Immunochemical analysis of illegal steroid growth promoters in cattle

A thesis submitted for the degree of Doctor of Philosophy
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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. 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.

Signed: _____

ID No.: _____

Date: _____

This thesis is dedicated to my Mum and Dad, without whom I could not even have started this journey.

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"To the generous mind the heaviest debt is that of gratitude when it is not in our power to repay it."

- Benjamin Franklin

Table of Contents

Abbreviations

2xTY	double strength tryptone yeast extract media
A/A_0	absorbance divided by the absorbance reading for the blank
ab	antibody
Abs	absorbance
Ag	antigen
Amp	ampicillin
BCA	bicinchoninic acid
BIA	biomolecular interaction analysis
bp	nucleotide base pairs
BSA	bovine serum albumin
Cam	chloramphenicol
cDNA	copy DNA (made from mRNA)
CDR	complementarity determining regions (of an antibody)
cfu	colony forming units
C_H	constant region of an antibody heavy chain
C_L	constant region of an antibody light chain
CMO	carboxymethylated oxime
conc.	concentration
CP	mono carboxypropyl
CV	coefficient of variation
DES	diethylstilbestrol
DESAb	anti- diethylstilbestrol polyclonal antibody
DEX	dextran
dH_2O	distilled water
DMF	dimethylformamide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotidyl triphosphates
EDC	N-ethyl-N'- (dimethylaminopropyl) carbodiimde
EDTA	ethylenediaminetetra-acetic acid
EE	ethynyl estradiol
EEAb	anti- ethynyl estradiol polyclonal antibody
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EtOH	ethanol
EU	European Union
Fab	binding region of the antibody above the hinge region
Fc	constant region of the antibody below the hinge region
FCA	Freunds' complete adjuvant
Fv	variable region of the antibody containing the binding areas
HAMA	human anti-murine-antibody immune reponse
HAT	hypoxanthine aminopterin thymidine (cell media)
HBS	hepes buffered saline
HGPRT	hypoxanthine guanidine phosphoribosyl transferase
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HS	hemisuccinate
IgA	immunoglobulin class A

IgD	immunoglobulin class D
IgE	immunoglobulin class E
IgG	immunoglobulin class G
IgM	immunoglobulin class M
IMAC	immobilised metal affinity chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
Kan	kanamycin
kbp	kilo base pairs
Mab	monoclonal antibody
MHC	major histocompatibility complex
MRL	maximum residue limit
mRNA	messenger RNA
MS	mass spectrometry
MW	molecular weight
NEB	New England Biolabs
NHS	N-hydoxysuccinimide
Ni	nickel
NTA	nitrotriacetic acid
OD	optical density
OPD	o-phenylenediamine dihydrochloride
OVA	ovalbumin
Pab	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
pAK	plasmid AK
PBS	phosphate buffered saline
PBST	PBS supplemented with 0.05% (w/v) Tween 20
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	log of the hydrogen ion concentration
ppb	parts per billion
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcriptase PCR
RU	response units
SAS	saturated ammonium sulphate
scFv	single chain antibody variable fragment
SD	standard deviation
SDS	sodium dodecyl sulphate
SOC	super optimal catabolites
SOE-PCR	splice-by-overlap extension PCR
SPR	surface plasmon resonance
TAE	tris-acetate-EDTA buffer
TBA	trenbolone acetate
TE	tris EDTA buffer
TES	tris EDTA sucrose buffer
Tet	tetracycline
THY	thyroglobulin
TLC	thin layer chromatography
TMB	tetramethylbenzidine
TR	trenbolone

TRAb	anti-trenbolone polyclonal antibody
UV	ultra violet
V_H	heavy chain variable fragment
V_{HL}	variable heavy and light chains spliced together
V_L	light chain variable fragment

Units

µg	micrograms (10^{-6} gram)
µl	microlitre (10^{-6} litre)
µm	micrometre (10^{-6} metre)
g	grams
h	hours
(k)Da	(kilo) daltons
l	litre
M	molar
mA	milliampere (10^{-3} ampere)
mg	milligram (10^{-3} gram)
min	minute
ml	millilitre (10^{-3} litre)
mM	millimolar (10^{-3} molar)
ng	nanograms (10^{-9} gram)
nm	nanometre (10^{-9} metre)
nmol	nanomole (10^{-9} mole)
°C	degrees Celsius
ppb	parts per billion
rpm	revolutions per minute
s	second
U	unit of enzyme activity
V	volts
v/v	volume per unit volume
w/v	weight per unit volume
ρg	picogram (10^{-12} gram)

Publications

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Daly, S.J., Dillon, P. Brennan, J., Dunne, L., **Fitzpatrick, J.** and O'Kennedy, R., (2001). Production and analytical applications of scFv antibody fragments. *Analytical Letters,* **34 (11)**.

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"Results! Why, man, I have gotten a lot of results. I know several thousand things that won't work"

- Thomas Edison

Abstract

Steroid hormones and their analogues can be used as growth promoters in cattle. The use of these substances is banned in the EU, and hence each member state must ensure that cattle are not being dosed with these drugs. This study focuses on the immunochemical detection of three steroidal growth promoters, ethynylestradiol (EE), diethylstilbestrol (DES) and trenbolone (TR).

Polyclonal antibodies were raised against EE and TR. The anti-TR antibodies were applied to two competitive ELISA formats, producing assays as sensitive as other reported systems while needing very little pre-treatment of the bile samples. The anti-EE antibodies were also applied to these ELISA systems and also used to develop a biosensor-based assay using a BIAcore 3000®. These assays allowed the detection of EE at low ng ml^{-1} quantities in bovine bile.

As an alternative antibody supply the production of scFv antibody fragments was investigated. Two naive phage display libraries, one murine and one human, were panned for the presence of binding fragments specific for the target steroid molecules. A number of pre-immunised libraries were also constructed and selected against the antigens of interest. None of the approaches provided specific clones that could be used as immunoreagents in analysis.

