ( $f$ ), retained by the first series of wells can be determined by the following equation:

$$f = \{ A_1(c) - A_2(c) \} / A_1(c) \quad \text{Equation 5.10}$$

Where  $A_1(c)$ ,  $A_2(c)$  are the absorbances in the first and second set of wells, respectively.

An example of such an assay is shown in Figure 5.14 for the anti-amphetamine antibody. The  $f$  value obtained was 0.18 which is acceptable. It shows that the amount of antibody retained in the ELISA represents a small amount of the free antibody. Therefore, the ELISA does not cause a significant displacement of the antigen:antibody equilibrium.

Another prerequisite is that the incubation time of the mixture in the coated wells should be kept at a minimum in order to minimise the solution phase equilibrium. The incubation period used for these assays was 15 minutes. The final prerequisite is that the total antigen concentration is in large excess over the total antibody concentration.

The standard antibody curve is shown in Figure 5.15. From this curve, the amounts of free antibody in the equilibrium solutions was calculated. The Klotz Plot for the anti-amphetamine monoclonal antibody and amphetamine is shown in Figure 5.16. The line has a  $r^2$  value of 0.99. The equilibrium dissociation constant for the antibody was calculated to be  $1 \times 10^{-9}$  M.

Stevens,(1987), introduced a correction factor into the Friguet determination of the dissociation constant. Antibodies whether monovalently bound or unbound to an antigen in solution will be able to bind to the wells of the ELISA. The correction factor introduced by Stevens is that instead of plotting  $1/V$  in the Klotz Plot,  $(1/V)^{1/2}$  is plotted. This takes into account the bivalent nature of the antibody.

Figure 5.17 shows the corrected Klotz Plot for the anti-amphetamine antibody and amphetamine, giving an equilibrium dissociation constant of  $6 \times 10^{-10}$  M. Table 5.9 lists the equilibrium constants for the interaction between the anti-amphetamine antibody and amphetamine and MDA.



Figure 5.18 and 5.19 show the Klotz Plots for the anti-amphetamine antibody and MDA, giving a  $K_D$  of  $2 \times 10^{-9}$  M, and a corrected  $K_D$  of  $6 \times 10^{-9}$  M and  $6 \times 10^{-10}$  M.

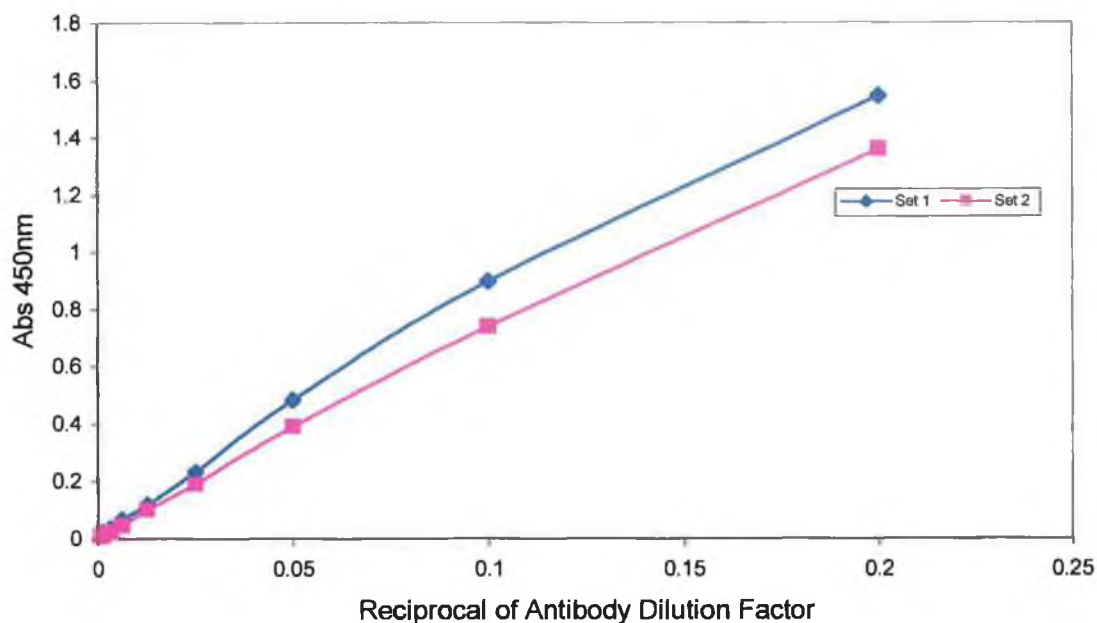
For the anti-methamphetamine monoclonal antibody, similar plots were constructed as shown in Figure 5.22 – 5.29, for the determination of the equilibrium dissociation constant of the antibody with methamphetamine, MDMA, MBDB, and MDEA. Table 5.10 lists the equilibrium dissociation constants determined for each derivative.

#### ***5.2.5.2 Determination of affinity constant of anti-amphetamine antibody and anti-methamphetamine antibody by BIAcore solution phase real-time interaction.***

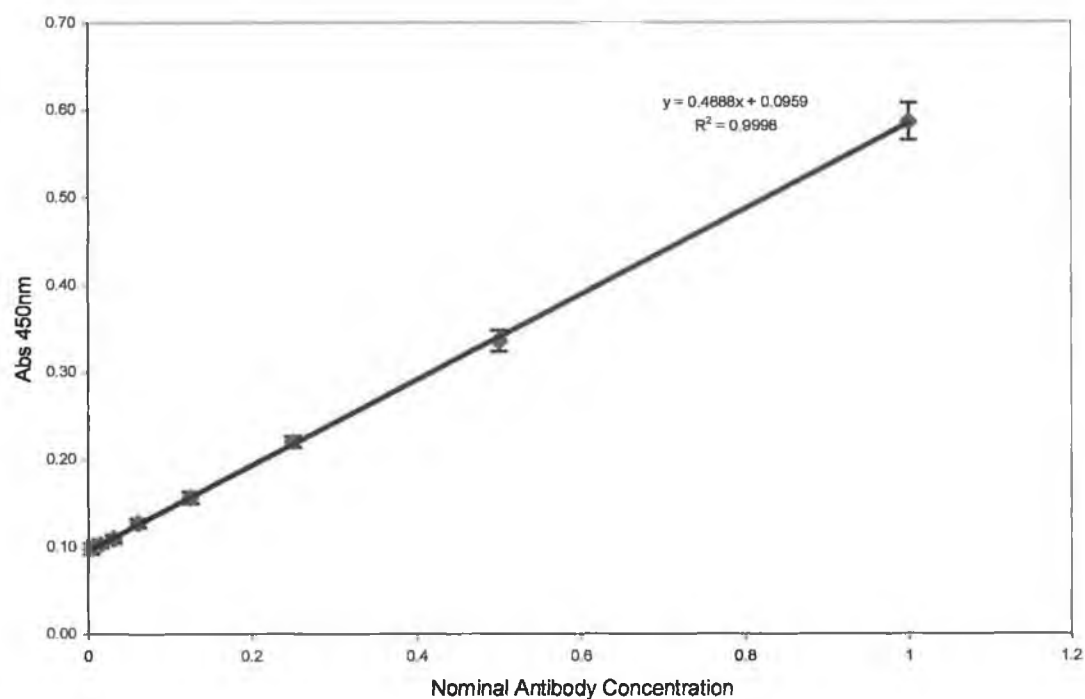
The method of BIAcore for the determination of affinity constants is based on the same principle as the ELISA based Friguet method. The drug protein conjugate is immobilised on the sensor chip and serial dilutions of a known concentration of antibody are passed over the surface. A calibration curve is constructed of mass bound versus antibody concentration. A known concentration of antibody is incubated with a range of antigen concentrations and allowed to reach equilibrium overnight. The equilibrium mixtures were then passed over the immobilised surface and the response units measured. The response units were used to calculate the amount of free antibody in the equilibrium mixtures, from the calibrated curve. A graph was then constructed of the drug concentration versus the free antibody concentration and using the solution phase interaction model in the BIAevaluation software, the overall affinity constant was determined.

Figure 5.20, and 5.21 show the curve obtained from plotting the free antibody concentration against amphetamine and MDA concentration, respectively. The equilibrium dissociation constant obtained was  $2.25 \times 10^{-9}$  M, and  $4.24 \times 10^{-9}$  M, respectively. Table 5.11 lists the equilibrium dissociation constants obtained for the interaction between the anti-amphetamine antibody and amphetamine and MDA.

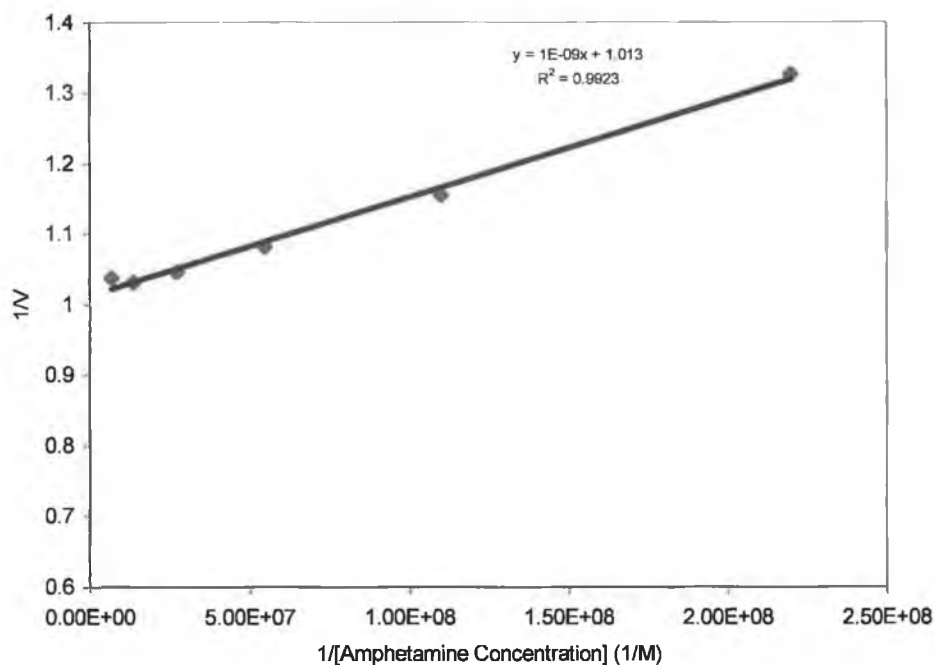
For the anti-methamphetamine monoclonal antibody, similar plots were constructed as shown in Figure 5.30 - 5.33, for the determination of the dissociation constant of the antibody with methamphetamine, MDMA, MBDB, and MDEA. Table 5.12 lists the equilibrium dissociation constants obtained for the interaction between the anti-methamphetamine antibody and the different derivatives.



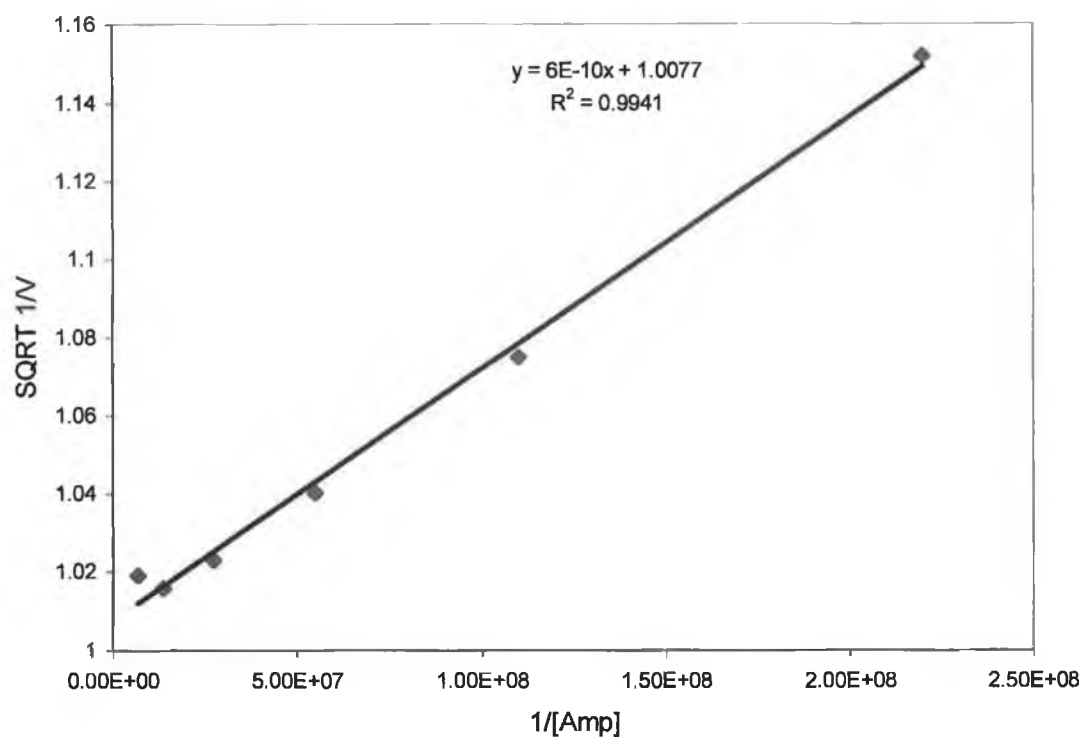
**Figure 5.14:** A prerequisite of the Friguet Assay is that there is no readjustment of the equilibrium between the antibody and antigen, during the incubation of the mixture in coated wells. This can be verified by incubating the antibody at various known concentrations in the coated wells for the specified incubation time of the assay, (Set 1). The contents of the wells are then transferred into another set of coated wells (Set 2) and incubated for the same time. The captured antibody is then detected by the anti-mouse enzyme labeled antibody. The value for,  $f$ , is then calculated as per equation 5.10. The value for  $f$ , represents the small amount of antibody that is captured in the ELISA and should represent a small fraction of the total free antibody. This, therefore, would prove that no readjustment of the antibody-antigen equilibrium occurs during the ELISA. The above figure shows that for the anti-amphetamine antibody, negligible readjustment occurred at the dilution range used, (1/300), for the determination of the affinity constant by the Friguet method.



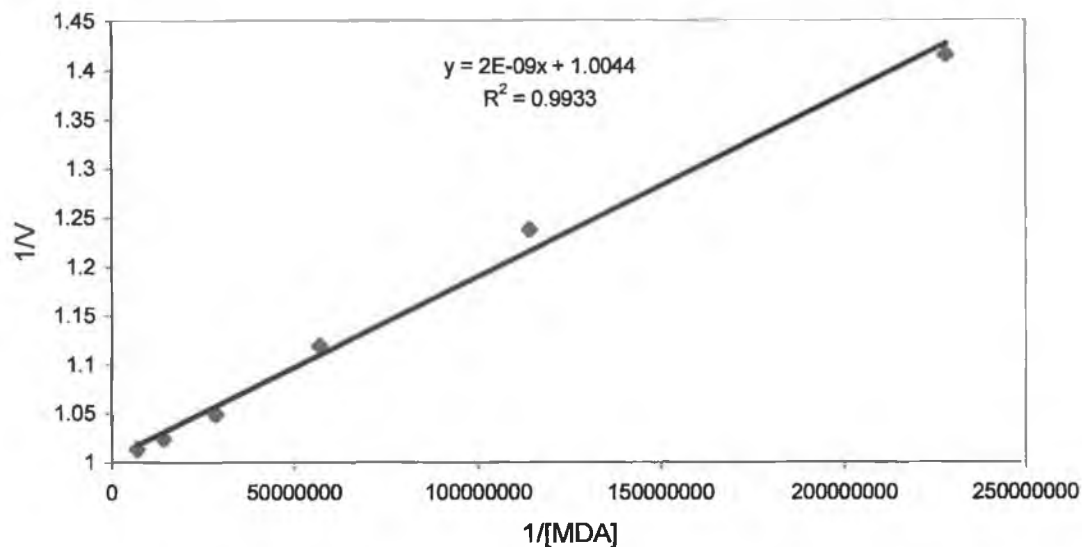
**Figure 5.15:** The standard curve of the nominal antibody concentration versus absorbance at 450nm. The highest antibody concentration was assigned the nominal antibody concentration of 1. Serial doubling dilutions were made and assigned a nominal concentration value. The antibody dilutions were applied to the wells of the ELISA plate for 15 minutes. They had been incubating on the bench for the same time period as the antibody-drug mixtures. A linear plot of nominal antibody concentration versus absorbance at 450nm was used to determine the bound and unbound fraction of antibody at equilibrium in these drug-antibody mixtures. The results shown are the average of triplicate measurements  $\pm$  standard deviation.



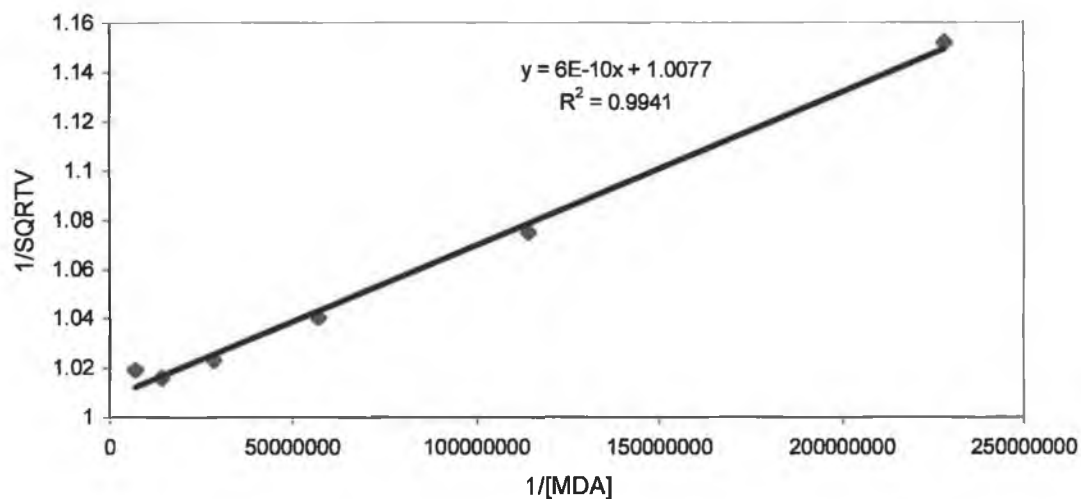
**Figure 5.16:** A plot of the reciprocal of the amphetamine concentration against the reciprocal of the bound antibody nominal concentration for the Friguet assay for the determination of the equilibrium dissociation constant. The value for the free nominal antibody concentration (NC), at each amphetamine concentration was determined from the antibody standard curve. The value of V, the bound antibody concentration, was determined as 1-NC. The slope of the above plot describes the  $K_D$  of the overall interaction. The  $K_D$  for the interaction of the anti-amphetamine monoclonal antibody and amphetamine is  $1 \times 10^{-9}$  M.



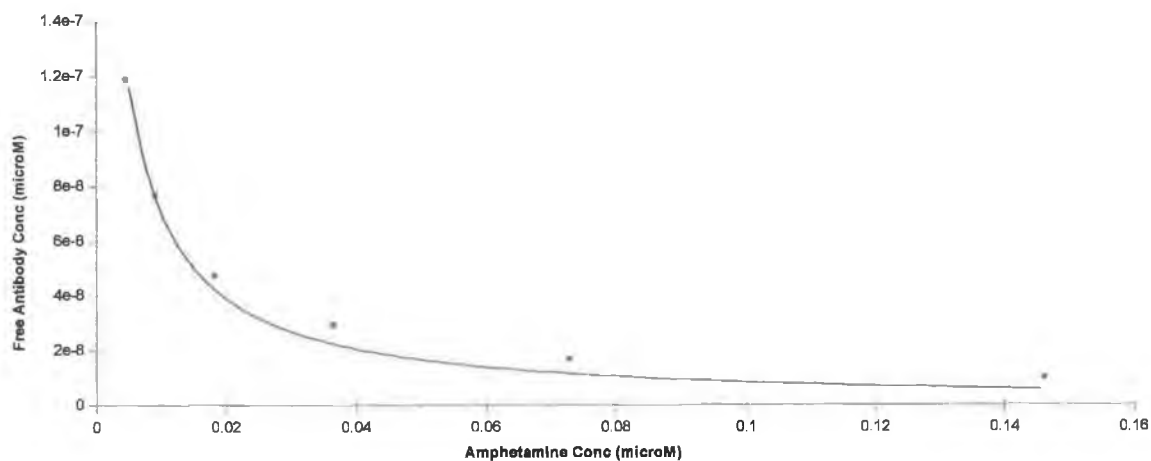
**Figure 5.17:** An adjustment of the antibody concentration, as described by Stevens, (1987), to account for the bivalency of IgG. A plot of the reciprocal of the amphetamine concentration against the reciprocal of square root of the bound antibody nominal concentration for the Friguet assay for the determination of the equilibrium dissociation constant. The bound antibody nominal concentration,  $V$ , was determined as described in legend to Fig 5.16. The slope of the above plot describes the  $K_D$  of the overall interaction. The  $K_D$  for the interaction of the anti-amphetamine monoclonal antibody and amphetamine is  $6 \times 10^{-10}$  M.



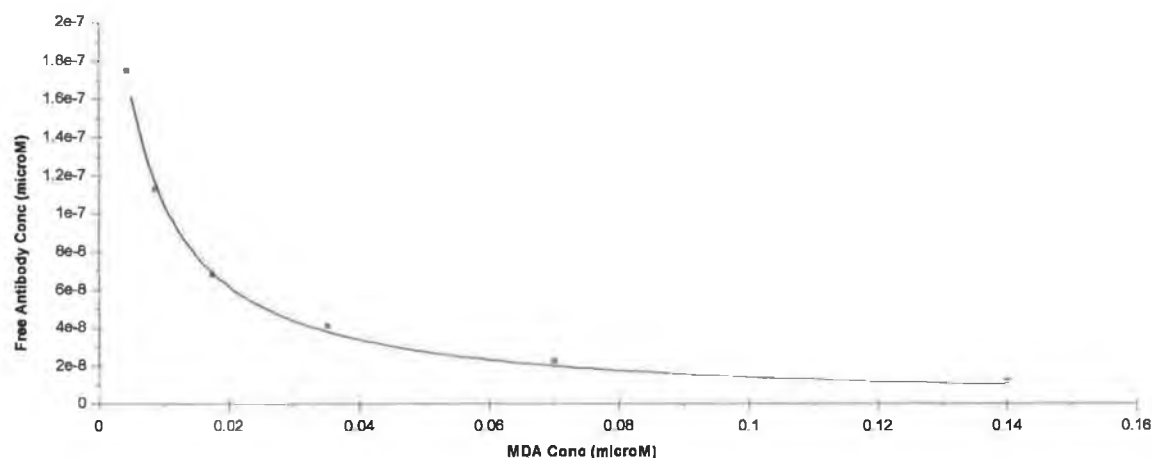
**Figure 5.18:** Friguet affinity determination of equilibrium dissociation constant for the interaction between anti-amphetamine monoclonal antibody and MDA. The  $K_D$  for the interaction of the anti-amphetamine monoclonal antibody and MDA is  $2 \times 10^{-9}$  M.



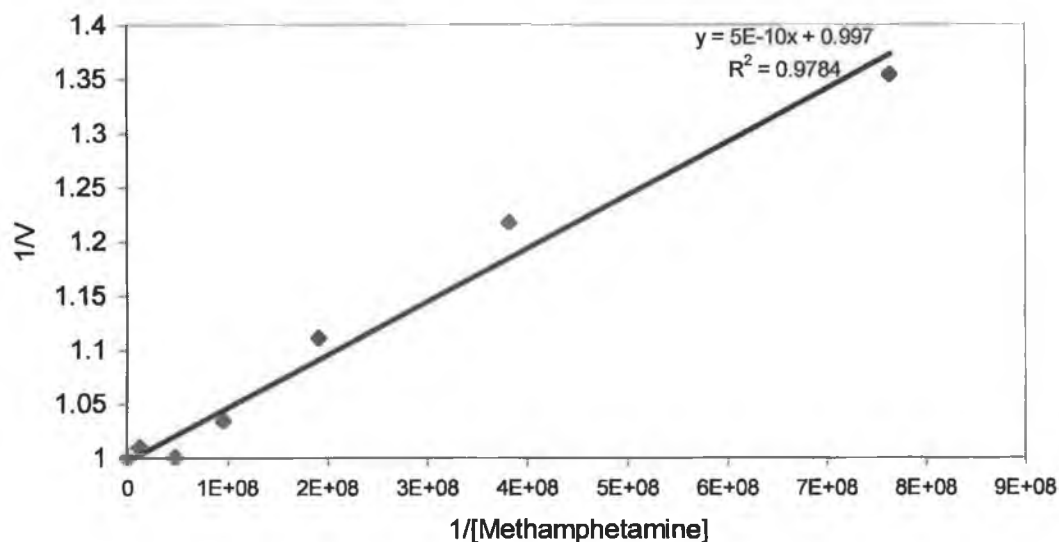
**Figure 5.19:** Friguet affinity determination of equilibrium dissociation constant using corrected IgG nominal concentration to account for bivalency of IgG. The  $K_D$  for the interaction of the anti-amphetamine monoclonal antibody and MDA is  $6 \times 10^{-10}$  M.



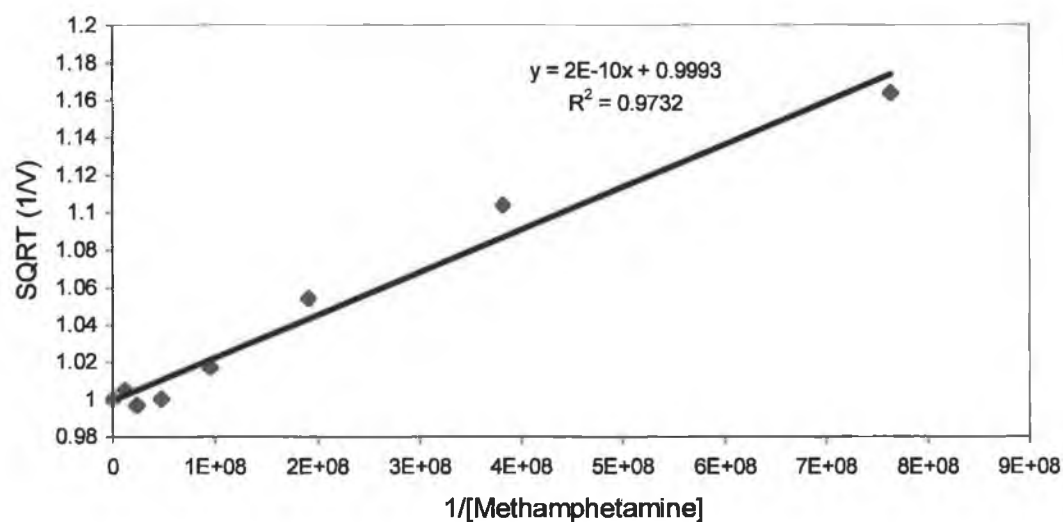
**Figure 5.20:** Determination of equilibrium dissociation constant of the anti-amphetamine monoclonal antibody and amphetamine on an amphetamine-BSA coated chip surface. The solution phase affinity model was fitted to the data using BIAevaluation software. The  $K_D$  value determined was  $2.25 \times 10^{-9}$  M, with a standard error of  $1.09 \times 10^{-10}$  M.



**Figure 5.21:** Determination of equilibrium dissociation constant of the anti-amphetamine monoclonal antibody and MDA on an amphetamine-BSA coated chip surface. The solution phase affinity model was fitted to the data using BIAevaluation software. The  $K_D$  value determined was  $4.24 \times 10^{-9}$  M, with a standard error of  $6.90 \times 10^{-11}$  M.

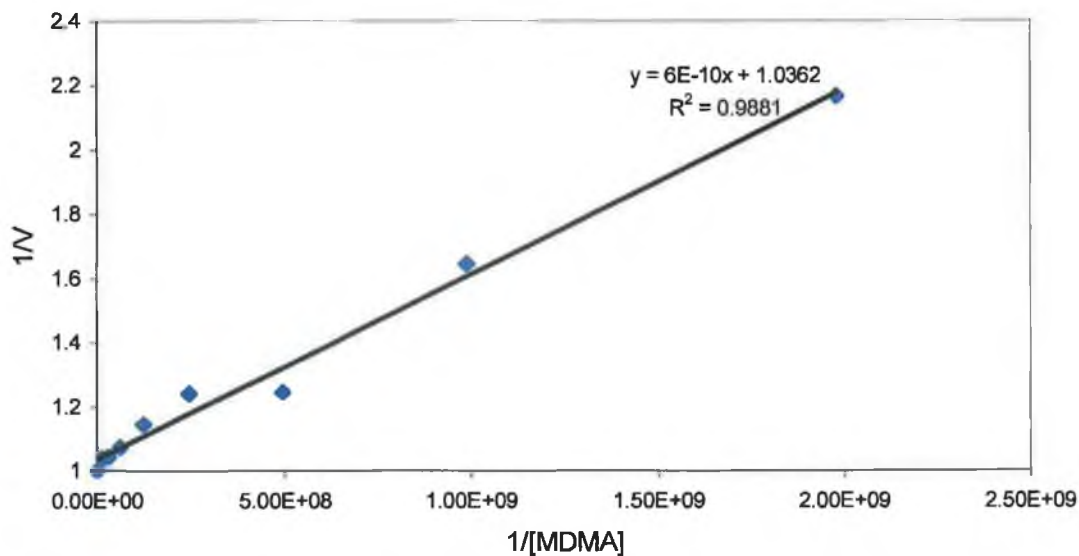


**Figure 5.22:** Friguet affinity determination of equilibrium dissociation constant for the interaction between anti-methamphetamine monoclonal antibody and methamphetamine. The  $K_D$  for the interaction of the anti-methamphetamine monoclonal antibody and methamphetamine is  $5 \times 10^{-10}$  M.

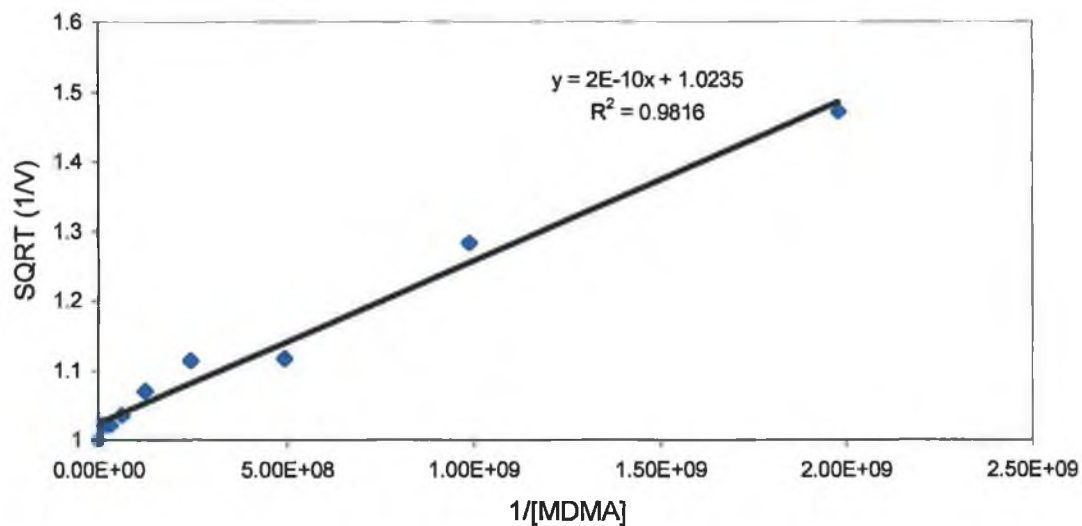


**Figure 5.23:** Friguet affinity determination of equilibrium dissociation constant using corrected IgG nominal concentration to account for bivalency of IgG. The  $K_D$  for the interaction of the anti-methamphetamine monoclonal antibody and methamphetamine is  $2 \times 10^{-10}$  M.

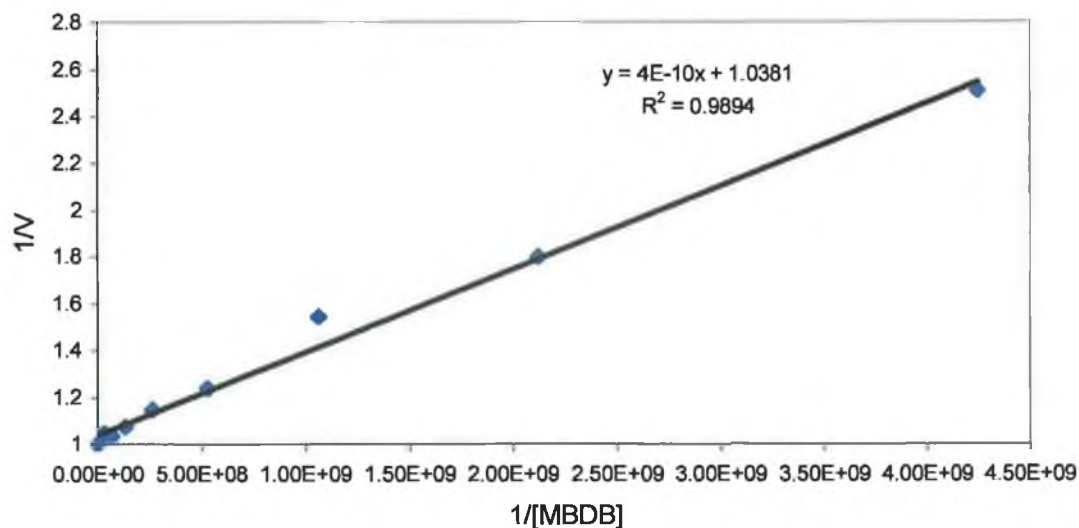




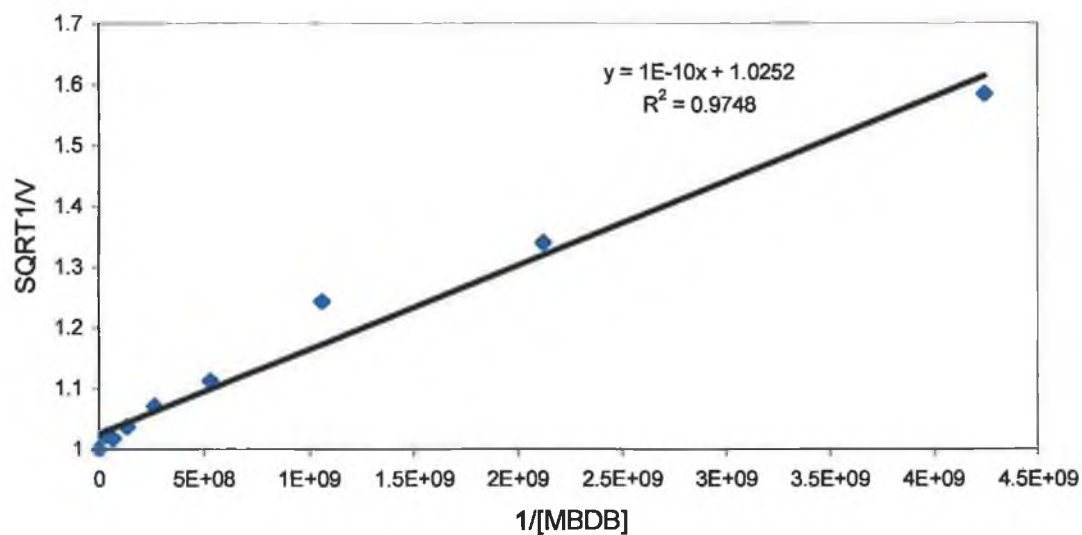
**Figure 5.24:** Friguet affinity determination of equilibrium dissociation constant for the interaction between anti-methamphetamine monoclonal antibody and MDMA. The  $K_D$  for the interaction of the anti-amphetamine monoclonal antibody and MDMA is  $6 \times 10^{-10}$  M.



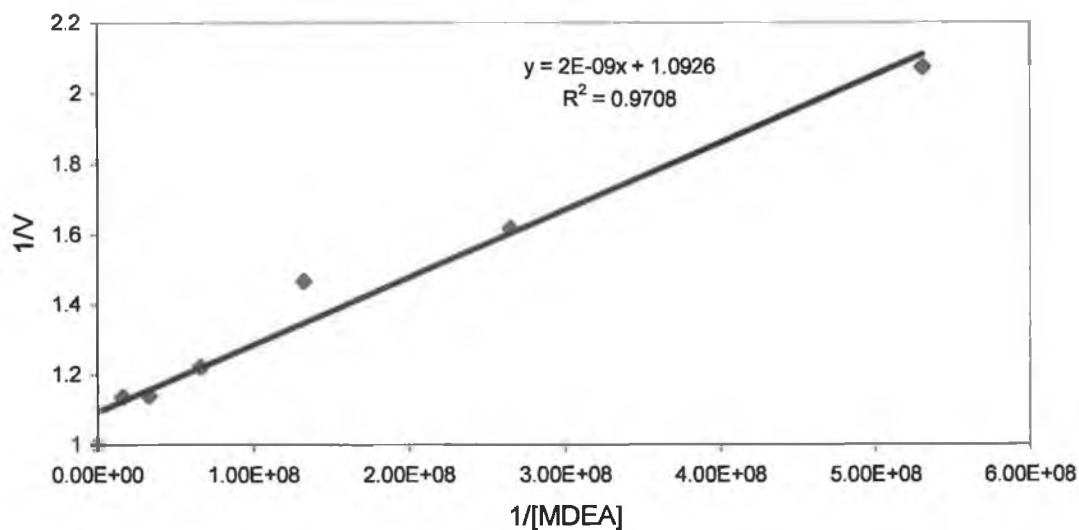
**Figure 5.25:** Friguet affinity determination of equilibrium dissociation constant using corrected IgG nominal concentration to account for bivalency of IgG. The  $K_D$  for the interaction of the anti-methamphetamine monoclonal antibody and MDMA is  $2 \times 10^{-10}$  M.



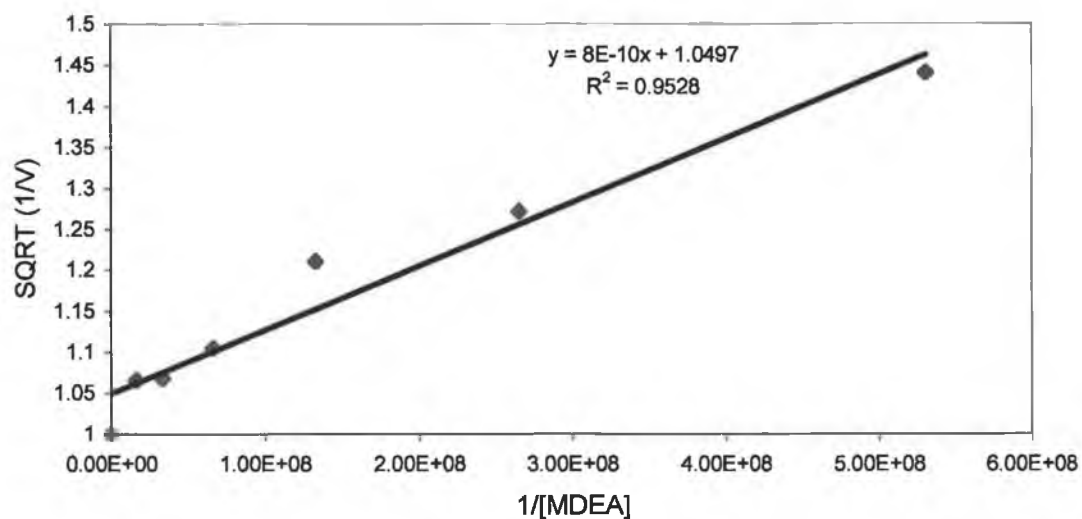
**Figure 5.26:** Friguet affinity determination of equilibrium dissociation constant for the interaction between anti-methamphetamine monoclonal antibody and MBDB. The  $K_D$  for the interaction of the anti-amphetamine monoclonal antibody and MBDB is  $4 \times 10^{-10}$  M.



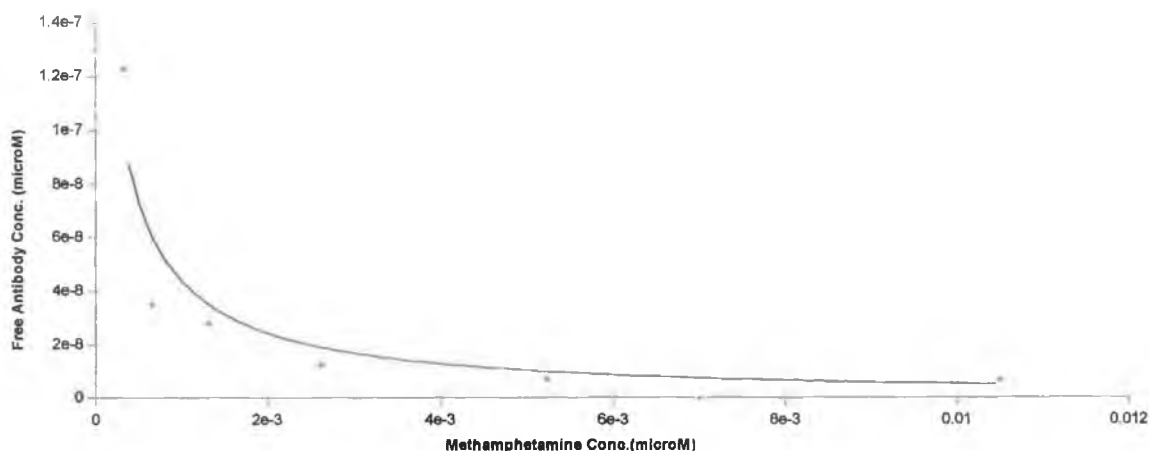
**Figure 5.27:** Friguet affinity determination of equilibrium dissociation constant using corrected IgG nominal concentration to account for bivalency of IgG. The  $K_D$  for the interaction of the anti-methamphetamine monoclonal antibody and MBDB is  $1 \times 10^{-10}$  M.



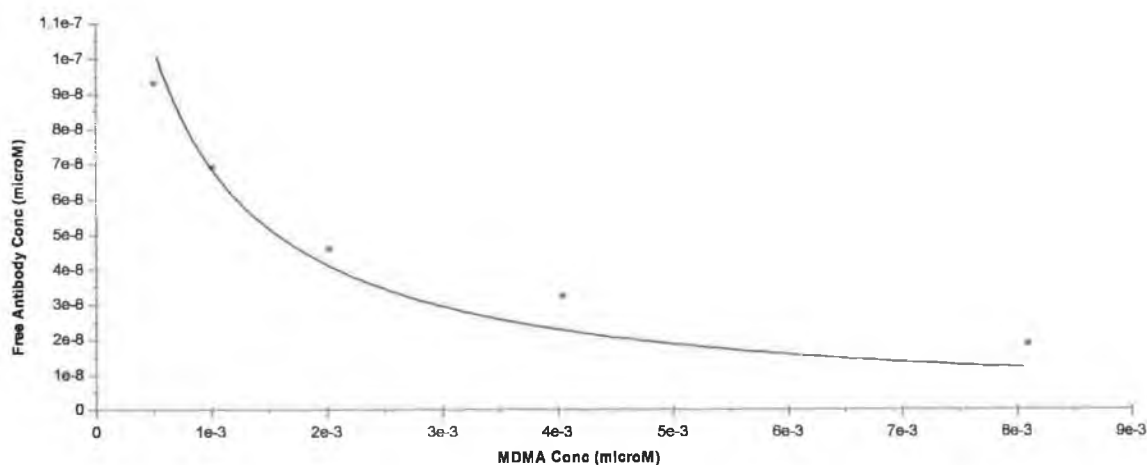
**Figure 5.28:** Friguet affinity determination of equilibrium dissociation constant for the interaction between anti-methamphetamine monoclonal antibody and MDEA. The  $K_D$  for the interaction of the anti-amphetamine monoclonal antibody and MDEA is  $2 \times 10^{-9}$  M.



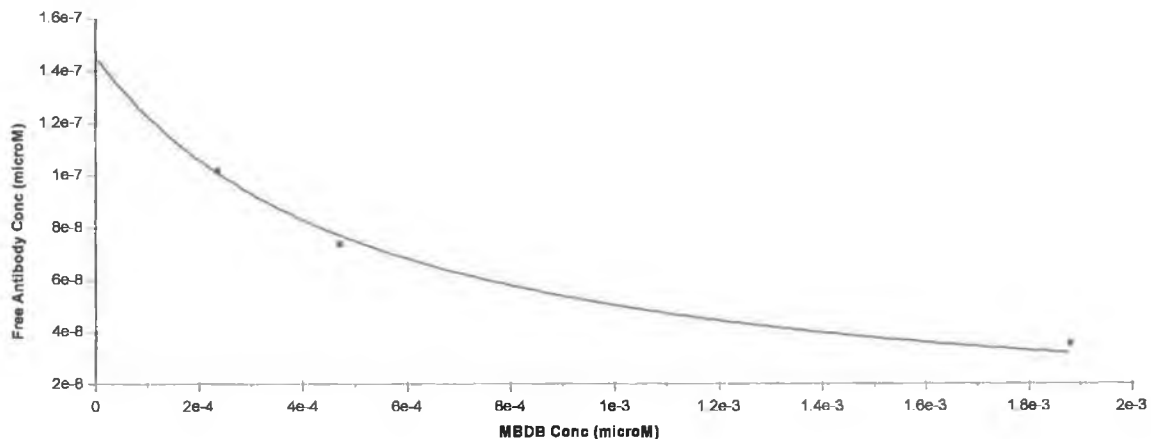
**Figure 5.29:** Friguet affinity determination of equilibrium dissociation constant using corrected IgG nominal concentration to account for bivalency of IgG. The  $K_D$  for the interaction of the anti-methamphetamine monoclonal antibody and MDEA is  $8 \times 10^{-10}$  M.