An investigation into the reduction of the incubation time required to perform a competitive ELISA was carried out. This method was shown to be effective for some of the polyclonal antibodies tested. The successful assays were applied to a prototype rapid ELISA machine. The ability of this device to detect low quantities of steroids in both a single and mixed sample was studied. A preliminary, prototype assay for the detection of two separate steroid growth promoters in a single sample was shown.

CHAPTER 1

INTRODUCTION

1.1 The Immune System

All living entities in nature must have the ability to protect themselves from elements in the environment that could cause them harm such as viruses, bacteria, fungi and parasites. It is the function of the immune system to protect against these foreign invaders by preventing them from multiplying and causing serious damage to the host. The immune system is made up of many different parts but it is usually separated into two functional divisions, innate and acquired immunity. These two parts have distinct functions and yet they are interdependent, complementing the tasks of each other, to provide effective protection from disease, (Davey, 1992).

1.1.1 Innate Immunity

Innate immunity operates non-specifically during the early phases of an immune response and it is sometimes referred to as natural immunity (Elgert, 1996). It is the body's first line of defence and some of the components it includes are the skin, the cough reflex, fever, inflammation response and phagocytic cells (Benjamini *et al.,* 2000). It can be found at differing levels of sophistication in vertebrates, such as mammals, reptiles and cartilaginous fish, and in some invertebrates, such as arthropods, earthworms and sponges.

The innate response is the primary immune response to any infection. It can respond rapidly and deal with an invading pathogen many days before the acquired immune response can begin to stage a response. This is one of the reasons why the two systems work so well together. The cells of the innate immune system, such as macrophages and neutrophils, have evolved receptors that can recognise common bacterial surface components. When these receptors are bound the cell is triggered to engulf and degrade the bacterial cell and also to release inter-cellular messengers called cytokines (Janeway *et al.,* 1999).

The innate system is relatively non-specific and this is typified by the response of some of the physiological and chemical barriers to infection. These barriers, such as hydrolytic enzymes in saliva, are not directed against any one type of invader but will break down any substrates they encounter. If a microorganism penetrates the barriers it is then attacked by the cellular defences, which can either engulf the invader and destroy it or kill it extracellularly. Some phagocytic cells contain

'enzyme-rich' lysosomes and produce peroxide or superoxide radicals to aid in the destruction of infectious cells once they have been phagocytosed.

Natural-killer cells will recognise cells that do not express a 'self-protein' on its surface called major histocompatibility complex I (MHC I) and may destroy them by releasing cytotoxic agents (Benjamini *et al.,* 2000). In some diseased cells MHC I is downregulated when compared to normal cells. This allows the natural-killer cells to recognise the altered cell and destroy it. Some cytotoxic agents can cause pores in the cell membrane of the target, while others can enter the cell and cause programmed cell death or apotosis. Another component of the immune system, called complement, can also destroy invaders by enhancing the pathogen's uptake by phagocytic cells or by initiating the complement cascade, which will lyse the cell. Complement can also be used as part of the adaptive system.

1.1.2 Adaptive Immunity

Adaptive immunity is a more specialised form of immunity that is only found in vertebrates, which develops during the lifetime of the organism (Benjamini *et al.*, 2000). The one aspect that differentiates the adaptive from the innate systems is the specificity that the adaptive immune system shows towards the targets. Each cell involved in the adaptive or acquired immune response has a specific receptor that will bind to a single epitope. It is specific and so time is required for the response to assert itself. In this lag period the innate system engages and destroys the foreign cell if possible. This system can be further divided into cell-mediated and humoral immunity

1.1.2.1 Cell-Mediated Immunity

Acquired immunity mediated by the T lymphocyte or T-cell is called cell-mediated or cellular immunity (Elgert, 1996). T-cells are lymphocytes that mature in the thymus and once they are mature they migrate to the peripheral lymphoid tissues such as the lymph nodes and the spleen. It is in these peripheral lymphoid tissues that the T-cells encounter and respond to foreign antigens.

The ability of the T-cell to recognise an antigen comes from its T-cell receptor (TCR). This specific receptor is acquired during the maturation of the cell and each cell has specificity for a single epitope or antigen structure. There are two

main types of T-cells, the cytotoxic T-cells (Tc) and helper T-cells (Th). To enable the T-cells to be activated they must have an antigen presented to them; they cannot interact with the antigen unless it is associated with the MHC proteins. When the antigen is degraded in some cells, the component epitopes are associated with the self-protein MHC I or II and expressed on the surface of the cell.

Th cells recognise the MHC II and epitope complex and release a set of chemical signals known as cytokines. A subset of the Th cells, Th-1, secrete interleukin 2 (IL-2), interferon gamma (IFN-γ) and tumour necrosis factor beta (TNF-β) and these in turn activate Tc cells (Male *et al.,* 1996). While Th-1 cells can activate B-cells this function is usually carried out by the Th-2 cells. Th-2 cells secrete IL-4 and IL-5, which activate the B-cells.

Tc cells recognise antigen epitopes in conjunction with MHC I proteins, which are found on virtually all normal nucleated cells. The Tc cells bind to cells expressing the MHC I and an antigen recognised by its TCR. When bound the Tc cells are able to deliver a lethal blow by releasing perforin to lyse the cells (Benjamini *et al.,* 2000). By destroying the host cell the pathogen is then released and is susceptible to attack by circulating antigens.

1.1.2.2 Humoral Immunity

The humoral immune response is mediated by serum antibodies that are secreted by the B-lymphocytes or B-cells. These cells are so called because they were found to be produced by the Bursa of Fabricus in birds. In experiments where this organ was removed from a bird, the creature was unable to mount a normal antibody response to an antigen. In mammals the B-cells are linked to gut associated lymphoid tissues, the fetal liver and, after birth, the bone marrow (Eales, 1997). The B-cells have a structure similar to the TCR on their surface. Each mature B-cell bears antigen specific receptors that have a structure and specificity identical to the antibody later synthesized by that B-cell.

To activate these cells the antigens must be presented to them just as in the case of T-cells. The cells that present these epitopes on their surface in conjunction with MHC II are called antigen presenting cells (APC) and include macrophages, dendritic cells and other B-cells. The recognition and subsequent binding to the

specific epitope will activate the cell and initiate its clonal expansion. The immune system is genetically able to produce a vast array of receptors specific for an estimated 10^{10} different epitopes (structure recognised by receptor). Only a small number of cells expressing each specificity are maintained. Large numbers of a single specificity are only produced after the original cell has been activated (Eales, 1997).

Once an epitope has been presented to the B-cells and clonal expansion has occurred the immune system can produce cells and antibodies specifically able to recognise that epitope. The epitope may be a surface protein of a bacteria or a coat protein of a virus or any structure that was presented to the B-cells. The cells resulting from the clonal expansion differentiate into effector and memory cells. Most of the B-cells, after contact with the epitope or antigen, will differentiate into plasma cells that synthesise and secrete proteins called antibodies. These antibodies have the ability to bind to the same epitope as the parent cell. The antibodies are then able to bind to the same pathogen that activated the original B-cell and enhance its destruction by cell lysis through complement or by phagocytosis. The secreting B-cells have a short lifespan of 5-8 days but can produce large amounts of antibody during this time. The memory cells give the immune system the ability to respond to the same invader much more quickly if it is encountered for a second or subsequent time. They have much longer life-spans and stay with the host for a life-time (Janeway *et al.,* 1999).

T-cells do not produce antibodies but they aid the B-cells in this function by providing growth factors and cytokines and they are also involved in the regulation of the immune response through the release of the cytokines (Elgert, 1996).

1.1.3 Immunogenicity

In order for the immune system to produce antibodies against a particular invader the pathogen must possess the following characteristics of foreignness, high molecular weight, chemical complexity and degradability (Eales, 1997).

An immune system does not mount an immune response to proteins that it recognises as 'self'. These antigens would cause an immune response in another species but not in the species of origin, e.g. a rabbit albumin would not elicit an immune response in rabbits but antibodies would be produced if a goat were

exposed. The more foreign the molecule is the greater the immunogenicity will be. The size and weight of the molecule also has some bearing on the immune response to it. Any compounds with weights less than 1,000 Da, such as the steroids investigated in this thesis, are not immunogenic and need to be altered and linked to larger carriers to increase their immunogenicity (Lieberman *et al.,* 1959). Compounds that have a molecular weight between 1 and 6 kDa may or may not elicit an immune response and those larger than 6 kDa are generally immunogenic (Benjamini *et al.,* 2000).

A certain degree of physiochemical complexity is required in an immunogen to elicit an immune response. Homopolymers, long chains of a single repeating unit, tend not to be immunogenic even if they have large molecular weights. If these structures are altered and smaller compounds are added to the structure this will enhance the immunogenic nature of the molecule allowing the generation of antibodies directed against both the large polymer and the smaller hapten. By increasing the chemical complexity of a compound its ability to raise an immune response can be enhanced (Janeway *et al.,* 1999).

To stimulate an immune response many antigens must be degraded so that they can be presented by antigen-presenting cells (Elgert, 1996). If the antigen presenting cells cannot easily degrade a compound the epitopes of the antigen are then not available to the immune system as a target. Peptides composed of D-amino acids have been shown not to be immunogenic and they are resistant to enzymatic degradation, while the same L-isomers are susceptible and are immunogenic (Benjamini *et al.,* 2000).

Others issues that relate to the production of an immune response are practical in nature and cover such topics as the dose, form and route of administration (Janeway *et al.,* 1999). The amount of immunogen given in a dose is important as too little will not cause a response and too much may induce tolerance. In general, if the immunogen is particulate and denatured, it will be more immunogenic than soluble and native forms. An immunogen may be mixed with an adjuvant before immunisation. Adjuvants are mixtures that will enhance the animal's response to the immunisation. Many of these mixtures contain oil and water, which delay the release of the antigen and cause it to deposit locally at the site of injection. They can also contain some treated bacterial or viral antigen,

which can stimulate macrophages and enhance the local immune response such as inflammation.

There are a number of routes that can be used to immunise an animal and some of these are considered to be more effective than others. It is generally found that subcutaneous injection is more effective than the intraperitoneal route, both of which provide better immune responses than intravenous or intragastric immunisation.

Repeated boosts are also required to obtain a strong immune response. A single dose will invoke the primary immune response and is known as priming the immune response. Further immunisations evoke increasingly intense responses leading to a heightened state of immunity called hyperimmunisation.

1.1.4 Antibody Structure

Antibodies are glycoproteins belonging to the immunoglobulin supergene family (Davey, 1992). This gene family contains genes located on many parts of the genome and has many proteins involved in the immune system (Roitt, 2001). There are five isotypes called classes of immunoglobulin, namely IgA, IgD, IgE, IgG and IgM. Each isotype possesses similar structural components as typified by the immunoglobulin G structure, (Figure 1.1). The amount of carbohydrate can vary widely between different immunoglobulin from 2-3% in IgG to 14% in IgD. The carbohydrate chain is usually covalently linked to the constant region of the heavy chain, (Figure 1.1) through asparagine residues, although serine or threonine residues can also be utilised. The carbohydrate moiety seems to have three different functions; to aid the secretion of the antibody from antibody synthesising plasma cells, to enhance the solubility of the antibody and to protect the molecule from degradation (Davis, 1994).

The basic immunoglobulin structure consists of four peptide chains linked with inter-chain disulphide bonds. These chains are separated into two heavy chains and two light chains. The constant regions of their heavy chains differentiate the isotypes of the immunoglobulins (Eales, 1997). These constant regions are given the designations C_α for IgA, C_δ for IgD, C_ε for IgE, C_γ for IgG and C_μ for IgM. The light chains that are linked to the heavy chains can be either kappa, L_κ or lambda, L_λ, but in any one immunoglobulin only one type is present. No natural

antibodies have both kappa and lambda light chains, although antibodies can now be engineered with one chain of each.