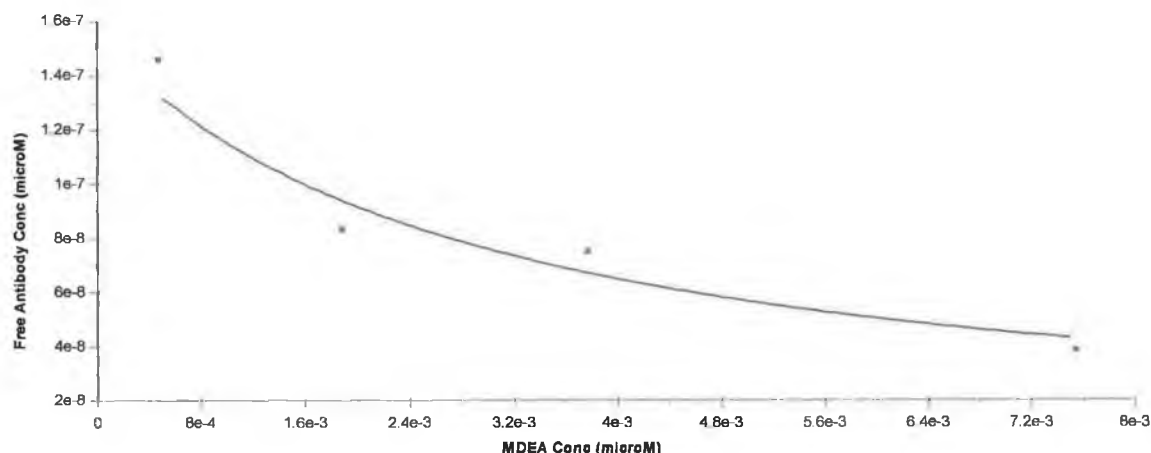
**Figure 5.30:** Determination of equilibrium dissociation constant of the anti-methamphetamine monoclonal antibody and methamphetamine on a methamphetamine-BSA-coated chip surface. The solution phase affinity model was fitted to the data using BIAevaluation software. The  $K_D$  value determined was  $2.42 \times 10^{-10}$  M, with a standard error of  $5.32 \times 10^{-11}$  M.



**Figure 5.31:** Determination of equilibrium dissociation constant of the anti-methamphetamine monoclonal antibody and MDMA on a methamphetamine-BSA coated chip surface. The solution phase affinity model was fitted to the data using BIAevaluation software. The  $K_D$  value determined was  $5.12 \times 10^{-10}$  M, with a standard error of  $5.08 \times 10^{-11}$  M.



**Figure 5.32:** Determination of equilibrium dissociation constant of the anti-methamphetamine monoclonal antibody and MBDB on a methamphetamine-BSA coated chip surface. The solution phase affinity model was fitted to the data using BIAevaluation software. The  $K_D$  value determined was  $5.3 \times 10^{-10}$  M, with a standard error of  $2.7 \times 10^{-11}$  M.



**Figure 5.33:** Determination of equilibrium dissociation constant of the anti-methamphetamine monoclonal antibody and MDEA on a methamphetamine-BSA coated chip surface. The solution phase affinity model was fitted to the data using BIAevaluation software. The  $K_D$  value determined was  $2.9 \times 10^{-9}$  M, with a standard error of  $4.89 \times 10^{-10}$  M.

**Table 5.9:** Equilibrium dissociation constants,  $K_D$ , as determined by the method of Friguet *et al.*, (1985), for the interaction between amphetamine, and MDA and the anti-amphetamine monoclonal antibody (Clone 4EP18E).

	$K_D$	Corrected $K_D$
Amphetamine	$1.0 \times 10^{-9}$	$6.0 \times 10^{-10}$
MDA	$2.0 \times 10^{-9}$	$6.0 \times 10^{-10}$

**Table 5.10:** Equilibrium dissociation constants,  $K_D$ , as determined by the method of Friguet *et al.*, (1985), for the interaction between methamphetamine, MDMA, MBDB, and MDEA and the anti-methamphetamine monoclonal antibody (Clone P18D).

	$K_D$	Corrected $K_D$
Methamphetamine	$5.0 \times 10^{-10}$	$2.0 \times 10^{-10}$
MDMA	$6.0 \times 10^{-10}$	$2.0 \times 10^{-10}$
MBDB	$4.0 \times 10^{-10}$	$1.0 \times 10^{-10}$
MDEA	$2.0 \times 10^{-9}$	$8.0 \times 10^{-10}$

**Table 5.11:** Equilibrium dissociation constants,  $K_D$ , and standard error (SE), as determined by the BIAcore solution phase assay, for the interaction between amphetamine, and MDA and the anti-amphetamine monoclonal antibody (Clone 4EP18E).

	$K_D$	SE
Amphetamine	$2.25 \times 10^{-9}$	$1.09 \times 10^{-10}$
MDA	$4.24 \times 10^{-9}$	$6.90 \times 10^{-11}$

**Table 5.12:** Equilibrium dissociation constants,  $K_D$ , and standard error (SE), as determined by the BIAcore solution phase assay, for the interaction between methamphetamine, MDMA, MBDB, and MDEA and the anti-methamphetamine monoclonal antibody (Clone P18D).

	$K_D$	SE
Methamphetamine	$2.24 \times 10^{-10}$	$5.32 \times 10^{-11}$
MDMA	$5.12 \times 10^{-10}$	$5.08 \times 10^{-11}$
MBDB	$5.3 \times 10^{-10}$	$2.70 \times 10^{-11}$
MDEA	$2.9 \times 10^{-9}$	$4.89 \times 10^{-10}$

### 5.3 Discussion

This chapter complements Chapter 4 which described the production and characterisation of an anti-amphetamine monoclonal antibody and an anti-methamphetamine monoclonal antibody, and the subsequent application of these antibodies in an ELISA format for the determination of amphetamine and derivatives in human salivary samples. It investigates the application of these antibodies in a BIAcore assay format, and additional characterisation studies to assess the affinity of the antibodies to amphetamine and derivatives. These affinity measurements were made using two different methods; the classic Friguet ELISA, and the solution affinity measurement using BIAcore technology.

Successful BIAcore-based competitive assays were developed in the model buffer system and also in the spiked saliva samples. For the model assay with the anti-amphetamine monoclonal antibody, Clone 4EP18E, the range of detection was 24.4 to 12,500ng/ml amphetamine. The percentage coefficients of variation ranged from 1.66 to 6.81%. The percentage recoveries ranged from 93.78 to 108.41%. When the assay was applied to saliva samples, the range of detection changed to 97.7 to 25,000 ng/ml, the percentage coefficients of variations also increased with a range from 7.40 to 29.36%. The percentage recoveries ranged from 91.46 to 114.16%, with value of 72.55% for the 6,250ng/ml standard, which is considered outside an acceptable range. The increased coefficients of variation reflect decreased accuracy in the saliva assay as compared to the model buffer assay. This could be accounted for by the extraneous proteins and substituents of saliva that may have an adverse effect on the antibodies and on the fluid mechanics of the BIAcore assay system. A similar pattern regarding the level of detection was seen in the methamphetamine assay. Clone P18D, was established in an inhibition BIAcore-based assay for the detection of methamphetamine. The range of detection in the model assay was 48.8 to 1562.5 ng/ml methamphetamine, whereas when the assay was applied to saliva samples, the range of detection was 97.7 to 6,250 ng/ml. In the model assay, the coefficients of variation ranged from 12.7 to 23.79% compared to 5.13 to 22.47% in the saliva-based assay. Both assays exhibited very good percentage recoveries, ranging from 95.49 to 102.52% for the model assay and 89.41 to 108% for the saliva-based assay. Overall, these results show that the assays developed using the anti-amphetamine monoclonal antibody, Clone 4EP18E, and

the anti-methamphetamine monoclonal antibody, Clone P18D, are accurate and reproducible. These clones are currently being evaluated for application in a commercial dip-stick test for the detection of amphetamines in human biological fluids.

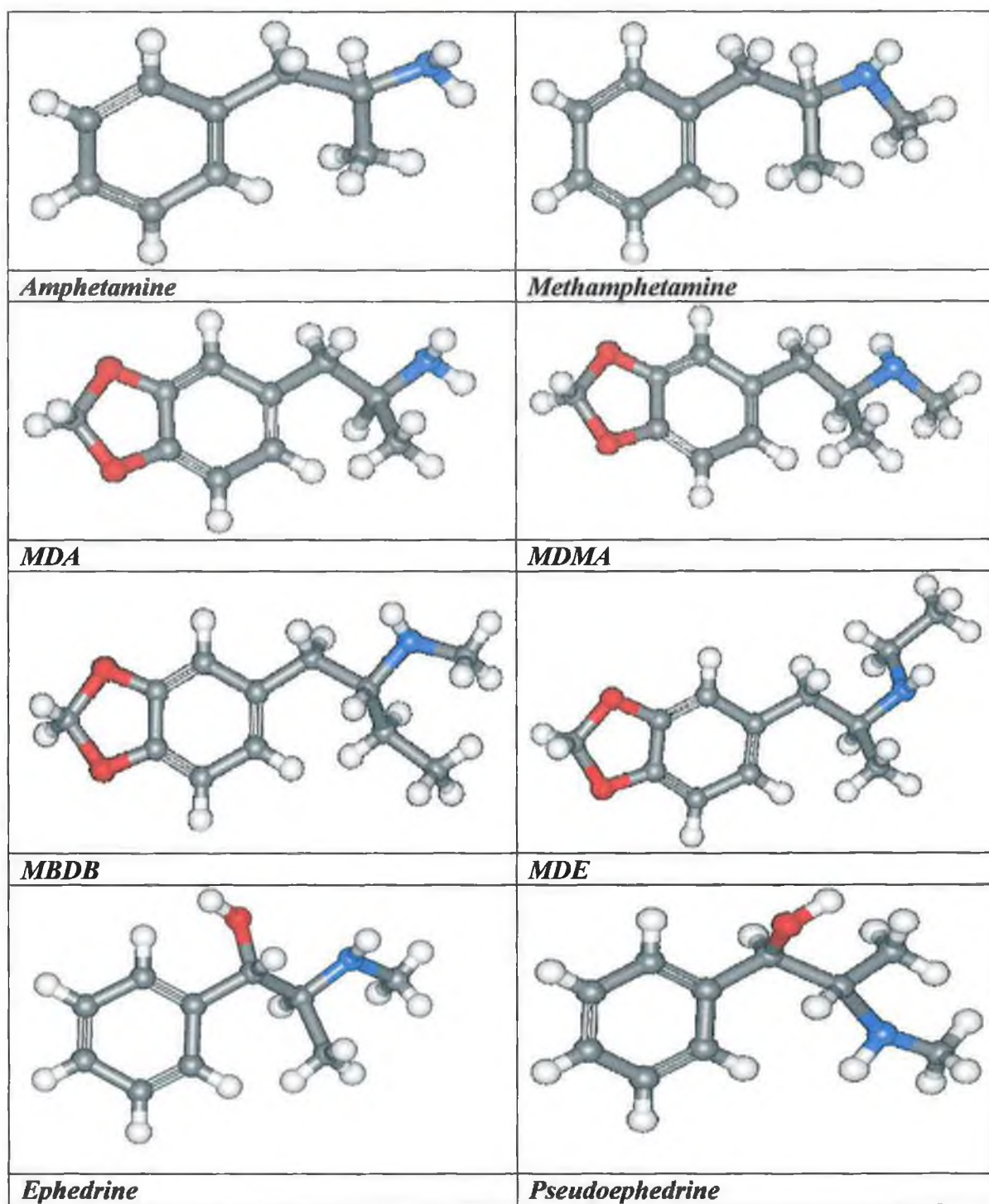
In the affinity studies of the antibodies, there was general agreement between the results obtained from the Friguet method and those obtained from the BIAcore-based solution affinity measurement. The same conjugates were used for immunisations and screening. In the case of the anti-amphetamine it was amphetamine-BSA and in the case of anti-methamphetamine it was methamphetamine-BSA. Both were linked through the phenyl para position. These conjugates were considered appropriate as the amphetamine structures are differentiated through the other side of the molecule at the carbon side chain. The anti-amphetamine antibody showed similar affinity for amphetamine and MDA. This is an expected result as the structural difference between the molecules is the methylenedioxy group situated at the para-position of the phenyl ring, which is used as the point of conjugation with the protein. The anti-methamphetamine antibody showed similar affinity for methamphetamine, MDMA, and MBDB. However, as the structure modified further, the affinity decreased as can be seen from the value for MDEA, which showed a difference in an order of magnitude from the value for the methamphetamine. This is expected as the original fusion was performed using a spleen from a mouse immunised with methamphetamine-BSA. So, as the structure of the derivative becomes less like the methamphetamine structure the value of the equilibrium dissociation constant increases, meaning a decrease in affinity. Overall, the interactions of the antibodies with compounds differing from the amphetamine and methamphetamine through substitutions on the side chain were directly proportional to the similarity of their structure to amphetamine or methamphetamine (Figure 5.34). This, therefore, confirms that the antibodies are recognising the carbon side chain of the amphetamine structures.

The issue of affinity determination based on monovalent and bivalent theories was investigated in the ELISA Friguet method. It is presumed that an antibody is bivalent and so can bind two molecules of the drug. However, steric interference may prevent this, so a definitive bivalent model cannot be presumed. The determination of the affinity constant by ELISA was calculated using a monovalent and bivalent model. The BIAcore solution phase affinity model assumes a monovalent fit. The graphical fit of the data for the anti-methamphetamine antibody to this model, shows that it is not a



very ideal fit. From this, it could be concluded that the interaction between the anti-methamphetamine and the methamphetamine structures is somewhere between a monovalent and bivalent.

The establishment of the successful BIAcore-based assay for the detection of amphetamine and methamphetamine is a good example of the usefulness of antibodies of intermediate affinities. This medium affinity allowed a successful regeneration cycle that is intrinsic for the development of the assay.



**Figure 5.34:** Three-dimensional representations of the structure of amphetamine, methamphetamine, and structural derivatives.

## *Chapter 6*

### *Development of ELISA, BIAcore Assay, and Envitec Device Assay for Detection of Drugs in Saliva Samples*

## 6.1 Introduction

### 6.1.1 Background to Envitec Device

DCU collaborated with Envitec-Wismar GmbH on the European Commission Standards, Measurement and Testing Project, entitled, 'On-site measurement of drugs of abuse in a saliva sample'. The aim of the project was to develop a new solid-phase format for the rapid detection of drugs of abuse in a saliva samples. Envitec developed an automatic device that could be used for this purpose. To achieve a safe and easy to use assay, the critical steps of the laboratory procedures for completing an immunoassay have to be simplified and the incubation steps shortened in time. The aim behind the development is that a simple test would be available whereby a non-scientific person could apply the saliva sample, press a button and within minutes have a result of positive or negative for the drugs under investigation.

DCU obtained a prototype of the Envitec device and concentrated on the development of an assay for detection of THC in saliva samples, using the anti-THC polyclonal antibody that was produced and characterised as described in Chapter 3.



**Figure 6.1:** Envitec prototype device for rapid analysis of drugs of abuse in saliva samples.

### 6.1.2 *Stability of drugs in storage samples*

The reliable detection of drugs in saliva samples is dependent on the internal controls in a particular assay. The importance of the accuracy of the standards cannot be overstated for obvious reasons. To reliably interpret analytical results the stability of the target molecule must be understood and, in particular, the stability of the molecule in the matrix used for the investigation. In the clinical and forensic setting, many samples need to be retained for additional confirmatory testing or retesting. In the case of forensic testing the samples sometimes need to be stored for long periods of time. This means information regarding the stability of the drugs in different biological matrices must be available so that an informed interpretation of the analytical result can be made.

#### 6.1.2.1 *THC*

Our first experience with the assay development for detection of THC using commercial antibodies did suggest that the robustness of the THC assay was not as good as the other assays such as morphine, cocaine and amphetamines. A contributory factor to this was that the stock solution of THC was prepared in ethanol and it did not go fully into solution in aqueous buffers such as PBS. This situation was resolved by using dilute solutions with a final ethanol solution of 0.2% (v/v) in PBS. For one assay using a commercial clone of anti-THC monoclonal antibody, the percentage coefficients of variation approached 20% and even greater, which is not an acceptable level. Part of the problem may have been the antibody (as suggested by the supplier, Fitzgerald Industries; personal communication). An additional complication contributing to the variation may have been the instability in THC samples on storage. Further experience in the development and optimisation of the THC assays demonstrated that there was an inherent problem with all of the assays with respect to the robustness and reproducibility of the results both with standard solutions, spiked saliva samples and 'real' patient samples.

Christophersen *et al.* (1986) examined the stability of THC in whole blood, in samples stored in glass vials and plastic tubes, after storage for four days at room temperature followed by storage at  $-20^{\circ}\text{C}$  for four weeks. They found that in THC spiked blood samples, stored in glass vials, there was no significant difference in the concentration of THC detected after storage. However, for the samples stored in plastic vials, there was a significant decrease in the THC concentration detected after storage, from 60 to 100%

of the original amount was not being detected. Likewise, in a set of samples from cannabis users, they showed a significant difference between the aliquots that were stored in glass vials compared to the duplicate aliquot stored in plastic tubes. This study clearly showed that in the case of blood, the samples should be stored in glass vials.

Joern (1987), also expressed frustration at attempts to demonstrate linearity in quantifying THC by a modified GC/MS procedure. He suggested that if the THC-COOH was stored either in strongly basic solution, or in an organic solvent, the adsorption onto plastic and glass surfaces seemed to be minimised. For all his working stock solutions, he added the stock solution in ethanol to a 0.1M sodium hydroxide solution. He found that spiked urine samples prepared in this way were stable for at least 18 months when stored at  $-80^{\circ}\text{C}$ . This is appropriate for his chromatographic assays. However, in the case of immunoassays, this could interfere with the activity of the antibodies and therefore would not be suitable. Giardino (1996) found that storage of a solution of THC in control urine at a concentration of 75ng/ml under refrigerated conditions, for up to 40 days, were appropriate storage conditions and the results were certified to be within 20% of 75 ng/ml after such time. This is put forward as valid control standard for the analysis of urine samples. Golding *et al.* (1998) examined the stability of cannabinoids in urine samples after storage in freezing conditions for 40 days, 1 year and 3 years. They found a decrease in the concentrations of the cannabinoids found of 8.0, 15.8 and 19.6% after these periods. Fairbairn *et al.* (1976) found that light exposure had an adverse effect on cannabinoids. Johnson *et al.* (1984) investigated the stability of THC, and its metabolites, THC-OH and THC-COOH, in blood and plasma stored at  $-10^{\circ}\text{C}$  and  $4^{\circ}\text{C}$ , and at room temperature over a six month period. No significant difference was found in the concentrations stored at  $4^{\circ}\text{C}$  and  $-10^{\circ}\text{C}$ . Six months after storage at room temperature, the concentrations of THC and THC-OH had decreased by 90 and 44%, respectively, but the concentration of THC-COOH had remained stable. In the same lab, Moody *et al.* (1999) looked at the stability of drugs of abuse in blood and urine samples stored over hundreds of days at  $-20^{\circ}\text{C}$ , in silanised glass vials. In plasma they found that the concentration of THC and THC-COOH had decreased by 15% at both 304 and 354 days. In urine, they found THC-COOH to be stable over the period measured of 482 days. Dugan *et al.* (1994) also found an average decrease of 1% in urine samples stored frozen for 12 months. However, of these, 44% of samples did have a decrease in THC-COOH concentration of between 0 and 25%. This contrasts with Romberg & Post (1991) who found a

decrease of 19% in the concentration of THC-COOH after two months of storage in frozen conditions.

Another interesting finding regarding quantification of THC is the statistic from the CAP FUDT proficiency test survey, in which the average coefficient of variation of THC-COOH assay results was 25.1%. This instability of the THC in storage is probably contributing to this. An additional interesting point in the survey was that one sample had a target value of 100ng/ml, however, the average value determined by 72 of the survey participants was  $21.0 \pm 7.8$  ng/ml, (Joern *et al.*, 1992). The results presented in this chapter with regard to the 'real' and 'spiked' saliva samples would appear to support the theory that there is considerable adsorption to both plastic and glass vials. This is evident in the low percentage accuracies seen in the assays, and the compromised robustness of the assays compared to the other assays described in the previous chapters.

Another problem with the quantification of THC is the purity of the reference stock. Verification of the true purity content can be difficult for a standard laboratory. This results in subsequent errors in the preparing of working dilutions. Poortman-van der Meer & Huizer, (1999) reported on a new method for the quantification of THC, performed by gas chromatography with a flame ionisation detector. The effective carbon number concept was used to predict the GC/FID response factors. Cannabinol and cannabidiol are structurally very closely related to THC. Their work showed that the response factors of cannabidiol and cannabinol can be used for the calculation of the THC content of standards or samples. Interestingly, in this study, there was no degradation of the THC after storage at  $-70^{\circ}\text{C}$ .

### **6.1.2.2 Morphine**

Morphine is a frequently used drug and so there is a lot of information regarding the stability of morphine in aqueous solutions. Morphine is sensitive to oxygen and the products of oxidation are morphine-N-oxide and pseudomorphine. For clinical morphine injections, the addition of the antioxidant sodium metabisulphite protects the morphine. The shelf-life of the injections is around 15 months, (Gleditsch & Waaler, 2001). The situation with regard to the stability of morphine and opioids in saliva is more favourable to analytical testing than that for THC. Niedbala *et al.* (2001B) investigated the stability of morphine from the saliva collected on a device pad for

samples stored up to 90 days after collection, at  $-80$ ,  $4$ ,  $25$  and  $37^{\circ}\text{C}$ . The samples were analysed using the Orasure immunoassay kit (Orasure, Bethlehem, PA, USA). There was no significant decrease in the amount of morphine detected after storage. In this study, the pad used for the collection is stored in preservative fluid, provided with the Intercept Oral Specimen Collection Device (Orasure Technologies, Bethlehem, PA, USA), and, on arrival at the lab, the tube is centrifuged and the oral fluid collected.

The stability of morphine in urine stored at  $-20^{\circ}\text{C}$  for 12 months was investigated by Dugan et al., (1994). The average percentage change in the concentration of morphine detected by GC-MS was a 9% increase. However, the range of difference was  $-68\%$  to  $+63\%$ . Moody *et al.* (1999) found that the concentration of morphine in urine stored frozen had not exceeded a 15% decrease after 852 days of storage in frozen conditions.

Giogi & Meeker (1995) looked at the stability of morphine in blood stored at ambient temperature. The morphine was stable at 3 and 6 months but then the results became erratic and showed an increase at the 3 year time point and decrease at the 4 and 5 year interval. The ranges for the 3 and 4 year intervals were  $-56$  to  $153\%$  and  $-77$  to  $133\%$ , respectively.

Skopp *et al.* (2001A), examined the stability of morphine, morphine-6-glucuronide and morphine-3-glucuronide in spiked fresh blood and plasma and also in authentic postmortem blood samples. The samples were stored in glass vials at  $-20$ ,  $4$ , and  $20^{\circ}\text{C}$  for up to six months. Morphine and the glucuronide metabolites were not effected by storage at  $4^{\circ}\text{C}$ . In the postmortem blood, the analytes were only stable when stored at  $-20^{\circ}\text{C}$ . In the postmortem samples, the morphine levels were increased due to the hydrolysis of the glucuronide metabolites. It is suggested that this happens through the migrating bacteria from the gastrointestinal tract, as heart blood samples often have high activities of  $\beta$ -glucuronidase. For these reasons, it is important to obtain information regarding the stability of the parent drug and its metabolites, so a profile can be interpreted appropriately. The deterioration of the analytes in plasma samples exposed to light suggests that it is an oxidation process. It was noted also, that the samples of whole blood were not as affected by the light exposure, compared to the plasma samples. This could be due to the active oxygen species degradation by components in whole blood such as haemoglobin. So, overall, the stability of the morphine and its metabolites could be preserved by the appropriate storage conditions.



### 6.1.2.3 Cocaine

As discussed in Chapter 1 and Chapter 3, cocaine is hydrolysed to benzoylecgonine, (BEC), in aqueous solutions. In blood, the BEC found could be the result of the non-enzymatic hydrolysis and enzymatic hydrolysis, (Fletcher & Hancock, 1981). Isenschmid *et al.* (1992) propose that the loss of cocaine seen in blood samples can be accounted for by the detection of EME, as BEC and ecgonine methyl ester (EME) are the breakdown products of cocaine in unpreserved blood samples. Cocaine is hydrolysed to BEC at physiological pH range by non-enzymatic hydrolysis and to EME by liver and plasma cholinesterases, (Stewart *et al.*, 1977). Levine (1996) looked at the stability of EME in urine specimens and noted the decrease in the cocaine concentration over the storage time, but the EME concentration remained stable suggesting the conversion of cocaine to EME is an *in vivo* process.

Toennes & Kauert, (2001), investigated the importance of a vacutainer containing the cholinesterase inhibitor sodium fluoride and potassium oxalate, for the short transfer of blood samples from the police to the laboratory. The samples were tested using the Abbott fluorescence-polarization immunoassays followed by gas chromatography-mass spectrometry. The degradation of cocaine to ecgonine esters was inhibited in the fluoride containing samples. They also found that there was hydrolysis of benzoylecgonine (BEC) in the unstabilised samples. Brogan *et al.* (1992) also found that sodium fluoride, with or without potassium oxalate inhibited cocaine degradation up to 48 hours after storage. In this case, the cocaine was measured using gas-chromatography.

McCurdy *et al.* (1989), assessed the stability of cocaine, BEC, and THC-COOH in whole blood while stored at room temperature and refrigerated, for up to 30 days, in four different types of storage vials (EDTA-containing, heparin-containing, sodium fluoride-containing and preservative, anti-coagulant free). The samples were tested using the Roche Diagnostics Abuscreen RIA tests for BEC and cannabinoids. They found that cocaine was not stable in blood, particularly when stored at room temperatures. They found that the storage in the different tubes and at different temperatures had no significant effects on the stability and RIA detectability of BEC and THC-COOH. Skopp *et al.* (2001B) investigated the stability of cocaine in whole blood and plasma samples stored for up to 15 days. They included ecgonine in the

panel of analytes, along with cocaine, BEC and EME. They found the conversion of cocaine to BEC, EME and ecgonine to be stoichiometric at all time intervals.

There are many publications dealing with the retesting of drugs in frozen urine samples, (Dugan *et al.*, 1994; Romberg and Past, 1994). With regard to cocaine, Romberg & Past (1994) found an average decrease of 19% (range +20% to -100%) in BEC concentration detected in samples frozen for up to 8 months. The average change reported by Dugan *et al.* (1994) was +10% (range -56% to +73%) for BEC and -37% (-87% to +20%) for cocaine. Hippenstiel and Gerson, (1994) reviewed the optimum storage conditions for cocaine and BEC in urine samples. The ideal storage conditions were found to be -15°C in unsilanized glass containers in the dark, and the pH adjusted to 5.0 with ascorbic acid. Lui *et al.* (1982) found that in refrigerated blood samples there was a 7% decrease in the concentration of cocaine after 1 day and a 30% decrease after 36 days. Moody *et al.* (1999) found that BEC was stable in frozen urine samples for the time measured up to 852 days, whereas cocaine had decreased by 15% after 165 days of storage. In frozen plasma samples, cocaine and BEC had 15% decreases in concentration at 154 and 111 days, respectively. Giorgi & Meeker (1995) also found cocaine and BEC to be unstable in blood samples frozen at room temperature. The cocaine was not detected after three months of storage and the BEC concentration decreased steadily and was not detected in the half of the samples at the six month and 1 year time points. Both Isenschmid *et al.* (1989) and Moody *et al.* (1999) found that lowering the pH to 5 or 6, with the addition of an esterase inhibitor stabilises cocaine in solution.

The conclusion from the above is that care should be taken with regard to the storage of blood and urine samples for the analysis of cocaine. The analysis should include cocaine, BEC and EME and ecgonine. The concentration of cocaethylene should also be examined as this is the main metabolite of cocaine ingested with alcohol. Consideration must be given to the enzymatic degradation in postmortem samples and non-enzymatic processes in storage samples.

#### **6.1.2.4 Amphetamine and methamphetamine**

The stability of amphetamine and methamphetamine in biological samples seem to be very good in comparison to the other drugs discussed above. Dugan *et al.* (1994) assessed the stability of drugs in urine samples stored for 12 months at

-20°C and found that the average change from the initial concentration detected of amphetamine and methamphetamine was +10% and -15 respectively. The range of change for amphetamine was -35% to +30%, and -48% to +25% for methamphetamine. Similarly in frozen blood samples, the stability of amphetamines is good as reported by Giorgi & Meeker (1995). They examined the stability of the drugs over a period of five years in frozen blood samples. They concluded that the stability of amphetamine and methamphetamine can be attributed to the presence of the phenethylamine nucleus that does not contain functional groups that are susceptible to hydrolysis.

## **6.2 Results**

### **6.2.1 Rapid assay for THC detection**

#### **6.2.1.1 Development and format of THC assay**

The Envitec device is a laboratory prototype providing automation of a rapid ELISA for detection of drugs of abuse. The anti-THC polyclonal antibody described in Chapter 3 was applied to the assay on this device for the detection of THC in saliva samples. The anti-THC antibody was chosen as the company, Envitec, had found that they were having problems with the optimisation and stability of this assay using commercial antibodies. To establish the test the following criteria had to be met:

- Determine optimum concentration of capture antibody; goat anti-rabbit immunoglobulin.
- Determine optimum concentration of anti-THC polyclonal antibody.
- Determine optimum dilution of THC-HRP conjugate.
- Determine effects of saliva as a matrix on the assay.
- Establish minimum level of detection of 200ng/ml.
- Examine batch variations in the THC-HRP conjugate and antibodies in relation to their effects on the assay performance.

The assay was initially set up on as an immunoplate-based ELISA, as a lot of analyses were needed to determine the many different test parameters. This saved on the usage of the prototype specialised wells that were used on the device. Table 6.1 describes the different parameters that were examined and the final concentrations and dilutions that were used for the final assay.

**Table 6.1:** The parameters that were investigated in the optimisation of the Envitec based assay for the detection of THC.

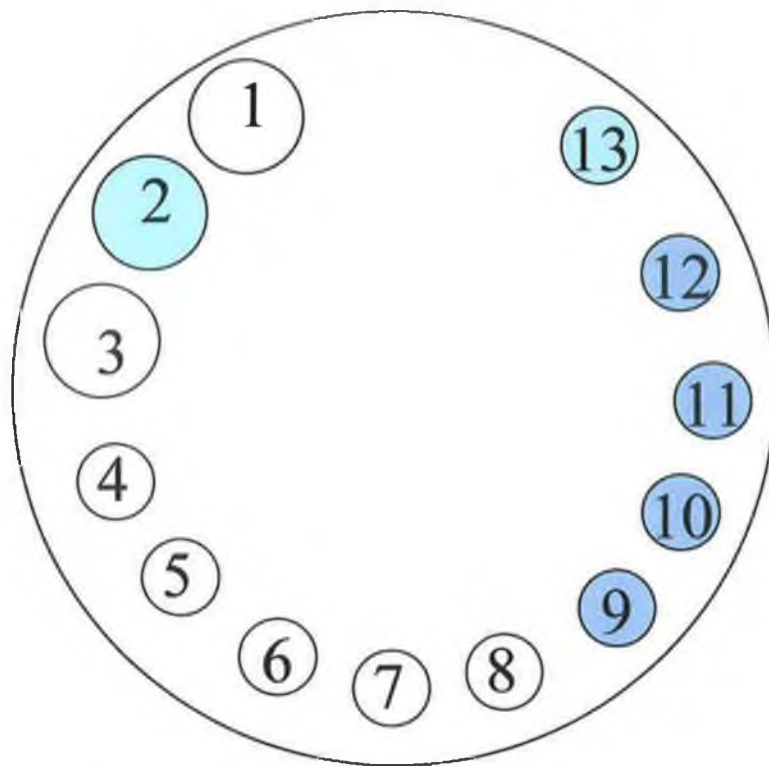
<b>Parameters</b>	<b>Variables</b>				<b>Assay Parameter Used</b>
<i>Dilution of capture antibody – goat anti-rabbit IgG (1mg/ml Stock)</i>	0	1/625	1/500	1/1000	1/500
<i>Dilution of anti-THC polyclonal antibody</i>	1/100	1/200	1/500	1/2000	1/100
<i>Dilution of THC-HRP</i>	1/50	1/100	1/250	1/500, 1/1000, 1/2000	1/500
<i>Incubation time with TMB</i>	5 mins	10 mins			5 mins

The above variables were examined over numerous assays and days. A new batch of THC-HRP was obtained from Fitzgerald industries as the first batch had been exhausted. This involved repeating many of the tests. All assays described below are used with the second batch.

The principles determining the parameters were as follows:

- to establish a minimum level of detection of 200ng/ml in saliva samples.
- to develop an assay to be as rapid as possible while maintaining 200ng/ml cut off .
- to test saliva samples with minimum preparation prior to analyses.

The well positions are shown in Figure 6.2, and the schedule of the final assay is described in Section 2.12 and outlined schematically in Figure 6.3.



**Figure 6.2:** Schematic diagram of the carousel of the Envitec device and the different wells.

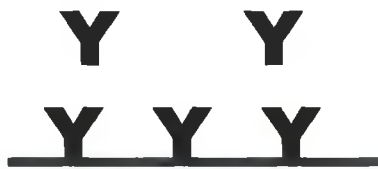
**Well 1:** Saliva sample mixed 1:1 with PBS (500 $\mu$ l total needed)

**Well 2:** TMB Substrate (1ml)

**Well 3:** Waste well

**Wells 4-8:** 100 $\mu$ l THC-HRP (Saliva sample is transferred to these wells for mixing with THC-HRP)

**Wells 9-13:** Reaction wells (coated with anti-THC antibody, after incubation step with sample and THC-HRP mixture, the TMB is transferred here and the transmission read



*Wells coated with 250 µl of 1/500 dilution of goat anti-rabbit IgG, in PBS, at 40°C overnight. Followed by washing with PBS-Tween.*



*Wells coated with 250 µl of 1/100 dilution of rabbit anti-THC polyclonal antibody, in PBS, at 4°C overnight. Followed by washing with PBS-Tween.*








*Wells blocked with 300 µl of 2% milk protein solution for 30 minutes at 37°C. Followed by washing with PBS-Tween.*



*Samples/standards and HRP labeled-THC are mixed in separate well and transferred to coated reaction well for 5 minute incubation. Followed by washing with tris buffer.*



*TMB substrate is added to reaction wells. An optical reading of the transmission at 620nm is recorded. After 5 mins the second reading is made, and the fractional change in transmission is recorded.*

-  Goat Anti-Rabbit Immunoglobulin
-  Rabbit Anti-THC Polyclonal Antibody
-  Blocking Solution (Milk Protein)
-  THC
-  HRP-Labeled THC

**Figure 6.3:** Schematic diagram of the assay for detection of THC on Envitec Device

## **6.2.2            *Optimisation of assay***

### **6.2.2.1        *Optimisation in PBS samples***

The initial optimisation tests were carried out using PBS as the sample diluent. The results were good and a limit of detection was obtained of 200ng/ml THC. However, these results were not easy to repeat. Figure 6.4a show the results obtained for the PBS samples spiked with THC from a working stock that had prepared greater than 24hours prior to experiment and stored at 4°C. Figure 6.4b shows the results obtained for PBS samples spiked with THC from a working dilution that had been freshly prepared. Table 6.2 shows the mean of four replicates  $\pm$  standard deviation for each point on the graphs. For each value, a control well, which did not contain the anti-THC antibody, was run in the same cycle. The reading for each value was normalised by dividing by the control well. The important point to notice from this table and Figures 6.4a and 6.4b is that the results obtained using THC solutions that had previously being stored did show the same cut off level of detection compared to the results from days when fresh stock solutions of THC were prepared. The different variables were examined including stability of THC, stability of antibody, and stability of THC-HRP. The antibody and THC-HRP were found to be stable under the conditions used for the assay. In Section 6.1.2.1, the issue of THC instability was discussed, and this is most probably the reason behind the non-reproducibility of the results. For the optimisation studies, using PBS as diluent, it was necessary to prepare a fresh working dilution of 1mg/ml THC in PBS daily, from a stock solution of 25mg/ml in ethanol. The plastic eppendorf tubes previously used for the storage of the working dilutions were substituted by glass vials, to eliminate any possible adsorption to the container surface, that could contribute to further inaccurate THC concentration determinations. As discussed previously, Christophersen *et al.* (1986) found a significant decrease in THC concentration after storage in plastic containers.

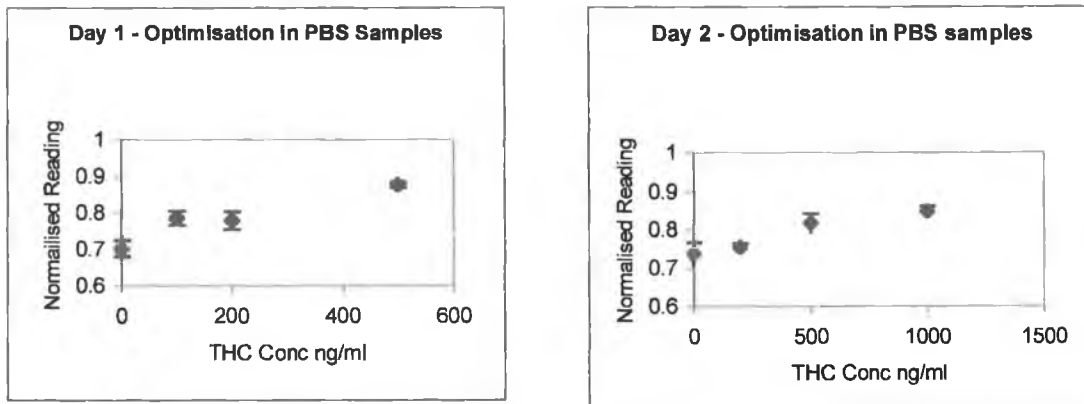
### **6.2.2.2        *Optimisation in saliva samples***

The samples of saliva used for the optimisation of the assay were drug-free and obtained from lab colleagues. The samples were spiked using freshly prepared stock solutions of THC in ethanol, ( $1 \times 10^{-3}$ ,  $1 \times 10^{-4}$ ,  $1 \times 10^{-5}$  g/ml). A control well that did not contain the anti-THC antibody was included in every run. The biggest difference seen

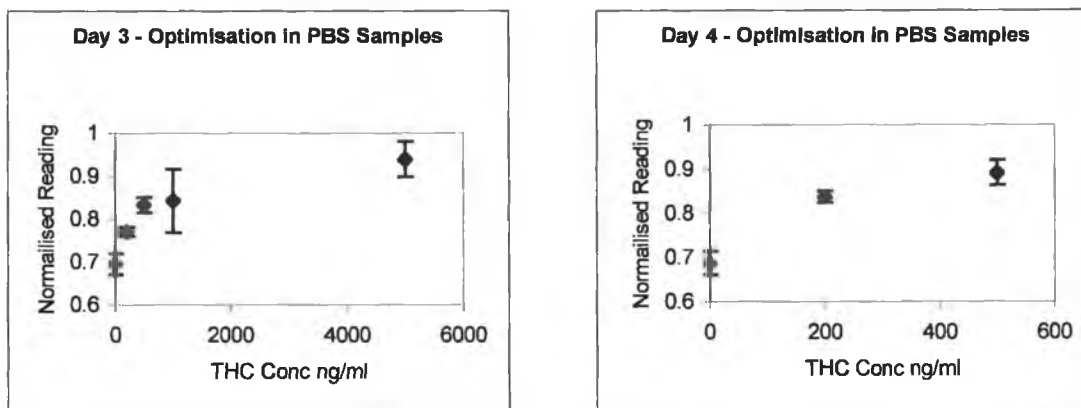


in the saliva samples, compared to the PBS samples, was the high background value as seen in the control well. These saliva results are not shown as the values are normalised by dividing by this control well. In the case of the samples in PBS, this control value was usually between 0.95 to 1.0, indicating negligible colour change in the control well, because there was no anti-THC antibody to bind the THC-HRP. However, in the saliva samples, this value could fall to 0.8. As there was no anti-THC antibody in the well to bind the THC-HRP, the change in colour in the well, has to be attributed to a background effect and the incomplete washing due to the viscosity and 'stickiness' of the saliva. The most likely reason is that the saliva could have caused residues of the THC-HRP to remain in the wash tubing. This is the same tubing that the TMB is dispensed through and the residues of THC-HRP would have reacted with the TMB that was being dispensed into the control well, causing a slight colour change. Another possibility is that the THC-HRP could be inadequately washed from the wells and so the residues in the well cause the colour change. As the value recorded is the fractional change in transmission of light at 620nm, this change in colour of the TMB would have caused the value to decrease to 0.8. To decrease this background value, the saliva samples were mixed 1:1 with PBS.

Since the saliva components of individuals differ so much, it was decided that the best way to account for these interferences was to normalise all values, by dividing the fractional transmission by the control well, as they have been plotted. For each four reaction well replicates, there was one control well. The results for the different days are shown in Figure 6.5. The values for each assay are displayed in Table 6.3 and clearly show that the presence of 200ng/ml THC in a saliva sample gives a significant decrease in the fractional normalised transmission, and so 200ng/ml is the cut off level of detection.



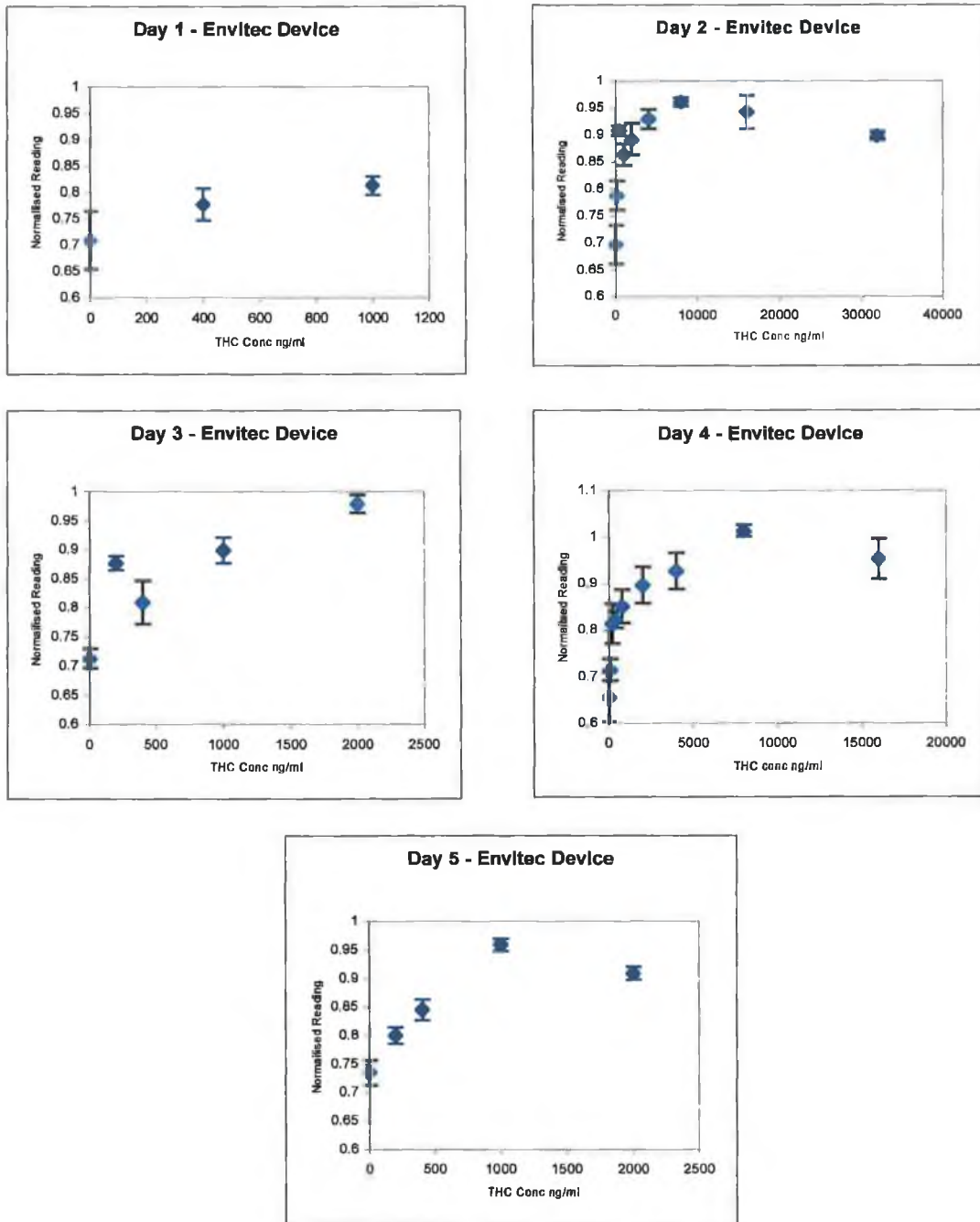
**Figure 6.4a:** Optimisation of the assay on the Envitec device using PBS as sample diluent. The above are the results obtained from two different days. These assays were run using PBS spiked with *THC that was prepared from a working dilution of 1mg/ml in PBS that had been stored at 4°C for greater than 24 hours*. The dilution of THC-HRP used was 1/500. Intra-day variation for the THC detection assay on the Envitec device. Each point on the graph is the mean of four replicates  $\pm$  standard deviation. For each value a control well, which did not contain the anti-THC antibody, was run in the same cycle. The reading for each value was normalised by dividing by this control well. All graphs show a value for a PBS sample containing no THC.



**Figure 6.4b:** Optimisation of the assay on the Envitec device using PBS as sample diluent. The above are the results obtained from two different days. These assays were run using PBS spiked with *THC that was prepared from a working dilution of 1mg/ml in PBS that had been prepared fresh on the day of the experiment*. The dilution of THC-HRP used was 1/500. Intra-day variation for the THC detection assay on the Envitec device. Each point on the graph is the mean of four replicates  $\pm$  standard deviation. For each value a control well, which did not contain the anti-THC antibody, was run in the same cycle. The reading for each value was normalised by dividing by this control well. All graphs show a value for a PBS sample containing no THC.

**Table 6.2:** Mean value  $\pm$  standard deviation for normalised readings of PBS samples spiked with THC. The results are plotted graphically in Figure 6.4a and 6.4b. The results show that the cut off level for the detection of THC by the assay is 200ng/ml. THC prepared from a freshly made working stock of 1mg/ml, provided better results than the results obtained from the assay using THC that was prepared from a working dilution that had been stored for over 24 hours.

	<i>Day 1 (Stored THC Spiked Sample)</i>	<i>Day 2 (Stored THC Spiked Sample)</i>	<i>Day 3 (Fresh THC Spiked Sample)</i>	<i>Day 4 (Fresh THC Spiked Sample)</i>
<i>Conc THC ng/ml</i>	<i>Mean <math>\pm</math> SD</i>	<i>Mean <math>\pm</math> SD</i>	<i>Mean <math>\pm</math> SD</i>	<i>Mean <math>\pm</math> SD</i>
<b>0</b>	0.702 $\pm$ 0.022	0.737 $\pm$ 0.03	0.695 $\pm$ 0.025	0.687 $\pm$ 0.026
<b>100</b>	0.786 $\pm$ 0.02	ND	ND	ND
<b>200</b>	0.779 $\pm$ 0.024	0.755 $\pm$ 0.01	0.77 $\pm$ 0.01	0.835 $\pm$ 0.013
<b>500</b>	0.876 $\pm$ 0.007	0.818 $\pm$ 0.024	0.833 $\pm$ 0.018	0.892 $\pm$ 0.028
<b>1000</b>	ND	0.848 $\pm$ 0.014	0.843 $\pm$ 0.074	ND
<b>5000</b>	ND	ND	0.939 $\pm$ 0.041	ND



**Figure 6.5:** Intra-day variation for the THC detection assay on the Envitec device. Each point on the graph is the mean of four replicates  $\pm$  standard deviation. For each value a control well, which did not contain the anti-THC antibody, was run in the same cycle. The reading for each value was normalised by dividing by this control well. All graphs show a value for a saliva sample containing no THC. All of the above tests were performed using real saliva spiked with THC. The samples were then mixed in a 1:1 ratio with PBS before running on the device. The results show that the assay can be used as a screening test with a cut-off level of detection of 200ng/ml. However, due to the variability of the results it is not suitable as a quantitative assay.