The variable regions of the antibody determine its specificity for binding of an epitope. Within the variable regions there are hypervariable regions called complementarity-determining regions (CDRs). It is at these points that most of the binding to the epitopes occurs. The CDRs are separated in the linear amino acid structure of the variable fragment but in the folded form of the peptide these areas come close to each other to form a pocket for the binding of the antigen. To maintain the epitope in this binding site the fit must be very close and cover a large region as the forces that control the binding are weak, non-covalent forces.

The constant or Fc portion of the Ig structure carries out the biological functions of the antibodies. These functions include

- Neutralisation of toxins
- Immobilisation of micro-organisms
- Neutralisation of viral activities
- Agglutination of micro-organisms particles
- Binding and precipitation of foreign particles
- Activating serum complement for lysis or phagocytosis
- Crossing the placental barrier to provide the foetus with immunity

The Fc region contains constant regions from only the heavy chains. Immunoglobulins have five different sequences in the constant region and these give each class of Ig a different biological function.

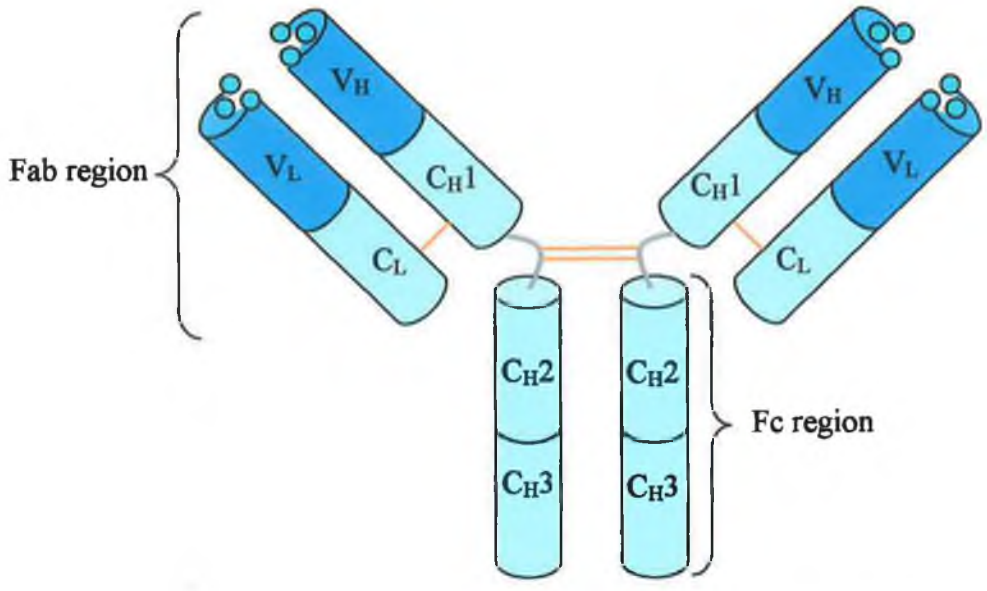


***Figure 1.1** : Structure of Immunoglobulin G (IgG) molecule. The structure contains two identical light chains (~25kDa) and two heavy chains (~50kDa). The heavy chain consists of five distinct domains usually known as the variable region, (V_H) the constant regions 1, 2 and 3, (C_H) and the hinge region represented by a grey line between C_H1 and C_H2. The light chain has two domains consisting of the variable and the constant regions. The two heavy chains are held together by two disulphide bonds between their hinge regions (orange lines). The light chains attach to the heavy chains through a single disulphide bridge between the C_L and the C_H1 domains. The C_L, V_L, C_H1 and V_H domains all contain intrachain disulphide bridges that give them their globular structure and these four domains make up the antigen-binding fragment (Fab) of the IgG. The antibody-binding site is coded for by the complementarity determining regions (CDR) at the end of both the heavy and the light chains. These are highly variable regions that confer the antigen specificity to the molecule. The Fc region of the molecule contains the C_H2 and C_H3 domains.*