**Table 6.3:** Mean value  $\pm$  standard deviation for normalised readings of saliva samples spiked with THC. The results are plotted graphically in Figure 6.4. The results show that the cut off level for the detection of THC by the assay is 200ng/ml. The THC was prepared from a freshly made working stock of 1mg/ml. The results show that the assay can be used as a screening test with a cut-off level of detection of 200ng/ml, but due to the variability, it is not suitable as a quantitative test. This is justified on the difference in one standard deviation between the positive and negative samples. It would be preferable to have a greater difference than this.

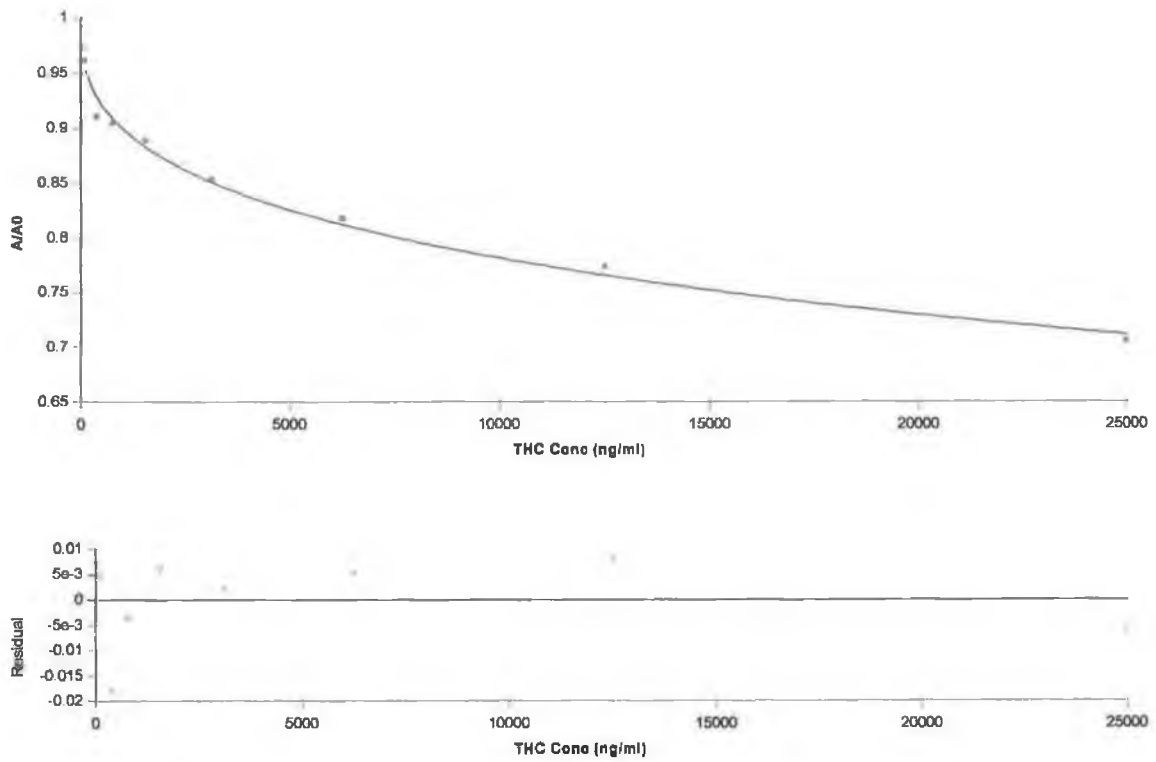
	<i>Day 1</i>	<i>Day 3</i>	<i>Day 5</i>
<b>Conc THC ng/ml</b>	<b><i>Mean <math>\pm</math> SD</i></b>	<b><i>Mean <math>\pm</math> SD</i></b>	<b><i>Mean <math>\pm</math> SD</i></b>
<b>0</b>	0.709 $\pm$ 0.055	0.713 $\pm$ 0.016	0.735 $\pm$ 0.022
<b>100</b>			
<b>200</b>	0.791 $\pm$ 0.012	0.877 $\pm$ 0.012	0.800 $\pm$ 0.014
<b>400</b>	0.778 $\pm$ 0.03	0.809 $\pm$ 0.037	0.845 $\pm$ 0.018
<b>1000</b>	0.813 $\pm$ 0.017	0.899 $\pm$ 0.022	0.959 $\pm$ 0.011
<b>2000</b>		0.979 $\pm$ 0.015	0.909 $\pm$ 0.011

	<i>Day 2</i>	<i>Day 4</i>
<b>Conc THC ng/ml</b>	<b><i>Mean <math>\pm</math> SD</i></b>	<b><i>Mean <math>\pm</math> SD</i></b>
<b>0</b>	0.697 $\pm$ 0.036	0.656 $\pm$ 0.053
<b>100</b>		0.715 $\pm$ 0.023
<b>200</b>	0.788 $\pm$ 0.027	0.814 $\pm$ 0.043
<b>400</b>	0.908 $\pm$ 0.01	0.823 $\pm$ 0.017
<b>800</b>		0.851 $\pm$ 0.036
<b>1000</b>	0.864 $\pm$ 0.02	
<b>2000</b>	0.892 $\pm$ 0.29	0.897 $\pm$ 0.039
<b>4000</b>	0.93 $\pm$ 0.018	0.927 $\pm$ 0.039
<b>8000</b>	0.961 $\pm$ 0.008	1.014 $\pm$ 0.013
<b>16000</b>	0.943 $\pm$ 0.031	0.953 $\pm$ 0.043
<b>32000</b>	0.899 $\pm$ 0.007	

### ***6.2.3 Development of ELISA for the detection of THC and morphine in saliva samples***

#### ***6.2.3.1 Development of ELISA for detection of THC in saliva samples***

The optimal coating concentration of THC-BSA and the optimal antibody dilution to use was determined by an indirect checkerboard ELISA. The characterisation of the anti-THC polyclonal antibody was described in Chapter 3. The concentration of THC-BSA used was 5µg/ml and the dilution of antibody was 1/200, for this assay. The competitive assay was performed as described in Chapter 2. The inter-assay plot of the normalised absorbance at 450nm ( $A/A_0$ ) and the concentration of free THC in the samples is shown in Figure 6.6. The values from five assays were used to calculate the inter-assay mean, coefficient of variation and percentage recovery, as shown in Table 6.4. The percentage recoveries are expressed as a percentage of the back-calculated value, obtained from the 4-parameter fit of the data, divided by the actual concentration of the standard. The percentage recovery is a measure of the accuracy of the assay. The percentage recoveries are quite acceptable for the range of detection of 96.7 - 25000ng/ml, with the exception of the value for 390ng/ml. One explanation for the higher variability at the lower concentrations of the detection range is simply that these samples are more prone to diluting errors and so show more variation from day to day. The asymptotes of the curve are more likely to have higher variability, as the concentrations of drug reach the highest and lowest levels.



**Figure 6.6:** Mean Inter-assay calibration curve for the detection of THC in saliva samples. The mean normalised value for each drug concentration from three replicates was calculated for each of the drug standards. These values from five assays were used to calculate the inter-assay mean, coefficient of variation and percentage accuracy. The calibration curve was plotted using BIAevaluation software.

**Table 6.4:** Inter-assay variation (degree of accuracy and reproducibility) for the detection of THC using the anti-THC polyclonal antibody based ELISA in saliva samples. The results presented are the mean values obtained from five intra-day assays, each assay was performed on three replicates.

<b>THC Standard Concentration (ng/ml)</b>	<b>Back-Calculated THC Concentration (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
96.7	71.3	8.53	72.95
390.6	724.9	3.99	185.57
781.1	865.8	1.32	110.82
1562.5	1339.1	0.89	85.70
3125	2983.2	3.71	95.46
6250	5737.9	4.46	91.81
12500	11197.6	5.86	89.58
25000	26703.3	0.33	106.81

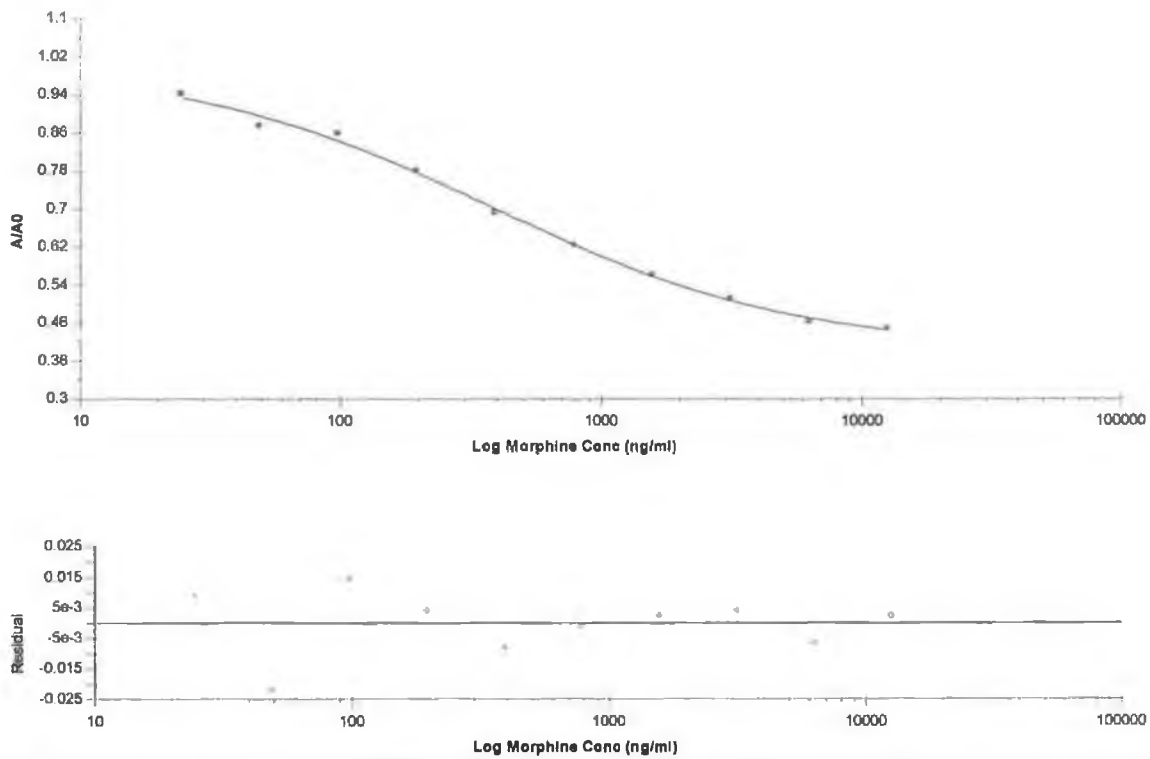


### 6.2.3.2 Development of ELISA for detection of morphine in saliva samples

The optimal coating concentration of morphine-3-glucuronide-ovalbumin and the optimal antibody dilution to use was determined by an indirect checkerboard ELISA. This was a repeat of the ELISA described in Section 3 for the characterisation of the anti-morphine polyclonal antibody. This was repeated as in this case the sample was a 25µls of saliva. The concentration of morphine-3-glucuronide-thyroglobulin used was 1µg/ml and the dilution of antibody was 1/400. The competitive assay was performed as described previously. The relationship between the normalised absorbance at 450nm (A/A0) and the concentration of free THC in the samples is plotted in Figure 6.7. The values from five assays were used to calculate the inter-assay mean, coefficient of variation and percentage accuracy, as shown in Table 6.5. Overall, a very good reproducible assay was produced with a range of detection between 24.4 - 12,500 ng/ml morphine.

**Table 6.5:** Inter-assay variation (degree of accuracy and reproducibility) for the detection of morphine using the anti-morphine polyclonal antibody based ELISA. The results presented are the mean values obtained from five intra-day assays, each assay was performed on three replicates.

<b>Actual Morphine Conc (ng/ml)</b>	<b>Back-Calculated Morphine conc. (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
24.4	19.7	7.75	80.72
48.8	66.7	5.47	136.61
97.7	82.1	13.32	84.10
195.3	187.6	14.05	96.04
390.6	419.1	13.91	107.28
781.3	787.0	8.72	100.74
1562.5	1520.8	7.82	97.33
3125.0	2926.1	0.54	93.64
6250.0	7312.8	5.35	117.00
12500.0	11446.8	7.55	91.57



**Figure 6.7:** Competitive ELISA for detection of morphine in saliva samples. Anti-morphine polyclonal antibody was added to a range of morphine standards. The data was fitted to a 4-parameter fit equation using BIAevaluation software. The results presented are the mean of 5 intra-day assays,  $\pm$  standard deviation. The coefficient of variation, and mean back calculated value for each standard within the accepted range for the equation was determined and the degree of accuracy determined .

## **6.2.4 Development of a model BIAcore-based competitive immunoassay for the detection of morphine**

### **6.2.4.1 Preconcentration studies**

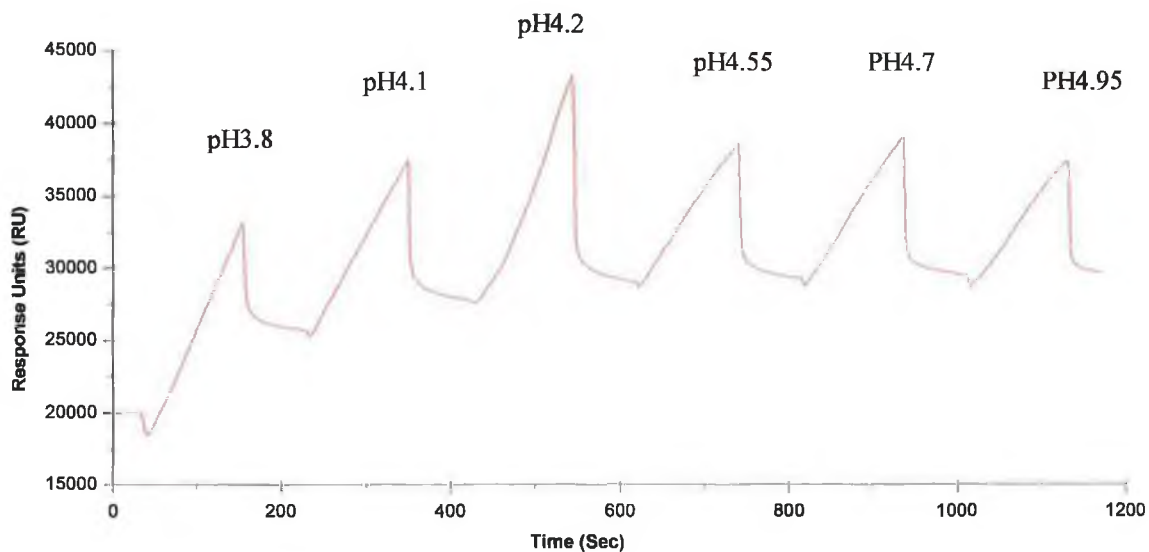
It is necessary to run a preconcentration step as a preliminary step to the immobilisation of a drug-protein conjugate on to the carboxymethylated dextran layer of the sensor chip. This step ensures that the immobilisation process is maximised. The preconcentration studies show the degree of electrostatic binding of the protonated amine groups on the conjugate to negatively charged carboxyl groups on the dextran matrix.

Different solutions of 50µg/ml morphine-3-glucuronide-thyroglobulin were prepared in 10mM sodium acetate of various pH, from 3.8 to 4.95. The pH of the sodium acetate was adjusted with 10% (v/v) acetic acid. Each protein solution was sequentially passed over an underivatised sensor flow cell at a flow rate of 5µl/min, as shown in Figure 6.8. Following the injection pulse of each solution, there is a pulse of Hepes buffered saline, (HBS), that is sufficient to dissociate the electrostatic attraction between the drug-protein conjugate and the carboxymethylated surface. The results of the preconcentration step are shown in Figure 6.8. The optimal pH determined for immobilisation of amphetamine-BSA is pH 4.2. All relevant immobilisations were carried out at this pH.

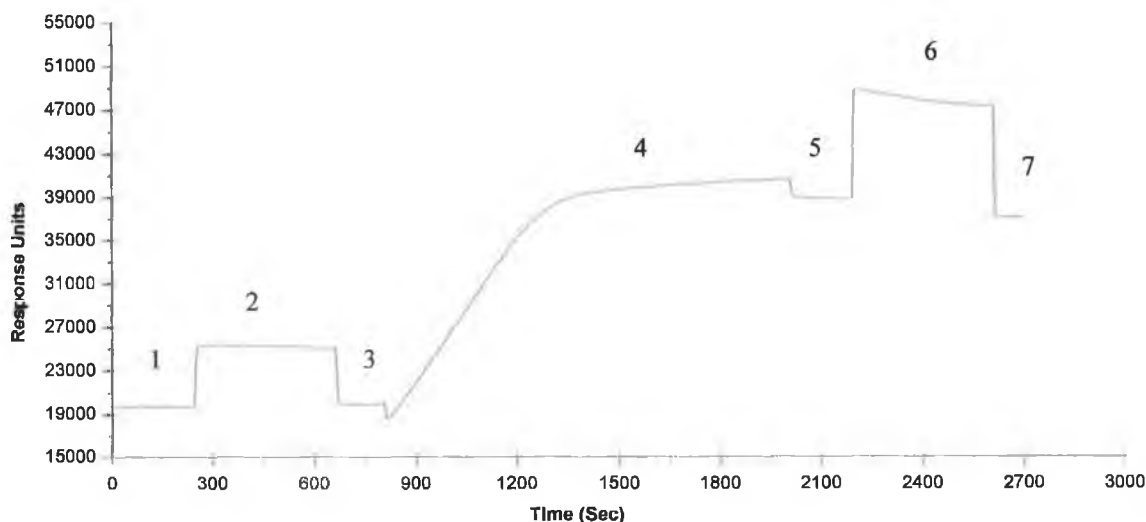
Although this pH is contributing to the immobilisation process, it is necessary to chemically modify the carboxymethylated dextran to achieve immobilisation.

### **6.2.4.2 Immobilisation of morphine-3-glucuronide-thyroglobulin**

The immobilisation of the morphine-3-glucuronide-thyroglobulin was performed as described in Section 2.10.2. A solution of EDC/NHS was passed over the chip. The carboxyl groups on the dextran layer of the sensor chip were converted into active ester functional groups by the EDC, and stabilized by the NHS. The morphine-3-glucuronide-thyroglobulin conjugate in 10mM sodium acetate, pH 4.2, was passed over the chip. The NHS esters then react with the available amine groups on the morphine conjugate. Figure 6.9 shows a typical immobilisation profile.



**Figure 6.8:** Preconcentration study of morphine-3-glucuronide-thyroglobulin in sodium acetate at a various pH onto the carboxymethylated dextran surface of the flow cell. The solutions containing 50 $\mu$ g/ml of morphine-3-glucuronide-thyroglobulin were passed over the surface for 2 minutes at a flow rate of 5 $\mu$ l/min. The response units for each solution is a measure of the electrostatic attraction between the negatively charged dextran and the positively charged protein conjugate. The ionic strength of the Hepes buffered saline is sufficient to dissociate the protein conjugate from the dextran layer. The optimal pH was determined to be pH 4.2, as shown on the figure.



**Figure 6.9:** Sensorgram of a typical immobilisation of morphine-3-glucuronide-thyroglobulin onto a CM5 dextran chip surface.

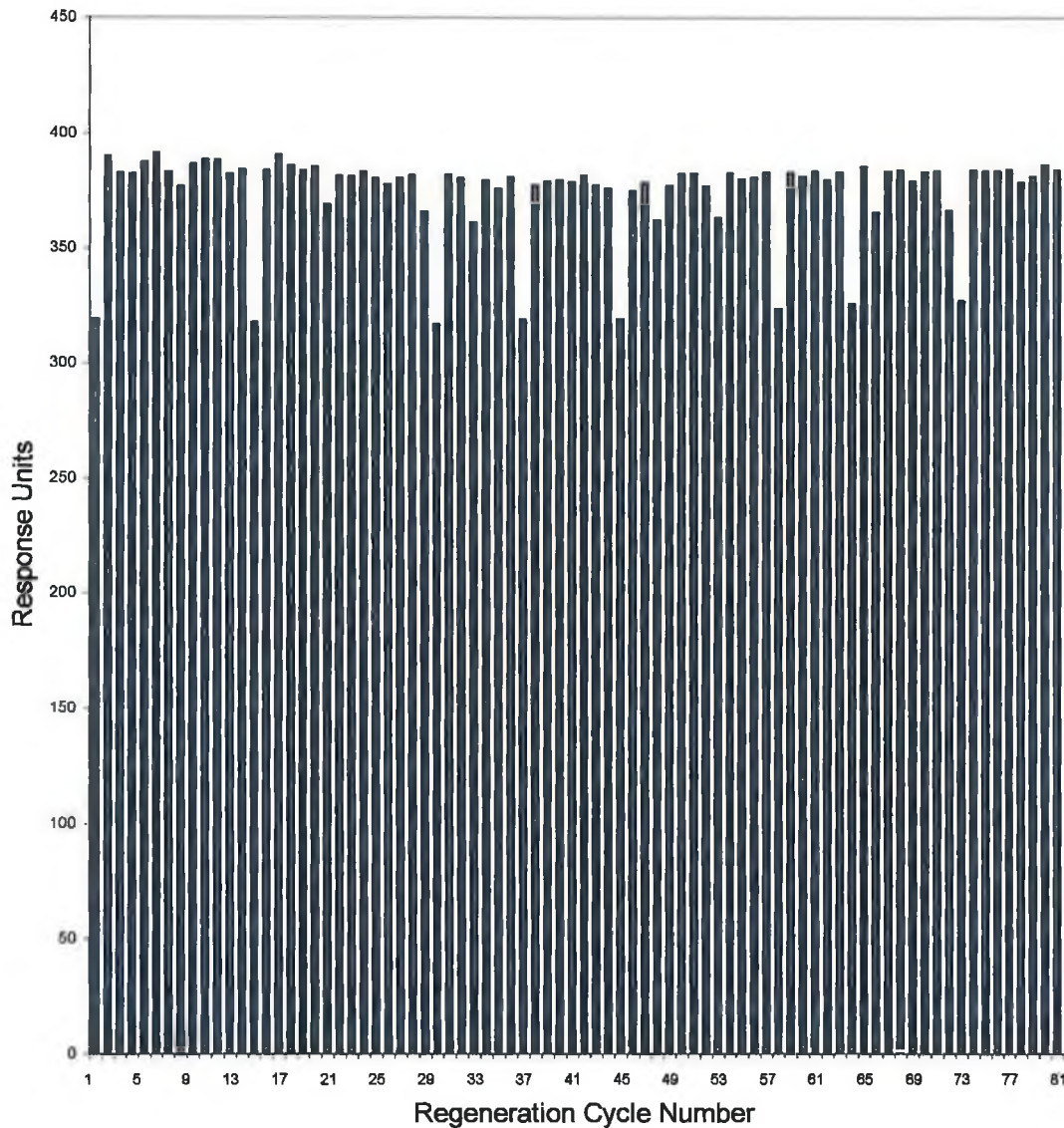
1. HBS buffer was passed over the surface and baseline measurement recorded.
2. A solution of EDC and NHS, final molarity 0.2M and 0.05M, respectively, was passed over the surface for seven minutes at a flow rate of 5 $\mu$ l/min to activate the carboxymethylated groups.
3. After the pulse of EDC/NHS, the HBS buffer was run over the surface again. The activation of the surface was seen by the small change (approx 120-200) in response units.
4. A solution of 50 $\mu$ g/ml of morphine-3-glucuronide-thyroglobulin in 10mM sodium acetate, pH 4.2, was passed over the surface for 20 minutes at a flow rate of 5 $\mu$ l/min.
5. The HBS buffer was run over the surface and the excess conjugate was eluted. The amount of bound conjugate was recorded as the change from baseline, in response units (RUs).
6. The surface NHS-esters were deactivated by a pulse of 1M ethanolamine hydrochloride, pH 8.5. This also removes any excess non-covalently bound conjugate.
7. The HBS running buffer resumes flow over the surface and the amount of bound morphine-3-glucuronide-thyroglobulin can be seen from the change in the response units. Approximately 18,000RUs of morphine-3-glucuronide-thyroglobulin were bound on the chip surface.