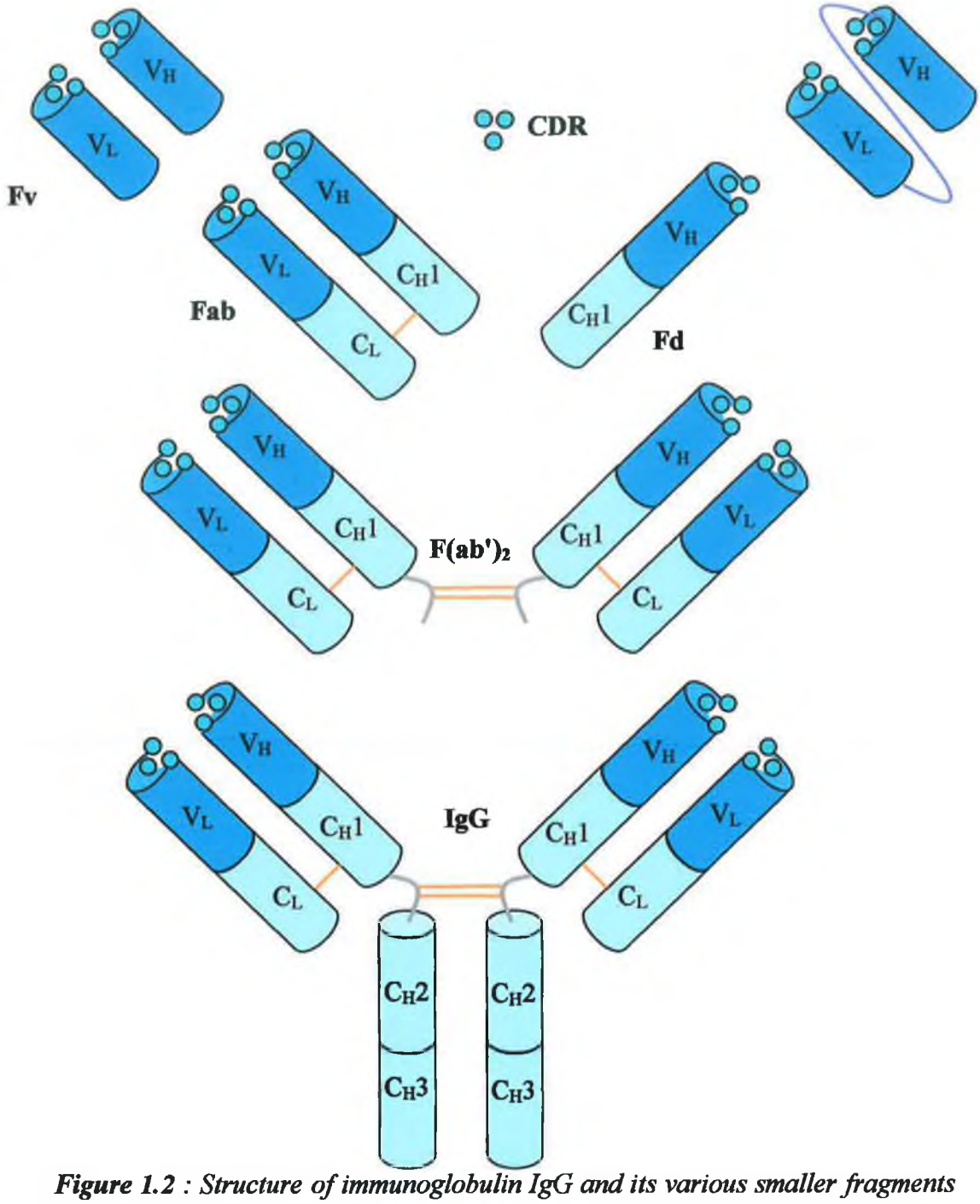


***Figure 1.2** : Structure of immunoglobulin IgG and its various smaller fragments that can be used to bind antigen. The pepsin enzyme degrades the IgG molecule below the inter heavy chain disulphide bridges giving the $F(ab')_2$ fragment and various Fc sub fragments. The $F(ab')_2$ fragment contains two antigen binding fragments linked by the disulphide bridges, while the Fab fragment has only one antigen binding fragment. Papain degrades the molecule just above the inter heavy chain disulphide link leaving 3 fragments, two Fab fragments and a Fc portion. A Fv molecule contains just the variable domains of the heavy and the light chains and a scFv (single chain Fv) contains the same domains stabilised by a peptide linker. An Fd fragment contains the varaible and C_H1 domains of the heavy chain. The smallest segment of the antibody that contains its binding properties is the CDR fragment. These are the actual peptides that are involved in the interaction with the antigen. They must, however, have a framework to allow them to bind specifically.*

1.1.5 Generation of Antibody Diversity

The diversity of each antibody molecule is coded for in the variable fragments of the heavy and light chains. It would be impractical for the germline DNA of an animal to hold separate coding sequences for each antigen specificity that it may require during its lifetime. To avoid this huge number of genes a smaller number of genes are joined together to form the functional protein. The three groups of genes coding for the heavy chain, the κ light chain and the λ light chain are located on 3 different chromosomes in both the mouse and the human genomes (Eales, 1997).

Three separate gene segments known as VH (variable), DH (diversity), and JH (joining) code for the variable region of a heavy chain immunoglobulin molecule. These then join to a C_H gene to form the complete heavy chain for the immunoglobulin. There are 5 classes of C_H genes coding for the constant region for each of the distinct immunoglobulin isotypes. The light chains use both V_L and J_L to code for their variable segment and these are different from the V and J genes used by the heavy chain. There are a number of different versions of each of these genes in the germline and they are rearranged during the differentiation of the B-cell to provide a single VDJ_H and a VJ_L construct, both of which will now appear next to the genes coding for their respective constant regions (Zaleski *et al.,* 1983). The genes are then translated into mRNA and the non-coding DNA is spliced out and the resulting genes transcribe the functional heavy and light chains of the immunoglobulin (Figure 1.3).

Further diversity can be generated in the areas where the gene segments join. The positions where the gene segments are fused together are not constant and so imprecise DNA recombination can lead to changes in the amino acids at these sites. Bases can be inserted, deleted, copied or altered at the sites of combination and these can be part of the hypervariable regions that produce the antigen-binding site leading to greater diversity in the immunoglobulin specificity (Tonegawa, 1983).

Point mutations occur at a much higher frequency in the V(D)J gene structure than normal. This is termed somatic hypermutation and it allows the secondary and further immune responses to produce antibodies with increased affinity for

the antigen of interest. In the primary response the antibodies produced are usually of low affinity and are close to germline sequence but as the response matures the amino acid sequence diverges from the germline. This gives the immune system the ability to provide more active and specific antibodies as it encounters an antigen more than once.

Somatic gene conversion is a mechanism that is used in some animals, such as birds and rabbits to create diverse antibody repertoires (not used in the human or murine systems). This inserts sequences from pseudogenes, located near the VDJ gene segments, into the variable fragment of the antibody. This occurs in the DNA sequence itself after the VDJ recombination and before the gene is translated. Many species rely on this and somatic hypermutation to generate their diverse primary Ig repertoire. This allows animals that have limited numbers of antibody genes to still create vast antibody repertoires (Elgert, 1996).

In essence, the antibody repertoire is a function of

- the multiple inherited genes that make up the variable regions of both the heavy and light chains,
- rearrangement of the V, D and J gene segments in different combinations and the random association of the different heavy and light chains,
- diversity at the junction points between V, D and J genes,
- somatic hypermutation,
- somatic gene conversion, (Benjamini *et al.*, 2000).