#### **6.2.4.3 Regeneration Conditions**

The previous sections dealt with the immobilisation of the morphine-protein conjugate on to the sensor chip. Other conditions must also be optimised before a successful assay can be established. It is preferable to be able to run multiple samples usually greater than 40 on one sensor chip. To do this, the regeneration conditions must be examined and chosen carefully and a regeneration cycle set up to determine the effects of the regeneration solutions on the surface and on the binding of the antibody to the surface.

A 1/100 final dilution of anti-morphine polyclonal antibody was found to give a binding response of approximately 250 response units. A range of different molarities of HCl and NaOH were tried to determine the optimal condition for generation of the sensor chip surface. The optimum combination found to give reproducible results was a 15 second pulse of 5µl of 5mM NaOH and a 30 second pulse of 10µl 40mM HCl. The surface was found to be reproducible for over 80 cycles of antibody binding and regeneration with this protocol. Figure 6.10 shows the response units for each cycle and it can be seen that from the second cycle to the eighth cycle there is a decrease in response units of only 1.6%. A drop in RU was seen in approximately every 15<sup>th</sup> cycle. It is difficult to explain this, other than it may have been caused by the cycle changing in the BIAcore, as the program for the regeneration were arranged in 15 pulse cycles.

The interaction between the thyroglobulin portion of the conjugate and the antibody was examined by immobilising 50µg/ml thyroglobulin in 10mM sodium acetate, pH 4.2. There was no response of the antibody to this surface. The response to dextran was examined and also found to be zero. It was found later, however, that in the saliva samples, the addition of thyroglobulin and dextran contributed to decreasing the 'stickiness' of the saliva, and so for all assays, 100µg/ml thyroglobulin and carboxymethylated dextran were added to the antibody diluent. The 'stickiness' of the saliva was seen as non-specific binding of the saliva to the immobilised surface.

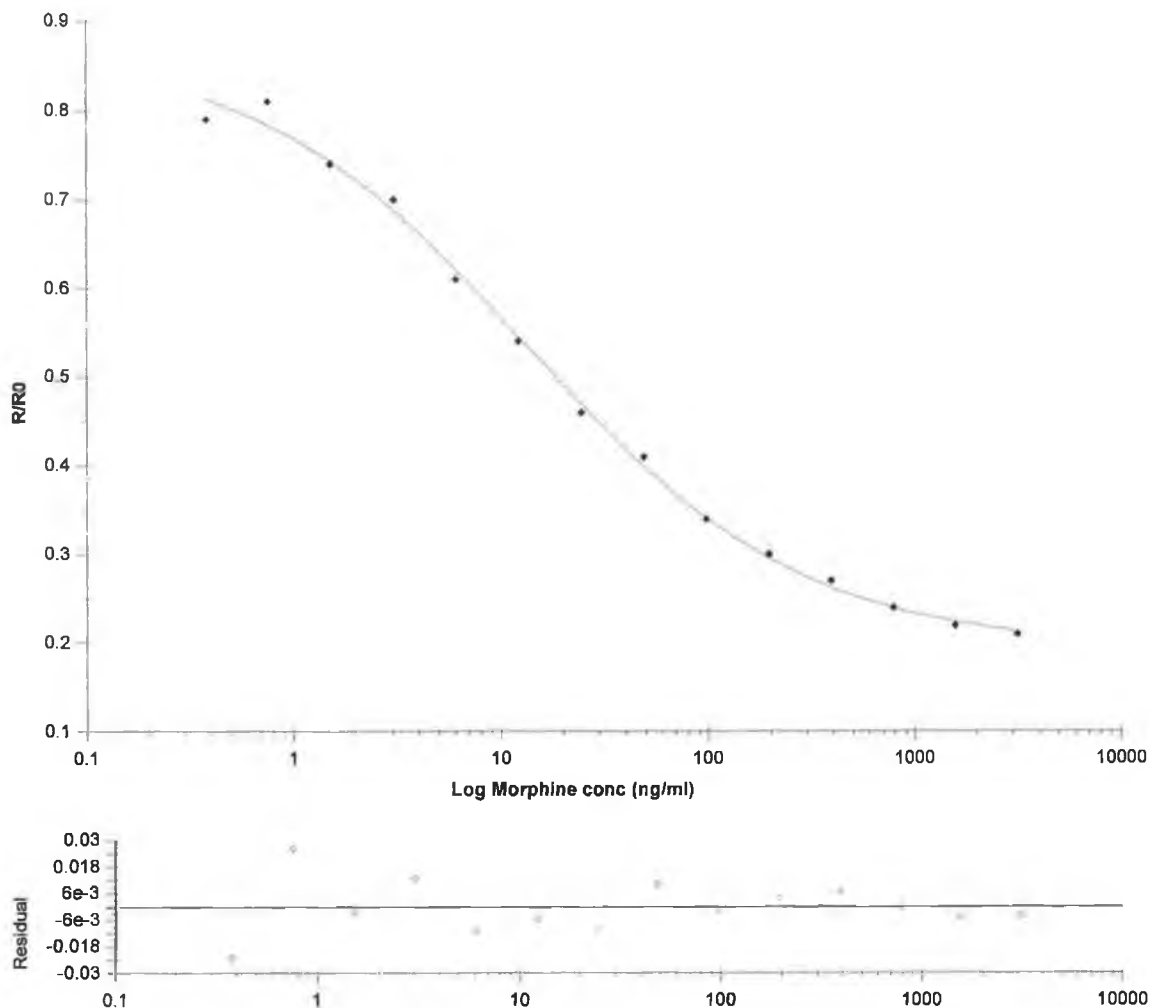


**Figure 6.10:** Regeneration profile of the anti-morphine polyclonal antibody binding to the morphine-3-glucuronide-tyroglobulin immobilised surface. A 1/100 dilution of antibody was passed over the surface for 4 minutes. The surface was regenerated with a 15 second pulse of 5  $\mu$ ls of 5mM NaOH and a 30 second pulse of 10  $\mu$ ls 40mM HCl. A drop in RU was seen in approximately every 15<sup>th</sup> cycle. It may have been caused by the cycle changing in the BIAcore, as the program for the regeneration were arranged in 15 pulse cycles.

#### ***6.2.4.4 Determination of range of detection of morphine in the BIAcore competitive assay***

To determine the working range of detection of morphine on this assay, standard morphine concentrations were prepared in HBS buffer, ranging from 0.09 to 25,000 ng/ml. The anti-morphine polyclonal antibody, diluted in HBS containing thyroglobulin and dextran, was mixed with equal volumes of each standard and allowed to come to equilibrium for 15 minutes on the bench before being placed in the BIAcore for the assay run. The samples were passed over the morphine-3-glucuronide-thyroglobulin immobilised surface in random order. Each cycle was followed by a regeneration cycle. Each drug-antibody solution was run over the surface three times in random order. This eliminated any possible bias that could have been incorporated into the assay. Each value was normalised for that intra-assay by dividing the RU obtained by the RU for the positive control that contained only antibody and no morphine. The intra-assay variability is shown in Table 6.6. The inter-assay calibration is the combination of three different assays run on three different days. The calibration curve for the inter-assay is plotted in Figure 6.11. The range of detection of the assay is 1.52 to 3125ng/ml. The back-calculated values as determined by the four-parameter fit of the calibration curve for the morphine standards show the assay to be accurate. The degree of accuracy can be determined by calculating the percent recovery of the known value for different concentrations. This percent recovery is a quantitative measure of the closeness of the observed result (back-calculated result) to its theoretical true value, expressed as a percent of the nominal, theoretical concentration. The high degree of precision of the assay is expressed in the percent coefficient of variation of the intra assay variation as shown in Table 6.6.





**Figure 6.11:** Inter-day curve for the detection of morphine using the anti-morphine polyclonal antibody on an morphine-3-glucuronide-thyroglobulin immobilised surface. The data was correlated to a four-parameter model fit and the plot constructed using BIAevaluation 3.1 software. Each point on the graph is the average of three results obtained on three different days from a set of three replicates. Each value was normalised for that intra-assay by dividing the RU obtained by the RU for the positive control that only contained antibody and no morphine. The coefficient of variation, back-calculated morphine concentration and the percentage recovery are shown in Table 6.7. The range of detection of the assay is 1.52 to 3125ng/ml.

**Table 6.6:** Intra-assay variation (degree of precision) for the detection of morphine in the BIAcore–based competitive assay using the anti-morphine polyclonal antibody.

The results presented are the mean values obtained from three replicates.

<b>Actual Morphine Conc (ng/ml)</b>	<b>Back-Calculated Morphine conc. (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
1.5	1.9	6.76	125.39
3.1	1.9	3.20	65.44
6.1	6.8	10.51	111.74
12.2	12.7	9.66	103.88
24.4	36.5	2.53	149.63
48.8	20.9	1.27	42.80
97.7	157.5	7.10	161.17
195.3	224.9	2.99	115.14
390.6	409.7	4.79	104.89
781.3	704.4	10.37	90.15
1562.5	1906.8	7.70	122.04
3125	2205.2	1.81	70.57

**Table 6.7:** Inter-assay variation (degree of accuracy and reproducibility) for the detection of morphine in the BIAcore –based competitive assay using the anti-morphine polyclonal antibody. The results presented are the mean values obtained from five intra-day assays, each assay was performed on three replicates.

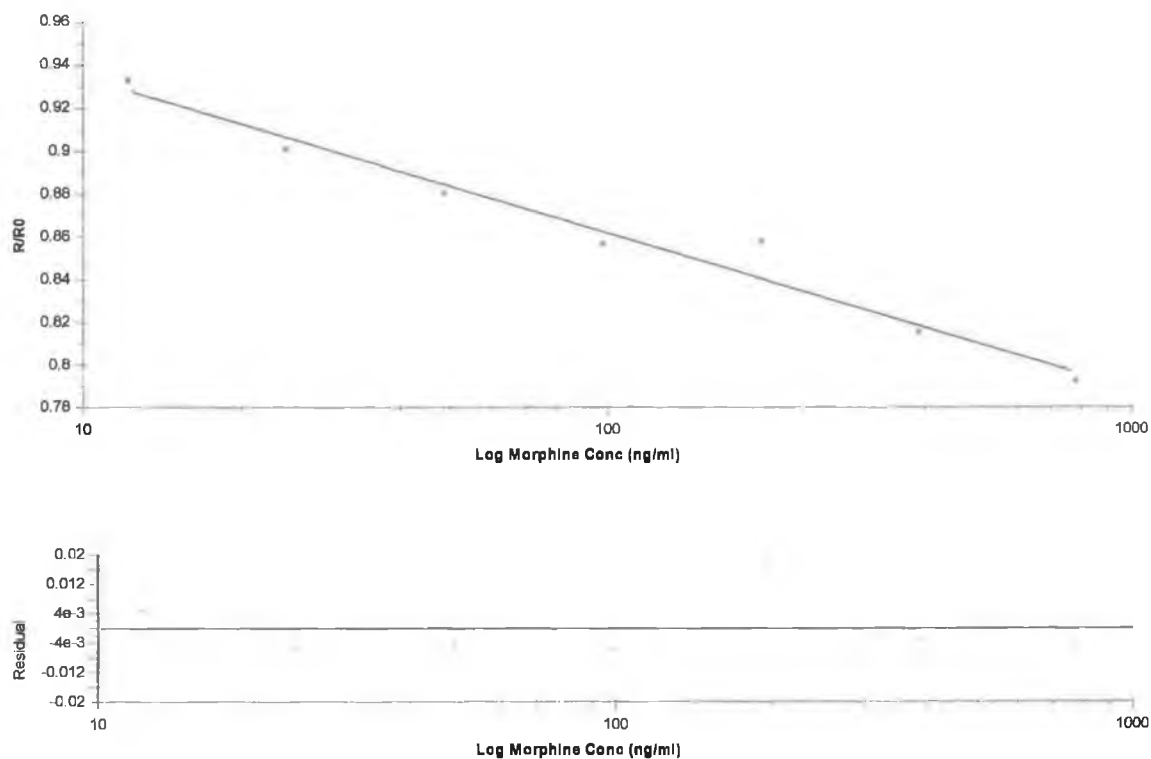
<b>Actual Morphine Conc (ng/ml)</b>	<b>Back-Calculated Morphine conc. (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
1.5	1.6	10.15	103.09
3.1	2.6	9.10	86.36
6.1	6.8	6.64	110.67
12.2	12.8	3.64	105.26
24.4	26.7	2.88	109.47
48.8	43.8	12.27	89.74
97.7	99.2	3.52	101.56
195.3	181.8	6.38	93.06
390.6	329.4	9.27	84.33
781.3	779.4	15.02	99.76
1562.5	2036.1	16.24	130.31
3125	4655.1	14.07	148.96

### ***6.2.5 Development of a BIAcore-based competitive immunoassay for the detection of morphine in saliva samples***

Saliva was applied to the BIAcore-based competitive assay for the determination of morphine. Negative control saliva samples were initially applied to the assay to determine the characteristics of the saliva with regard to the assay format. A huge difference was seen with regard to the response units obtained when saliva was passed over the morphine-3-glucuronide-thyroglobulin immobilised surface. Additions were made to the saliva to try and decrease this response, including the addition of thyroglobulin and dextran. Another problem experienced with the saliva samples was that different negative control saliva samples gave different responses with regard to the non-specific binding. The relatively high inter-assay coefficients of variation for the saliva assay express this variation also. The working range of detection within this assay was 12.2-781.3ng/ml (Figure 6.12, Table 6.8).

The possibility of using undiluted saliva directly in the BIAcore assay was ruled out. Attempts were made to optimise the saliva assays by looking at three different aspects as follows:

- Dilution of saliva sample
- Ionic strength of HBS diluent for antibody, that would be mixed with saliva sample
- Sensor chip surface



**Figure 6.12:** Inter-day curve for the detection of morphine in saliva samples using the anti-morphine polyclonal antibody on a morphine-3-glucuronide-tyroglobulin immobilised surface. The data was correlated to a four-parameter model fit and the plot constructed using BIAevaluation 3.1 software. Each point on the graph is the average of three results obtained on three different days from a set of three replicates. Each value was normalised for that intra-assay by dividing the RU obtained by the RU for the positive control that only contained antibody and no morphine. The coefficient of variation, back-calculated morphine concentration and the percentage recovery are shown in Table 6.8.

**Table 6.8:** Inter-assay variation (degree of accuracy and reproducibility) for the detection of morphine in saliva samples in the BIAcore –based competitive assay using the anti-morphine polyclonal antibody. The percentage coefficient of variation values are quite high. The results presented are the mean values obtained from five intra-day assays, each assay was performed on three replicates.

<b>Actual Morphine Conc (ng/ml)</b>	<b>Back-Calculated Morphine conc. (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
12.2	10.5	37.74	86.29
24.4	28.9	19.58	118.52
48.8	55.2	23.90	113.22
97.7	116.7	23.91	119.54
195.3	113.1	17.71	57.94
390.6	428.9	22.70	109.82
781.3	886.8	23.33	113.51

### ***6.2.5.1 Influence of saliva dilution on BIAcore assay***

Figure 6.13 shows the results of saliva sample dilutions on the degree of competition seen on the BIAcore assay, as compared to that of the model assay in HBS. As expected the degree of competition and so level of detection of morphine increases as the saliva content is decreased. This clearly shows the constituents of saliva are directly effecting the assay and decreasing the sensitivity.

### ***6.2.5.2 Influence of ionic strength of running buffer on saliva assay***

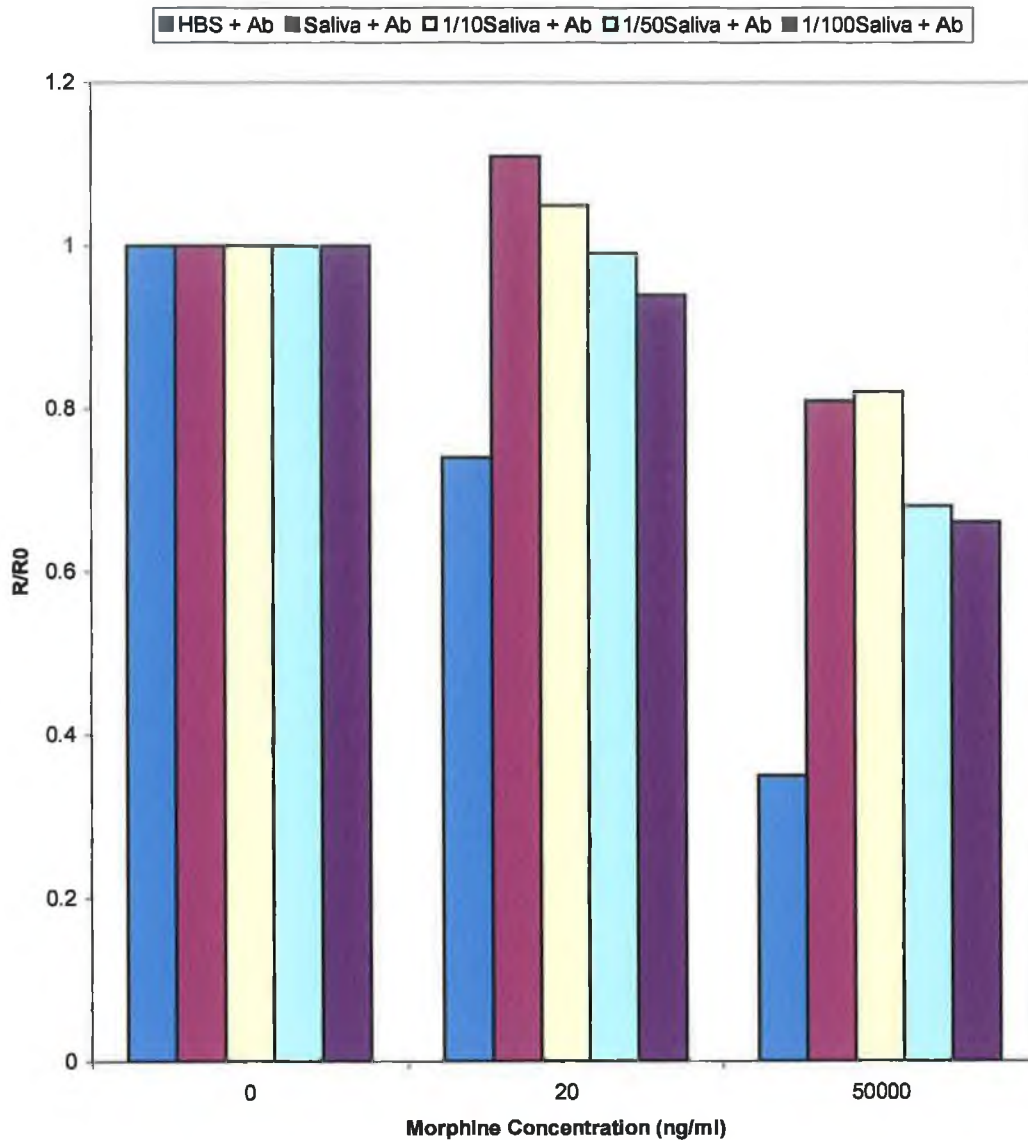
The ionic strength of saliva may vary and so as a means to standardise the assay, the effect of varying the ionic strength of the antibody diluent, HBS, was investigated. As a control, samples were also run in HBS alone, with no saliva. It was seen from these samples that as the ionic strength of the buffer increase the level of competition seen in the assay was decreased. In the case of the saliva samples, the change in ionic strength did not contribute to increasing the level of competition (Figure 6.14).

### ***6.2.5.3 Influence of different sensor chip surfaces on saliva assay***

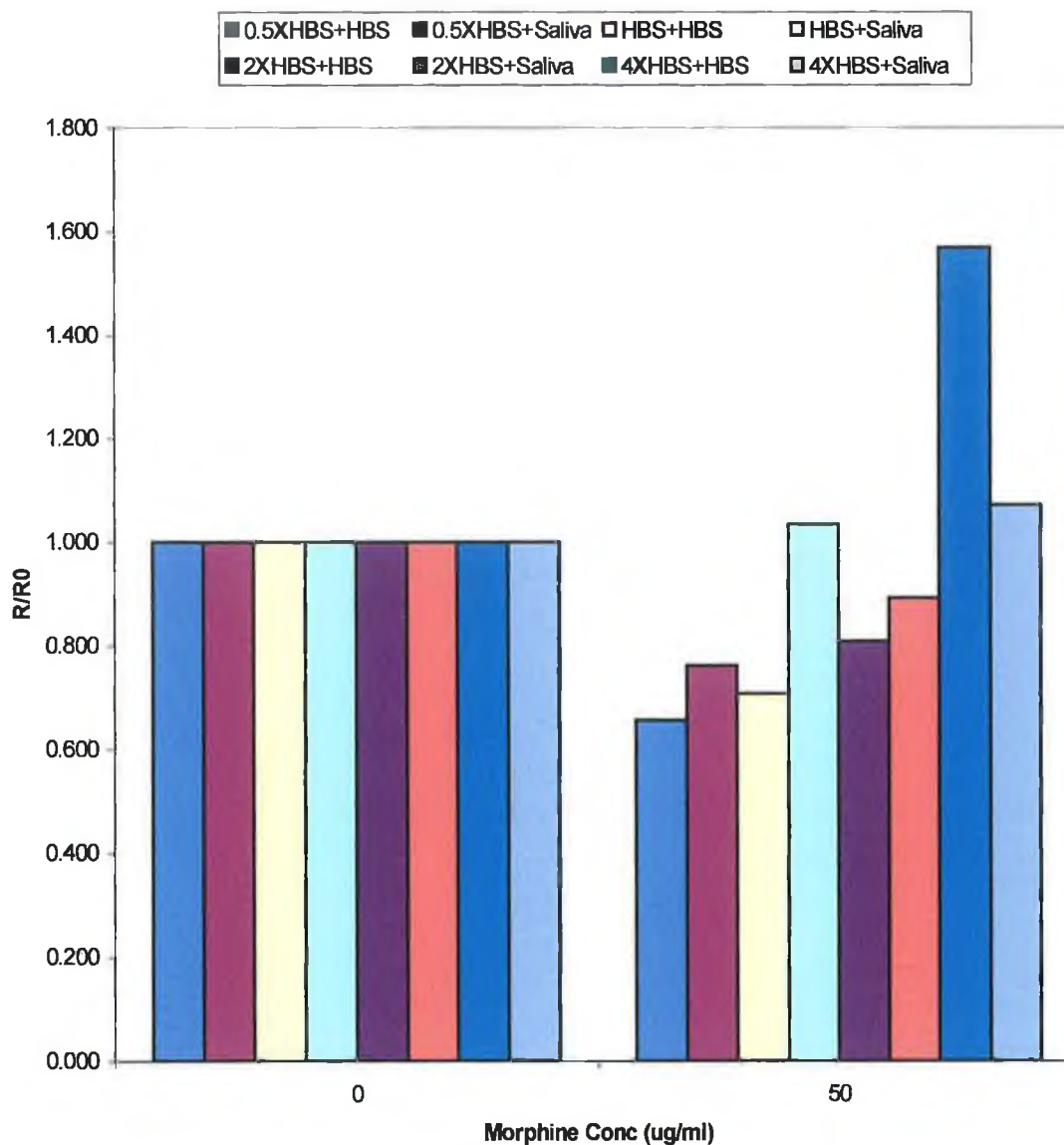
BIAcore design different sensor chip surfaces for different applications. The most common analytical chip is the CM5, and this is used for all the inhibition assays. The Pioneer range of products are available for more investigative applications, as these sensor chip surfaces are made of alternative matrices such as shorter dextran layers. As discussed above, the addition of 100µg/ml cm-dextran to the diluent did reduce the non-specific binding of saliva to the CM5 chip. To investigate if the dextran layer was a factor contributing to this non-specific binding, it was decided to try a different sensor chip that had a shorter dextran layer. The F1 chip provides the same functionality as the CM5 chip but has a shorter dextran matrix. Due to the shorter matrix the immobilisation yield is reduced to about 30% of that obtained with the CM5 chip. To examine the influence of the dextran layer, the morphine assay was applied to the F1 chip. The results were disappointing in that a inhibition assay was not achieved. The values for the intra-assay are displayed in Table 6.9. One reason for this lack of inhibition is the

lower amount of immobilisation of the conjugate, and so the sensitivity is reduced to the point where inhibition cannot be detected.