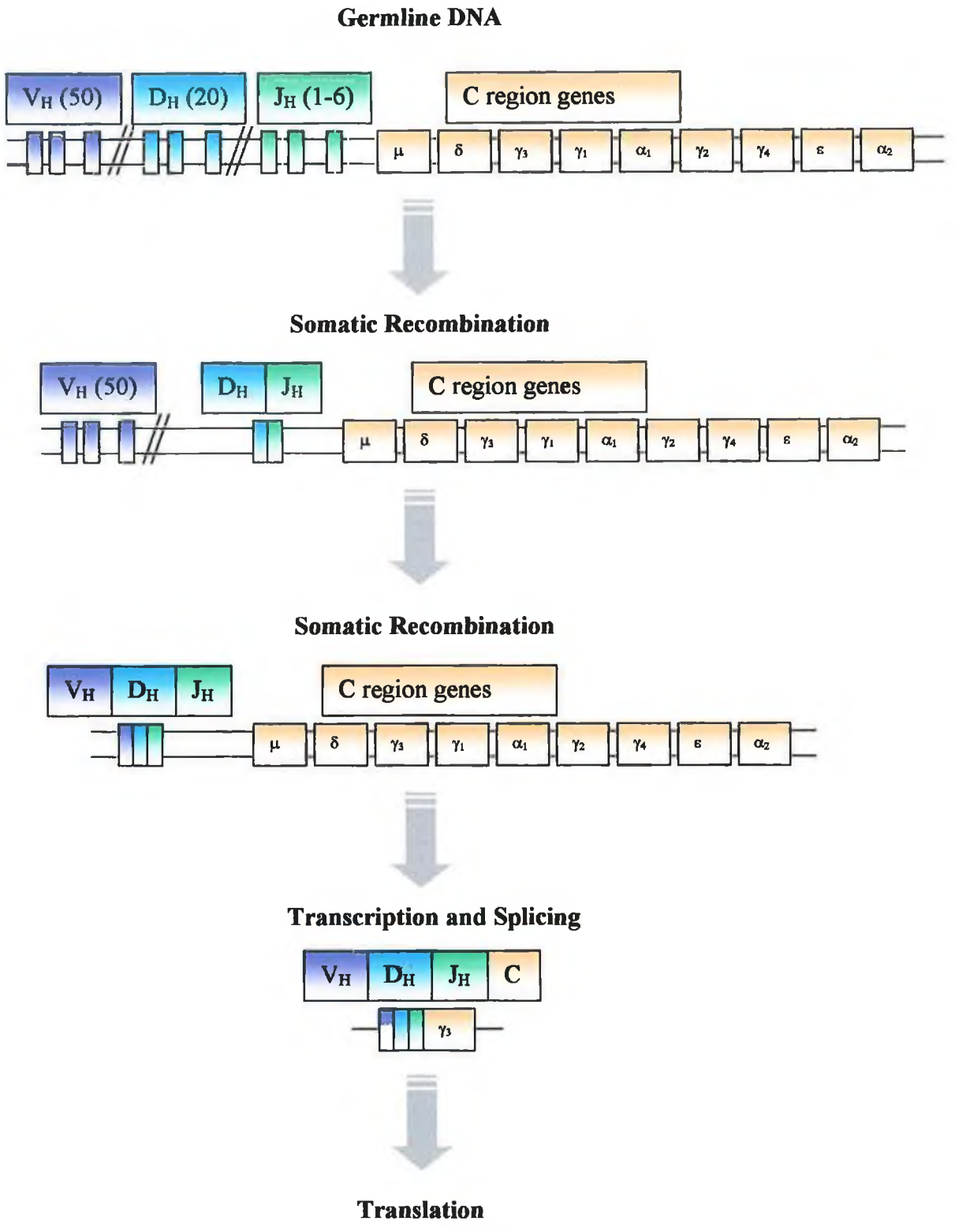


Figure 1.3 *: Genetic rearrangement of the human heavy chain to give the completed protein. Each of the segments that make up the variable domain has a number of different variances, i.e. V_H has 50 sequences, D_H has 20 and J_H has 6. Each one of these segments can recombine with any of the others leading to great variety in the transcripted sequence. The variable sequence can be linked to one of the constant regions to produce a specific class of antibody with distinct functions. The variable region can be grafted onto a different constant region to produce an antibody with the same specificity but different biological properties.*

1.2 Production of Antibodies

A number of methods can be employed for the production of antibodies. Some factors should be considered before antibody production commences including the immunogen, the type of antibody required and its final application. Antibody generation may require the production of either polyclonal, monoclonal or recombinant antibodies.

1.2.1 Immunogen

As previously mentioned antigens must have a number of features before they are able to elicit an immune response. These include a critical size, chemical complexity, foreignness and degradability. Many small haptens, including steroid hormones, do not have these traits. To overcome this problem it is possible to link the small molecule to a larger one and immunise with this new conjugate (Erlanger, 1980). By linking the hapten to a carrier it has a significantly larger size, greater chemical complexity, a greater degree of foreignness to its host and it is now susceptible to enzymatic degradation.

The small molecules studied in this thesis are synthetic steroid hormones having molecular weights of less than 300Da. To produce an immunogen these compounds must be linked to a carrier molecule. To do this a chemical handle is introduced to the molecule. This allows any chemical linkage to be carried out through a position that does not involve an active group of the steroid and so the linkage will not mask an area of the molecule that may be required for recognition during immunisation. The derivatised molecule, or hapten, is then chemically linked to a carrier, usually a protein but carbohydrates can also be used.

The chemical handle that is introduced usually contains a chain of molecules. This chain introduces a distance between the hapten and the carrier molecule permitting the epitopes of the target to lie away from the carrier itself. In this way the steroid is not hidden or masked by the much larger carrier protein and the immune system is able to mount a response against it. This chain of molecules is often referred to as the bridge or linker region. It is possible that the immune system will produce antibodies specific for this region and so in an attempt to minimise this response the linker cannot be too long or too complex. If the bridge

is chemically quite simple it is less likely to be immunogenic. One method to avoid any cross reactivity in the final antibody application is to use a different bridge in the conjugates used in the assays than the one used in the immunogen. In addition, if the immunoassays used a format where there was no need for a conjugate this problem of bridge recognition would be avoided.