**Figure 6.13:** The effect of saliva on the BIAcore competitive assay for the detection of morphine is demonstrated above. The model assay was established in HBS buffer as described previously in Section 6.2.4.4. The RU obtained at different morphine concentrations are normalised by expressing as a fraction of the RU obtained for the antibody in HBS, with no morphine. Undiluted saliva, and saliva diluted, 1/10, 1/50 and 1/100 in HBS were spiked with 20ng/ml and 50 $\mu$ g/ml morphine and mixed with antibody solution as per the assay described. The saliva interfered with the competition seen in the assay, with a decrease seen in the level of detection. This decrease was proportional to the dilution of the saliva sample used.



**Figure 6.14:** The effect of ionic strength of the HBS buffer on the BIAcore inhibition assay for the detection of morphine is demonstrated above. The samples were run with the antibody diluted in different buffers: normal strength HBS, 0.5X HBS, 2XHBS and 4XHBS. Samples of HBS and saliva were spiked with 50 $\mu$ g/ml morphine, and samples containing no morphine were used as controls. The RU obtained for a morphine-containing sample was normalised by expressing it as a fraction of the RU obtained for the antibody in the corresponding buffer and HBS or saliva, with no morphine.

**Table 6.9:** Intra-assay degree of competition (R/R0) and variation (degree of precision) for the detection of morphine in the BIAcore-based competitive assay on a F1 Biacore sensor chip, using the anti-morphine polyclonal antibody. Competition was not found in this assay, as can be seen from the results. The results presented are the mean value of three replicates.

<b>Morphine Conc (ng/ml)</b>	<b>R/R0</b>	<b>CV %</b>
0	1	14.23
3.05	0.867	6.97
6.1	0.927	18.91
12.2	0.942	15.72
24.4	0.967	20.71
48.8	0.914	7.67
97.7	0.848	11.31
195.3	1.001	22.49
390.6	0.896	5.15
781.3	0.879	1.67
1562.5	0.891	11.74
3125	0.887	15.32
6250	0.944	11.95
12500	0.903	8.58
25000	0.967	22.37

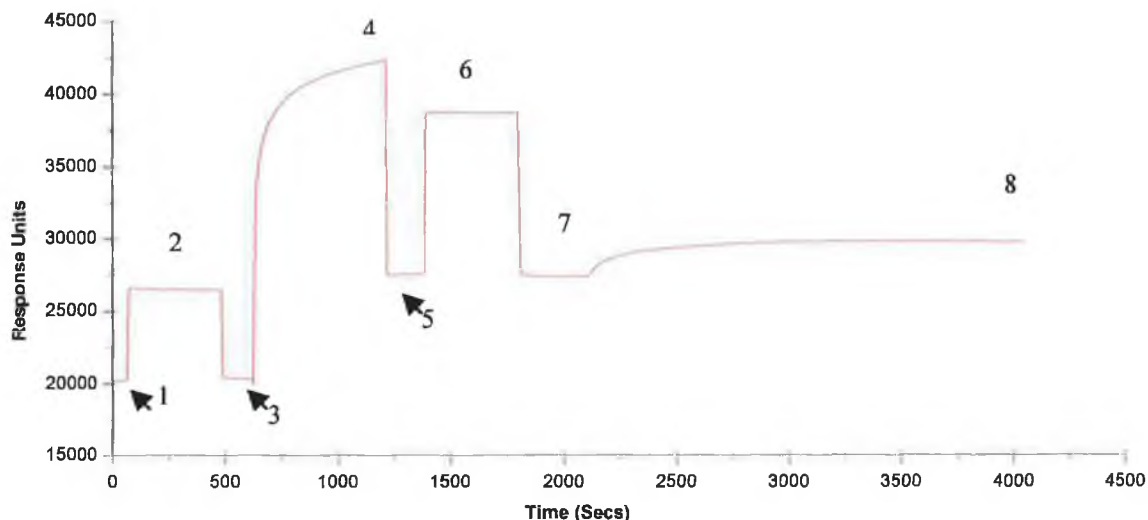
## **6.2.6 Development of a BLAcore-based competitive immunoassay for the detection of THC in saliva samples**

### **6.2.6.1 Preconcentration studies**

As described in a previous section, the preconcentration step is performed to assess the electrostatic binding of protonated amine groups on the conjugate to negatively charged carboxyl groups on the dextran matrix. Preconcentration can be facilitated by lowering the pH of the solution below the pI of the protein conjugate. The preconcentration studies were carried out for THC-thyroglobulin and THC-BSA by preparing a series of standards in 10mM sodium acetate buffer at pH from 4.0 to 5.3. The solutions were passed over the underivatized sensor chip and the degree of electrostatic binding was monitored. The preconcentration study with THC-thyroglobulin and THC-BSA showed negligible binding at the range of pH examined. It is not possible to lower the pH below 4.0 as the protonation of the carboxy groups on the dextran matrix occurs and this causes a collapse of the gel. It can be concluded from these studies, that the pI of the THC-thyroglobulin and THC-BSA is less than 4.0. This is probably a result of the high degree of conjugation of the THC to the amine groups of the thyroglobulin and BSA. To overcome this problem an alternative process for the immobilisation of the THC conjugate had to be investigated.

### **6.2.6.2 Immobilisation of THC-protein conjugate through biotinylation and use of streptavidin on the sensor surface**

The strong avidity between streptavidin and biotin has been utilised in many processes. In this case, the THC-protein conjugate was biotinylated through the use of a biotin-S-NHS ester. The streptavidin was successfully immobilised on the dextran layer of the sensor chip using EDC/NHS chemistry. The biotinylated THC-thyroglobulin was then passed over the surface and the subsequent binding of the biotin to the streptavidin occurred, resulting in the immobilisation of the THC-thyroglobulin. The sensorgram for this procedure is shown in Figure 6.15.



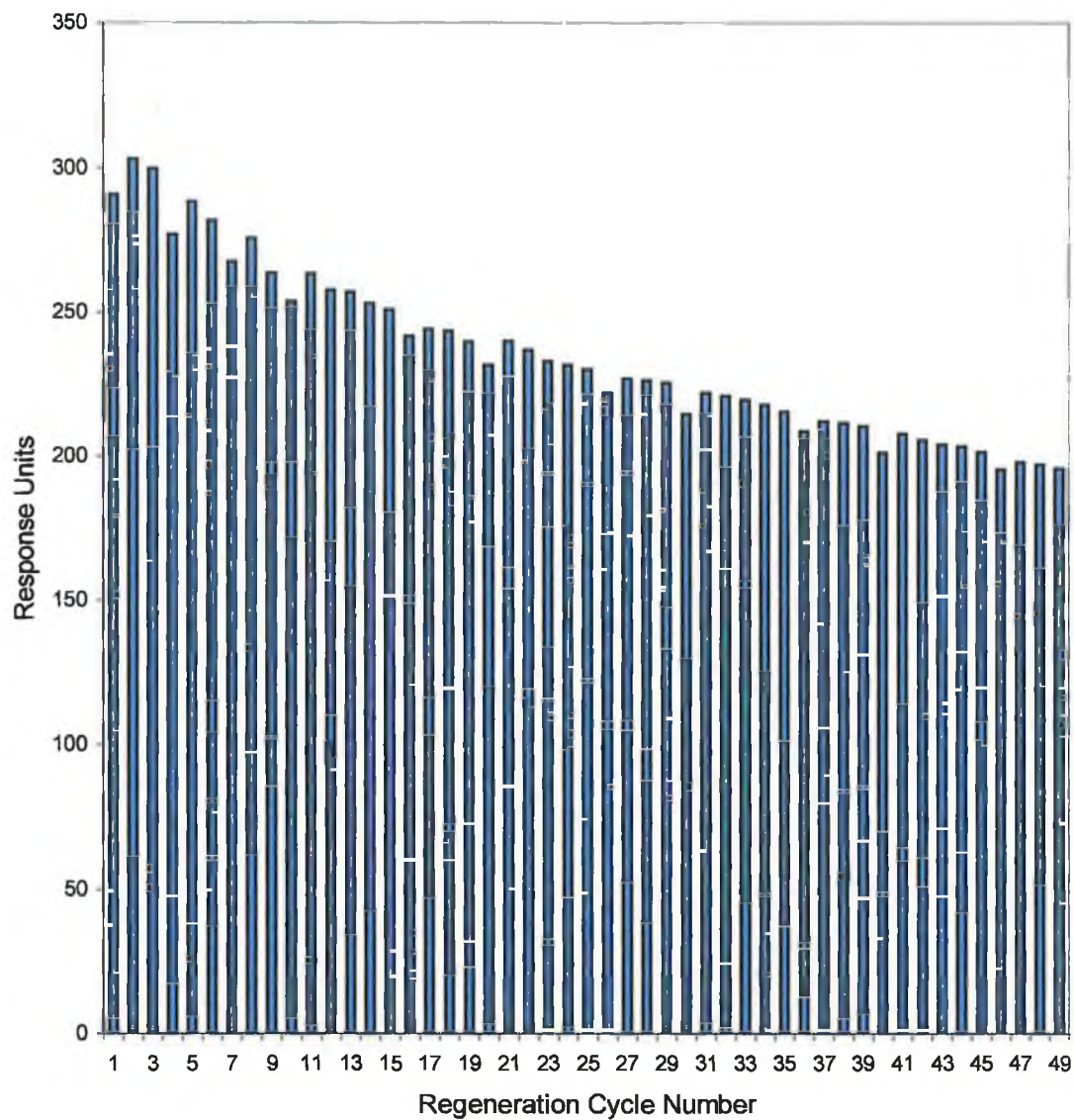
**Figure 6.15:** Sensorgram of a typical immobilisation of streptavidin followed by biotinylated-THC-thyroglobulin onto a CM5 dextran chip surface.

1. HBS buffer was passed over the surface and baseline measurement recorded.
2. A solution of EDC and NHS, final molarity 0.2M and 0.05M, respectively, was passed over the surface for 7 minutes at a flow rate of 5 $\mu$ l/min to activate the carboxymethylated groups.
3. After the pulse of EDC/NHS, the HBS buffer was run over the surface again. The activation of the surface is seen by the small change (approx 120-200) in response units.
4. A solution of 12.5 $\mu$ g/ml of streptavidin in 10mM sodium acetate, pH 5.0, was passed over the surface for 7 minutes at a flow rate of 5 $\mu$ l/min.
5. The HBS buffer was run over the surface and the excess streptavidin eluted away from the surface. The amount of bound streptavidin was recorded as the change from baseline. Approximately 5,000 –7,000RU of streptavidin were bound on the chip surface.
6. The unreacted surface NHS-esters were deactivated by a pulse of 1M ethanolamine hydrochloride, pH 8.5. This also removes any excess non-covalently bound streptavidin.
7. The HBS running buffer resumes flow over the surface and the amount of bound streptavidin can be seen from the change in the response units.
8. A solution of biotin-NHS-THC-thyroglobulin (1/100 dilution in HBS, approximately 10 $\mu$ g/ml) was passed over the surface for 30mins at a flow rate of 5 $\mu$ l/min. This was followed by the HBS running buffer resuming flow across the sensor surface. The amount of bound biotin-THC-thyroglobulin was seen by the change in response units. Approximately 3,000RU of biotin-THC-thyroglobulin were bound on the chip surface.

### **6.2.6.3 Regeneration Studies**

As discussed previously, the successful development of a BIAcore-based assay needs to establish the conditions for regeneration of the immobilised surface and demonstrate the reproducibility of the assay over a number of cycles of binding and regeneration.

Many different solutions at different molarities were tested for dissociating the anti-THC polyclonal antibody from the immobilised streptavidin-biotin-THC-thyroglobulin surface. These included ethanolamine, HCl and NaOH. The solution that gave the best results was one pulse of 60mM NaOH for 4 minutes at a flow rate of 5 $\mu$ l/min. To establish the regeneration profile for the assay, the antibody solution was passed over the surface for 4 minutes at a flow rate of 5 $\mu$ l/min. This was followed by the regeneration solution of 60mM NaOH for another four minutes. The study showed that there was a progressive decrease in the binding capacity as the cycles were repeated. This was the best solution of all those tested, but unfortunately only allowed 30 cycles of binding, after which, there was a >22% decrease in the binding capacity. This is shown graphically in Figure 6.16. From cycle 1 to 30 there was a 22% decrease in the binding capacity, from cycle 1 to 40, there was a 40% decrease in capacity and from cycle 1 to 50 there was a 36% decrease in binding. When this is compared to the regeneration studies for other assays, it does appear to be very low. However, in the case of this assay, it must be remembered that the immobilised surface has a number of different chemistries and interactions involved. This means that the regeneration solution has more potential interactions that it can effect. In this case the NaOH could be effecting the biotin-streptavidin interaction or the streptavidin-carboxydextran interaction. It could also effect the THC molecules on the immobilised conjugate.



**Figure 6.16:** Regeneration studies on the anti-THC polyclonal antibody on the streptavidin-biotin-THC-thyroglobulin. Pulses of 60mM NaOH were used to dissociate the antibody from the immobilised surface. The ligand binding capacity was shown to progressively decrease with the cycles. Only 30 cycles of regenerations are recommended for this assay.

#### ***6.2.6.4 Non Specific Binding***

The degree of non-specific binding of the anti-THC polyclonal antibody to the streptavidin-biotin-THC-thyroglobulin surface must be determined, as a control in establishing the BIAcore-based assay. To do this, the sensor surface must be activated the same way as for the THC conjugate immobilisation by immobilising streptavidin, followed by biotin-thyroglobulin. The immobilisation was successful and approximately 3,000 response units of biotinylated-thyroglobulin were immobilised. A solution of antibody was then passed over the chip surface and a response of approximately 90 response units was seen. In order to eliminate this non-specific binding from the assay, thyroglobulin, at a concentration of 50µg/ml, was included in the HBS used for the antibody diluent. This resulted in a decrease in the non-specific binding to 40 response units. The thyroglobulin was increased further, however this did not decrease the non-specific binding further. This can be explained by the polyclonal nature of the antibody. The production of polyclonal antibodies to an immunogen means that there are different antibodies recognising different epitopes of the immunogen. Antibodies could recognise regions between the THC and thyroglobulin and similar regions between the THC and biotin. These regions would not be on underivatised thyroglobulin and this would explain why the response was not eliminated completely by including the thyroglobulin in the diluent.

#### ***6.2.6.5 Determination of range of detection of THC in the BIAcore competitive assay***

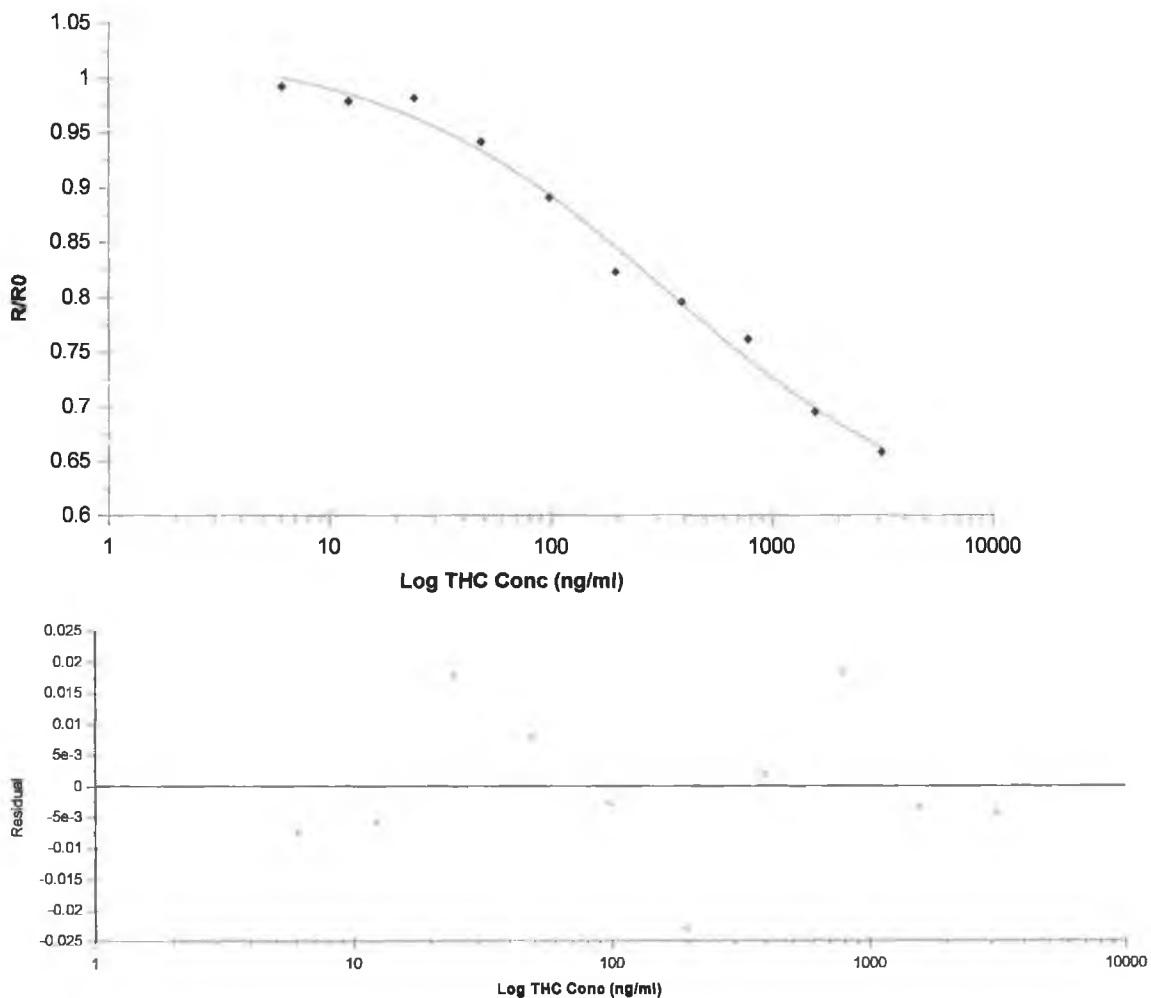
To determine the working range of detection of THC in this assay, a number of standard THC concentrations were prepared in HBS buffer, ranging from 0.09 to 25,000 ng/ml. The anti-THC polyclonal antibody, diluted in HBS containing thyroglobulin, was mixed with equal volumes of each standard and allowed to come to equilibrium for 15 minutes on the bench before being placed in the BIAcore for the assay run. The samples were passed over the streptavidin-biotin-THC-thyroglobulin immobilised surface in random order. Each cycle was followed by the regeneration cycle. Each drug-antibody solution was run over the surface twice in random order. This, therefore, eliminated any possible bias that could have been incorporated into the assay. Each value was normalised for by dividing the RU obtained by the RU for the control that contained



only antibody and no THC. An example of the intra-assay variability is shown in Table 6.10. The inter-assay calibration is the combination of five different assays run on five different days. The calibration curve for the inter-assay is plotted in Figure 6.17. The range of detection of the assay is 48.8 to 3125ng/ml. The back-calculated values, as determined by the four-parameter fit of the calibration curve for the THC standards, show the assay to be quite accurate for values between 48.8 and 3125ng/ml. The degree of accuracy can be determined by calculating the percent recovery of the known value for different concentrations. This percent recovery is a quantitative measure of the closeness of the observed result (back-calculated result) to its theoretical true value, expressed as a percent of the nominal, theoretical concentration. The degree of precision of the assay is expressed in the percent coefficient of variation of the intra assay variation as shown in Table 6.11.

**Table 6.10:** Intra-assay variation (degree of precision) for the detection of THC in the BIAcore –based competitive assay, using the anti-THC polyclonal antibody. The results presented are the mean of three replicates.

<b>THC Conc (ng/ml)</b>	<b>R/R0</b>	<b>%CV</b>
0	1	9.70
6.1	0.993	7.78
12.2	0.944	4.66
24.4	0.928	10.78
48.8	0.894	8.19
97.7	0.891	10.34
195.3	0.866	11.23
390.6	0.850	6.36
781.3	0.838	6.77
1562.5	0.821	3.32
3125	0.772	5.36
6250	0.783	8.01
12500	0.713	7.81
25000	0.708	7.64
50000	0.659	0.12



**Figure 6.17:** Inter-day curve for the detection of THC using the anti-THC polyclonal antibody on an streptavidin-biotin-THC-thyroglobulin immobilised surface. The data was correlated to a four-parameter model fit and the plot constructed using BIAevaluation 3.1 software. Each point on the graph is the average of two replicates obtained on five different days. Each value was normalised for that intra-assay by dividing the RU obtained by the RU for the control that contained antibody and no THC. The coefficient of variation, back-calculated THC concentration and the percentage recovery are shown in Table 6.11. The range of detection of the assay was 12.2 to 3125 ng/ml THC.

**Table 6.11:** Inter-assay variation (degree of accuracy and reproducibility) for the detection of THC in the BIAcore-based inhibition assay using the anti-THC polyclonal antibody. The results presented are the mean of five intra-day assays, each assay performed on two replicates.