The site of the linkage is also important as any antibody specificity is directed at the portion of the hapten that is most sterically accessible. This is known as Landsteiner's principle (Wild and Davies, 1994). If a compound is linked at a position next to a unique immunogenic site very little, if any, of the antibodies produced will be directed against it.

1.2.2 Polyclonal Antibodies

Polyclonal antibodies have been used successfully in research and clinical applications for many years. When an antigen is introduced to an immune system immunoglobulins specific for that antigen are produced. The immune system is then exposed to the antigen a number of times through booster injections until the amount of antibody in the serum reaches an acceptable level for use as an analytical or clinical reagent. The amount of antibody in serum is called the titre. The antibodies contained in polyclonal sera are not all of the same specificity or affinity and they react to different epitopes on the immunogen (Dankwardt, 2000). This is the reason for the name polyclonal as the antibodies arise from a number of different B-cells or clones.

The choice of animal for use in this procedure is important and the decision is based on a number of parameters. The facilities available for holding the animals must be adequate to house the species of choice. The animal must be easy to handle, produce the required amount of antibody, be able to mount a response to the immunogen and be able to do it with the amount of immunogen available. Some genetically in-bred strains are available for rodent and rabbits species and these can provide a more predictable immune response to a foreign antigen (Knott *et al.*, 1997).

Polyclonal antibodies combine the advantage of a simple production technique producing large amounts of antibody with the disadvantages of animal usage and a poly-specific reagent (Knott *et al.*, 1997).

1.2.3 Monoclonal Antibodies

The next step in the development of antibodies for use as reagents was the production of a homogeneous antibody preparation. This was made possible by the discovery that B-cells could be fused with myeloma cells to produce immortal cell lines that could secrete antibodies (Köhler and Milstein, 1975). The resulting hybridoma had two important traits that allowed it to be used as a source of monoclonal antibodies. It was immortal and therefore could be subjected to selection cloning and grown in culture and each hybridoma only secreted antibody of a single specificity (Dankwardt, 2000). The process for the production of the hybridoma and the selection of the relevant clones is outlined in Figure 1.4.

An animal, usually a rodent (mouse), is immunised with the antigen of interest. When the antibody titre is high enough the mouse is sacrificed and the spleen is removed. The lymphocytes are flushed from the spleen and fused with myeloma cells using polyethylene glycol (PEG). PEG is used as it forces the cells in solution close to each other and promotes membrane bridging, cell fusion and the transfer of nuclei (Hurrell, 1985). The resulting cells are grown in a media containing hypoxanthine, aminopterin and thymidine, (HAT). The aminopterin blocks the main synthetic pathway for nucleic acids and cells must use the alternative pathway utilising the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) to survive. The myeloma cells are altered so that they do not contain this pathway and the unfused spleen cells are unable to grow in culture and die within a few days. Hence, after a number of days the only cells growing in culture will be the fused cells or hybridomas combining the immortality of the myeloma cell line and the HGPRT enzyme from the spleen cells.

These hybridomas must then be screened to detect the cells that are producing antibody with the required specificity. The isolation of the antibody-producing cells is carried out using a procedure known as limiting dilution. This involves the dilution of the culture containing the cells and splitting it into smaller aliquots, ideally containing a single cell in each. The resulting culture will have cells with a single specificity derived from the original cell. These cultures are tested by checking the culture supernatants for antibodies that recognise the

antigen of interest. Once a particular culture is shown to be positive it is cloned out again, (diluted and split), and this process continues until the culture is totally monoclonal, i.e. all cells present are identical and secreting the same antibody (Goding, 1983).

The monoclonal cell line can then be used to produce limitless amounts of the antibody and the cell line can be stored under liquid nitrogen for use at a later stage. Monoclonal antibodies can be produced *in vivo* by injected the hybridomas into a mouse peritoneal cavity where they will be produced in a fluid called ascites (Knott *et al.,* 1997). This fluid can be drained either continuously or terminally, depending on mouse strain, to provide an antibody containing solution. At the present time many researchers wish to use fewer animals in their work and so the production of monoclonal antibodies *in vitro* is used in many cases. Large-scale cell culture facilities can produce gram quantities of antibody within a few weeks and the downstream processing is simple and reagents of high purity are achieved (Wild and Davies, 1994).

The advantages of monoclonal antibody production include the limitless supply, the singular specificity and the use of fewer animals than polyclonal antibody production. However, there are some disadvantages. Monoclonal antibodies tend to have lower affinities for the target antigen than the corresponding polyclonal antibodies. This is the main factor affecting sensitivity in competitive assay formats and so it may cause lower sensitivities in these assays. In addition, the fact that a monoclonal antibody is only reactive to one epitope may cause problems if the target has more than one isoform and the total amount of the protein is being assayed (Wild and Davies, 1994). In this case a polyclonal antibody that can recognise all isoforms may provide a more appropriate reagent.