<b>Actual THC Conc (ng/ml)</b>	<b>Back-Calculated THC Conc. (ng/ml)</b>	<b>CV %</b>	<b>Recovery %</b>
12.2	15.1	17.59	124.1
24.4	13.9	18.97	57.1
48.8	41.4	4.21	84.9
97.7	101.8	6.53	104.2
195.3	265.3	4.80	135.8
390.6	380.6	9.58	97.4
781.3	603.4	17.16	77.2
1562.5	1656.0	18.31	106.0
3125.0	3448.3	21.67	110.3

### **6.2.7 *Real sample analysis for detection of THC and opioids***

A pilot study was undertaken to assess the feasibility of collecting and testing 'real' saliva samples from drug abusers. A number of samples were taken from clients attending the Trinity Court Drug Centre. During our interview with the clients, the saliva sample was collected and they were asked about their recent use of drugs. The project, including the method of saliva collection, the drug tests on the saliva and the confidentiality of the study were described fully to the clients. Samples were only taken from clients who gave written informed consent. A copy of the consent form was given to the clients. The samples were only taken from clients who were currently thought to be using drugs to some degree. The staff at the clinic identified these clients from their experiences with them and prior laboratory urine analysis. All patients, with the exception of one, were on methadone at varying dosages. Client No. 1 was on full dose methadone on the rehabilitation program, whereas the clients who had previously failed to stay off drugs, were on the low dose methadone program. This client population had a higher risk of concurrent drug abuse. Client No. 6 was attending the clinic for the first time and was not receiving any methadone. All clients were asked to provide information regarding their recent drug use. This was completed in the absence of the Trinity Court personnel to encourage the clients to give more information to us. They also expressed their views on giving urine and saliva samples. A summary of the information obtained from interviewing the clients regarding their recent drug use is shown in Table 6.12. It is important to remember that this information was obtained from clients who are not always willing to share such information as it can effect the program they are on and how much methadone they are prescribed.

All clients expressed their dislike of giving urine samples. They also mentioned that on several occasions they are unable to urinate. All clients, with the exception of client No.8 were willing to use the saliva collection device. Client No. 8 did not like the texture of the material and gave a spit sample. On return to the lab this sample proved unsuitable for testing. The volume of sample provided by the clients varied from 1.8mls for Client no. 1 to less than 25µls Client no. 6. It was evident during our meetings with the clients that the greater the use of drugs the more likely they were to give smaller volumes of sample.

**Table 6.12:** Summary of clients interviews regarding recent drug use.

<b>Client Number</b>	<b>Program</b>	<b>Recent Drug Use</b>
1 (A-D)	Rehab – High dose Methadone	Heroin – last taken within 5 days Benzodiazepines Cannabis – within 24 hours (smoked) Medications: Methadone
2 (S-C)	Low dose methadone	Heroin – last taken 15-20 minutes Diazepam – 10mgs X 20 (just prior to visit) Dalmane – 30mgs X 10 (just prior to visit) Cannabis – not taken Medications: Methadone, Zimfine (Unable to give urine on day of saliva collection and interview)
3 (F-W)	Low Dose Methadone	Heroin – within 24 hours Benzodiazepines – within 24 hours (Unable to give urine on day of saliva collection and interview)
4 (JMK)	Low Dose Methadone	Heroin – within 24-48 hours Cannabis – within 24 hours Medications: Methadone, Valium
5 (JOD)	Low Dose Methadone	Heroin – within 24 hours Cannabis – within 24 hours Medications: Methadone, Valium
6 (R-H)	New client – first visit	Heroin – within hours DF8's – within hours
7 (AOR)	Low Dose Methadone	Heroin – within 48 hours Cannabis – not taken Medications: Methadone
8 (A-D)	Low Dose Methadone	Heroin – taken day before visit Cannabis – within 24 hours (smoked) Medications: Methadone, Stillnoc, Valium Benzodiazepines

#### *6.2.7.1 Detection of THC in 'real' saliva samples*

The saliva samples were collected as above and they were tested over several days for the presence of THC using the assay on the Envitec device and the conventional ELISA format as characterised above. Table 6.13 shows the results from the chronic drug users collected in Trinity Court Drug Treatment Centre. The samples were initially frozen at  $-20^{\circ}\text{C}$ , and were defrosted before each assay. There was no correlation between the result obtained on ELISA, and the normalised result from the Envitec device assay. The results presented show the instability of the THC to these conditions and this is most probably the reason behind the disparity in results from the ELISA and Envitec device. These sample were subject to more freeze-thaw cycles as additional testing for morphine was carried out on these samples. These repeated freeze-thaw conditions obviously degraded the THC between the time of the initial ELISA and being run on the Envitec device. As discussed at the beginning of the chapter, THC is a very unstable solution in storage. From the prospect of testing for drugs of abuse in a road-side or clinic environment, this should not pose a problem as the sample is tested immediately after it is taken. This was the case with the spiked samples that were run on the Envitec device. Positive results could be identified with a cut off level of detection of 200ng/ml. It does however, present a problem if samples are to be stored for a quantitative analysis, as these results show there is a definite decrease in the level of THC detected after prolonged storage and freeze-thaw cycles.

**Table 6.13:** Detection of THC concentration in samples from Trinity Court Drug treatment clients on the ELISA and Envitec device assay.

<b>Client Sample Number</b>	<b>THC Conc per ELISA (1)</b>	<b>THC Result from Envitec Device (2)</b>	<b>Repeat ELISA Results (3)</b>
1	196.6	<i>1/10 Dilution:</i> 0.66±0.03 versus 0.68±0.03 for blank <i>1:5 Dilution:</i> 0.65±0.004 versus 0.64±0.03 for blank	0
2	0	ND	0
3	625.1	ND	0
4	28385	<i>1/10 Dilution:</i> 0.78±0.006 versus 0.70±0.009 for blank <i>1/100 Dilution:</i> 0.70±0.009 versus 0.70±0.009 for blank	0
5	7886.8	<i>1/10 Dilution:</i> 0.73±0.02 versus 0.70±0.04 for blank	0
6	Too high to extrapolate	ND	0
7	0	ND	0
8	Not suitable for testing*	ND	0

(1) After storage for 3 weeks at -20°C and one freeze-thaw cycle in plastic vials

(2) After repeated freeze thaw cycles and storage at -20 °C in plastic vials

(3) After repeated freeze thaw cycles and storage at -20 °C in plastic vials

\* Sample from client no.8 was not suitable for testing because the sample was obtained by spitting and not using the collection device.



### ***6.2.7.2 Detection of morphine in 'real' saliva samples***

The samples collected from clients in Trinity Court were stored at  $-20^{\circ}\text{C}$ , until testing. The samples were initially tested on ELISA and dilutions were needed as some of the results from the original undiluted samples were off the scale of the assay. These samples were then retested at appropriate dilutions, such as, 1/10 and 1/50. The samples were also applied to the BIAcore assay. The results of the ELISA and BIAcore assay are shown in Table 6.14. The unexpected part of the results was the high level of morphine found in these saliva samples. These results were verified by a lab colleague who tested them using an immunoassay developed with an anti-morphine scFv antibody. Similar results were obtained with this assay. It should be remembered that these were the same samples as the ones tested for THC. Unlike, THC, it appears that the presence of morphine is not affected to the same degree as THC, by storage or freeze thaw cycles. The disparity in the results obtained between the ELISA and the BIAcore assay can be explained by two factors. The first relates to the dilution of the saliva sample applied to the BIAcore assay. As discussed above, it was impossible to optimise a successful morphine BIAcore assay using saliva samples, unless the sample diluted appropriately so that none of the interferences caused by saliva were evident. This means the saliva samples should be tested at a dilution of at least 1/10. This dilution in itself also introduces another possible error factor. The second factor contributing to the disparity was the very high levels of morphine found in some of the samples. These were simply off the scale of the assay and so a reliable quantitative result could not be obtained even with diluted samples. Morphine in aqueous solutions has been reported to be stable over different storage conditions, as discussed in Section 6.1.2.2, so this would not be considered to be factor involved in the disparity between the results. The samples were used for the THC and morphine testing and due to the limited volume available there was no more available for re-testing at more dilute concentrations.

**Table 6.14:** Detection of morphine concentration in samples from Trinity Court Drug Treatment Centre clients on the ELISA and BIAcore assay.

	Conc of morphine as determined by BIAcore assay (6 weeks post collection after multiple freeze-thaw cycles)		Conc of morphine as determined by ELISA (3 weeks post collection)		% Correlation
	<i>Dilution Used</i>	<i>ng/ml</i>	<i>Dilution Used</i>	<i>ng/ml</i>	
<b>Client No. 1</b>	1/10	168.4	Straight	99.9	168.58%
<b>Client No. 2</b>	1/10	542.4	1/10	866.7	62.58%
<b>Client No. 3</b>	1/100	13176.6	1/50	39469.4	33.38%
<b>Client No. 4</b>	1/10	89.3	Straight	128.1	69.72%
<b>Client No. 5</b>	1/10	767.9	1/10	2914.7	26.35%
<b>Client No. 6</b>	1/2000	28108.9	1/50	20430.1	137.59%
<b>Client No. 7</b>	1/20	1084.8	1/50	1439.2	75.37%

### 6.3 Discussion

This chapter described the development of the assay for the detection of THC on the Envitec device using the anti-THC polyclonal antibodies; the application of anti-THC and anti-morphine polyclonal antibodies for the detection of THC and morphine in saliva samples by ELISA, and by BIAcore. A pilot study to determine the feasibility of the assays was performed using 'real' saliva samples that were obtained from multi-drug users who also received methadone at a drug treatment centre. The samples were analysed by the different assays.

The assay developed on the Envitec device for the detection of THC in saliva samples was successful as a screening test. The cut-off level of detection of THC was 200ng/ml. The assay is not suitable as a quantitative test due to the high levels of variability that were seen. There are a couple of factors that could be contributing to this variability over the days of the assay. These include the incubation times, the washing cycles between steps, the matrix effect of saliva, and the instability of THC in solutions. The incubation time for the saliva sample and THC-HRP in the antibody coated well is only five minutes for this assay. This is not a long enough period for the mixture to reach equilibrium binding with the immobilised anti-THC antibody. This is compared to a 60 minute incubation period in the case of the ELISA developed for the quantitative measurement of THC, using the same polyclonal antibodies. The time restrictions for the assay run did not allow a longer incubation time. The washing of the reaction wells is automated and performed by the same pump that dispenses the fluids. This means that the washing steps are slow because greater volumes are needed for the wash steps. For the assay, the washing steps take about six minutes, which is considerable given the assay completion time is 20 minutes. The dual function of the pump to dispense smaller volumes accurately and larger volumes quickly have to be balanced and perhaps it could be improved upon by having two pumps operating for each function. This would increase the size of the device though, and portability is a characteristic that would need to be retained for this device, for road-side testing in a police car. Another factor that is hindering the washing steps is the saliva matrix. High background values were obtained in the control well for the assays using saliva samples. These background values were not seen during the development of the assay using PBS samples. Proteins and components in saliva are contributing to the stickiness of the saliva and residues of the

THC-HRP conjugate are not being fully washed through the tubing. To eliminate this effect, the saliva samples were diluted 1:1 with PBS before application to the well. The result from the control well was used to normalise the results from the reaction wells and so the physical effects of the saliva matrix could be eliminated. Section 6.1.2.1 discussed the reports in the literature concerning the instability of THC samples and the resulting errors that are seen in quantitative THC analytical assays. It is important to remember this inherent instability of THC. However, given that the ultimate aim for the device is road-side testing, the saliva samples will be collected and applied to the device immediately and so the assay should not be hindered by that problem. The issue will be important however, if a sample of the saliva is retained and stored for repeat laboratory-based testing. At this point, the possibility of a variant result from the original screening is highly likely. The only information that will be obtained from the Envitec device will be a positive or negative result based on the 200ng/ml cut-off level of detection. So, it will be samples that border on this limit that will need careful analysis and interpretation of the results in the context of the instability of THC in stored biological samples.

The ELISA for the detection of THC in saliva samples had a range of detection of 96.7 to 25,000ng/ml. The inter-day coefficients of variation ranged from 0.33% to 8.53%, which are acceptable. The percentage recovery ranged from 72.95% to 185.57% and, these values are outside an acceptable level for a reliable quantitative assay for the detection of THC. The most likely reason for the out of range recovery values are the instability of the THC in the samples.

The ELISA for the detection of morphine in saliva samples had a range of detection of 24.4 to 12,500ng/ml. The inter-day coefficients of variation ranged from 0.54% to 14.05%, which are acceptable. The percentage recovery ranged from 80.72% to 117%, these values are acceptable for a reliable quantitative assay for the detection of morphine.

The model BIA assay for the detection of morphine was successfully developed using HBS as the matrix. The range of detection of the assay was 1.52 to 3125 ng/ml, the coefficients of variation ranged from 1.27 to 10.37% for the intra-day assay and 2.88 to 16.24% for the inter-day assay. The percentage recovery, as a measure of accuracy of the assay ranged from 84.31 to 148.96%. However, when the assay was applied to saliva samples, the accuracy and reproducibility were reduced. For the range of detection of 12.2 to 781.3 ng/ml, the coefficients of variation for the inter-day assay in

saliva samples were 17.71 to 37.74%. This is above the 20% level, which is usually considered the cut-off level for a reliable assay. The percentage recovery of the assay ranged from 57.74 to 119.54%. Attempts were made to optimise the saliva BIAcore assays by looking at diluting the samples, alteration of ionic strength of the saliva by changing the ionic strength of the diluent HBS, and investigation of use of alternative sensor chip surface. It was clearly shown in Section 6.2.5.1 that the saliva is interfering with the degree of competition of the assay, most probably caused by the 'stickiness' of the saliva due to proteins and other components. The change in ionic strength of the saliva samples did not contribute to optimising the assay. It appeared that the underlying problem with the assay was the 'stickiness' and non-specific binding of the saliva components to the sensor surface. The addition of dextran and thyroglobulin to the saliva samples did not eliminate this, and so the final variable to be changed was the sensor chip surface. This was accomplished by using a Pioneer F1 chip, as it has a shorter dextran matrix than the CM5 chip, and so it was expected that there would be less non specific binding. However, the use of this chip was unsuccessful as no competition was observed. The reason for this was probably that not enough morphine-protein conjugate was immobilised on this shorter dextran surface and so there was no competition between the morphine immobilised and the morphine free in solution.

The BIAcore assay for the detection of THC was established by conjugating the THC-BtG with biotin and subsequently immobilising it through prior immobilisation of streptavidin on the sensor surface. Using these conditions, a competitive assay was established with a range of detection of 12.2 to 3125 ng/ml. The coefficients of variation for the intra-assay ranged from 3.32% to 11.23%, and 4.21% to 21.67% for the inter-assay variation. The degree of recovery for the inter-assay ranged from 57.1 to 135.8%. The main problem with the assay was the regeneration of the surface. Only 30 cycles of binding and regeneration with 60mM NaOH are possible, after that there is a greater than 22% degradation of the surface.

The final part of this chapter described the results obtained from the multi-drug abuser saliva samples. An important finding regarding the stability of THC in saliva in storage conditions could be seen in these experiments, from the concentrations measured in the initial ELISA test to the results seen on the Envitec test. Repeated freeze-thaw cycles of the real saliva samples of multi-drug users resulted in a significant decrease of the THC concentration. This supports other publications that have been reviewed in Section 6.1.2.1 concerning the instability of THC in biological fluids during storage.

The saliva samples from the multi-drug users were analysed for morphine using the ELISA and BIAcore assay. As previously mentioned, the BIAcore assay was not very successful due to the high coefficients of variation, 17.71 to 37.74%, and percentage recoveries ranging from 47.78 to 158.31%. With these in mind the samples were applied to the BIAcore assay. For the seven samples that were analysed, the degree of correlation between the two assays ranged from 33.38 to 168.58%. This was disappointing but can be explained by the high variability of the BIAcore, implying that this assay should really only be used as a screening assay. The other issues that came to light during this study was firstly the small volumes of saliva that could be provided by the users and secondly the very high concentrations of morphine that are found in these samples. The concentrations of morphine ranged from 99.9 to 39469.4 ng/ml. These results were independently confirmed using a different morphine assay by a lab colleague (Brennan, unpublished data, 2001).

Overall, successful screening methods were developed for the detection of THC, by applying the anti-THC polyclonal antibodies onto the Envitec device, and for the detection of morphine by applying the anti-morphine polyclonal antibodies in the BIAcore assay. The Envitec is suitable for road-side testing due to its rapid, portable nature. The important point is that it is only suitable as a screening test. The BIAcore assay for morphine is also suitable as a laboratory-based screening technique. The ELISAs developed for the detection of THC and morphine are of a sufficient standard to be used as qualitative tests for the detection of these drugs in neat samples of saliva.

*Chapter 7*  
*Conclusions*

## **7.1 Overall Conclusions**

The aims of this project were the production of anti-drug polyclonal and monoclonal antibodies and the development of novel specific assays for the detection of drugs of abuse in saliva. These aims were achieved through:

- The production of polyclonal antibodies against THC, cocaine and morphine and the application of these antibodies in ELISA tests for the detection of these drugs in saliva samples.
- The production of highly specific anti-amphetamine and anti-methamphetamine monoclonal antibodies that recognised amphetamine and its commonly abused ‘designer’ derivatives.
- The characterisation of these monoclonal antibodies and the development of ELISA and novel BIAcore assays for the detection of amphetamine and derivatives in saliva samples.
- The application of the anti-THC polyclonal antibody on the novel Envitec device for the rapid screening of THC in saliva samples.
- The testing of real clinical saliva samples on the assays developed.

The preliminary work involved production of morphine and cocaine protein conjugates for the immunisation procedures. These drug-protein conjugates were used for the production of polyclonal antibodies to morphine and cocaine. THC-BSA was also used for the production of anti-THC polyclonal antibodies. The purified antibodies were then successfully applied to an ELISA format for the detection of morphine, cocaine and THC in saliva samples. In all cases the assays developed gave good, reproducible results with a level of detection correlating to that agreed upon by the SMT project team. As discussed in Chapter 1, the international agencies and scientific community have not clearly established the concentrations of these drugs in saliva samples for the purposes of determining positive samples.

Anti-amphetamine and anti-methamphetamine monoclonal antibodies were produced. The production of these specific antibodies presented a serious challenge in that there are many different ‘designer’ amphetamine drugs, such as MDA, MDMA, MDEA,



MBDB that need to be recognised for an amphetamine test to be useful. On the other hand, there are closely related molecules such as ephedrine, found in common flu formulations, that must not be recognised by these antibodies in such an assay as they would lead to false positives. In order to generate such antibodies it was necessary to produce monoclonal antibodies against amphetamine and against methamphetamine. The specific antibodies were isolated using extensive screening procedures during the cloning out stage of the hybridoma development. The antibodies were applied to an ELISA for the detection of amphetamine, methamphetamine, and the other common designer derivatives, MDA, MDMA, MDEA, MBDB, in saliva samples.

Affinity constant measurements of the two monoclonal antibodies were determined using two different techniques, the classic ELISA-based Friguet method and the solution-phase BIAcore assay. The equilibrium dissociation constants obtained using the well-based system were of comparable magnitude to those determined using BIAcore, showing the appropriateness of both assays. The anti-amphetamine monoclonal antibody showed affinity for amphetamine and MDA of the same order of magnitude ( $K_D = 1.0 \times 10^{-9}$  and  $2.0 \times 10^{-9}$ ). This can be explained by the immunogen used. It was an amphetamine-BSA conjugated through the para phenyl position of the amphetamine. This is the point of differentiation between amphetamine and MDA, the MDA having a methylenedioxy group at the 3,4 position. The anti-methamphetamine antibody had equilibrium affinity constants also of similar magnitude for methamphetamine, MDMA and MBDB ( $K_D = 5.0 \times 10^{-9}$ ,  $6.0 \times 10^{-9}$ , and  $4.0 \times 10^{-9}$ ). The interactions of the antibodies with substituted derivatives followed an expected pattern, the more the structure differed from the parent amphetamine or methamphetamine molecule, the less reactive the antibody was towards it. It can also be concluded from the cross reactivity and affinity studies, that the anti-amphetamine and anti-methamphetamine monoclonal antibodies are reacting at the substituted carbon chain side of the molecules.

A prototype of the Envitec device was used, and a rapid test for THC using the anti-THC polyclonal antibody was developed. This test fulfilled a number of prerequisites for a 'road-side' test, that could be used by law-enforcement agencies for screening saliva samples. The test was rapid being approximately 20 minutes in total from time of application of saliva sample to result. It was specific for THC with a cut-off level of

detection of 200ng/ml and was easy to use. The only preparatory step involved in using the saliva samples was a 1:1 dilution in PBS. The ease of collection of saliva, coupled with this simple preparation step is significantly advantageous compared to the current situation of using urine or blood samples as a preliminary screening step.

A pilot clinical study, involving collection of saliva samples from drug users, was conducted to investigate the application of the antibodies produced for the detection of drugs of abuse. A number of different assays were used for the analysis of THC, and morphine. BIAcore assays were also investigated for the detection of morphine in saliva samples. Real saliva sample samples were collected from drug users and analysed in the different formats. The results obtained from the ELISA show surprisingly high levels of morphine and THC in these samples. The BIAcore assay using the anti-morphine assay was not suitable for saliva samples. This was in contrast to the two other BIAcore assays developed using the anti-amphetamine and anti-methamphetamine monoclonal antibodies, which were found to be suitable for analysis of saliva samples. Overall, saliva provided a suitable matrix in all assays developed with the exception of the BIAcore assay using the anti-morphine polyclonal antibody. The only preparatory step used for the other BIAcore assays was one freeze-thaw cycle followed by a centrifugation step.

The current interest in rapid screening tests for drugs of abuse has placed immunology-based tests in centre stage. The success of such tests is reliant on the quality of the antibodies that are used, as illustrated in the results described in this thesis. The developments in the field of biosensors provide a synergistic advancement in this area of rapid testing. The Envitec automated device assay and the BIAcore assays described in this thesis are examples of the successful co-application of good antibodies and technology.

*Chapter 8*  
*References*

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## **Appendix A**

### **Study on the analysis of saliva for drugs of abuse**

#### **Subject Information Sheet and Informed Consent Form**

##### **Collection of Saliva**

You are being asked to take part in a study that will test your saliva for drugs of abuse. At your routine clinic visit, when you give a urine sample, you will also be asked to give a saliva sample. To collect the saliva you will chew on a piece of material like cotton wool given to you by a nurse. These are special saliva collecting devices and there are no known risks associated with using them. After a couple of minutes of chewing when the material has absorbed the saliva, it will be placed in a container and the saliva taken for testing.

##### **Confidentiality**

Labelling the saliva sample with your initials and a number ensures your confidentiality. Your name will not appear on the sample container. The results will be compared to those of the urine test. Your name will not appear on any associated paperwork or publications.

##### **AGREEMENT TO CONSENT**

The research project has been fully explained to me. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that participation is voluntary and that I may withdraw my consent at any time. I am aware that my decision not to participate or to withdraw will not restrict my access to health care services normally available to me. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. When required the records of this research will be reviewed by the sponsors of the research.

I, the undersigned, hereby consent to participate as a subject in the above described project conducted at the National Drug Treatment Centre. I have received a copy of this consent

form for my records. I understand that if I have any questions concerning this research, I can contact the doctor at the clinic.

After reading the entire consent form, if you have no further questions about giving consent, please sign where indicated.

\_\_\_\_\_  
**Client's Name (Block capitals)**

\_\_\_\_\_  
**Client's Signature**

\_\_\_\_\_  
**Date (to be dated by the patient)**

\_\_\_\_\_  
**Investigator name (Block capitals)**

\_\_\_\_\_  
**Investigator's signature**

\_\_\_\_\_  
**Date (to be dated by the investigator)**

**If applicable:**

\_\_\_\_\_  
**Signature of Parent or Guardian**

\_\_\_\_\_  
**Date:**