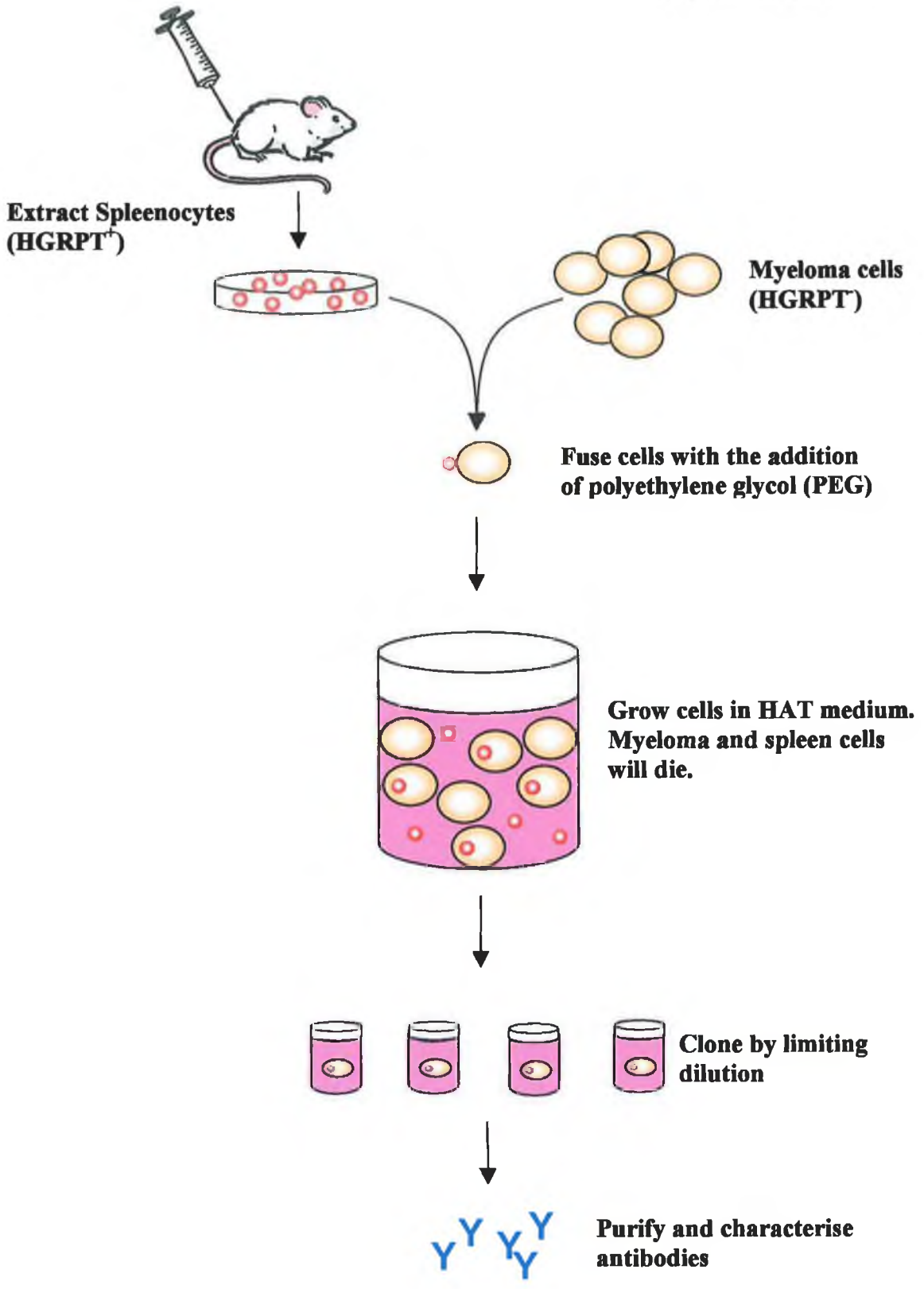


Figure 1.4 *: Flow diagram of the production of monoclonal antibodies. The spleenocytes are fused with myeloma cells using PEG resulting in the production of hybrid cells (hybridomas) that are HGPRT+. The hybridomas are grown in media so that only immortal HGPRT+ cells survive. The antibody-secreting cells are cloned out to give a monoclonal source and the antibodies are purified and characterised.*

1.2.4 Recombinant antibodies

The production of recombinant antibodies uses molecular biology techniques to isolate, and so immortalise, the genes coding for antibodies of interest and express them using one of the expression systems available (Marks *et al.,* 1991). It is these genes that are manipulated and screened to find the antibody that will bind to the target antigen. This technique intends to isolate all the genes coding for the variable fragments of the antibodies, express them and select from them antibodies with the required specificity (Lu and Sloan, 1999).

Recombinant antibodies can be produced from any source once the antibody genetics of the host species is known. As discussed in section 1.1.4 antibodies have their major variations in the variable sections of their structure but each end of this fragment are relatively conserved. Primers can be designed that anneal to these conserved areas (Wind *et al.,* 1997). These primers will amplify the variable fragments regardless of their sequence between the primers. In most cases a single set of primers will not bind to many of the variable sequences due to slight base pair differences. Usually sets of these primers are designed and used in multiplex polymerase chain reactions (PCR).

Once these fragments have been amplified the genes can be expressed in a number of different ways. They can be expressed separately and allowed to combine in solution (Fv), linked together by a peptide chain and expressed (scFv), grafted onto a short framework of one heavy and one light constant region (Fab) or they can be grafted into an intact antibody gene, (Figure 1.2). Using this technology human antibodies can be constructed with a binding site derived from a murine source and so avoiding the problem of human anti-mouse antibodies (HAMA) if the antibodies are to be administered to humans (Maynard and Georgiou, 2000). The small scFvs can be linked together to provide diabodies, triabodies or tetrabodies with the same or sometimes different specificities (Todorovska *et al.,* 2001).

The primers for the heavy and the light chain will be different and they will also change depending on the host species and source of the DNA. The DNA can be derived from hybridomas secreting an antibody of interest, the spleens of animals immunised with the target antigen (pre-immunised) or cells from a host that has not been immunised at all (naïve). Once the variable sequences, both heavy and

light, have been amplified they can be used to construct a library of antigen binders (Figure 1.5). It is expressed proteins from this library that are screened to find the gene sequence with the required specificity.

Pre-immunised libraries have the advantage that the immune system has been exposed to the antigen. This implies that the cells of the immune system will contain more B-cells producing antibodies specific for the antigen of interest. When the mRNA is extracted from the cells, usually splenocytes, they should contain a higher proportion of sequences specific for the immunogen. This will make the isolation of the required antibody gene sequence easier as the library has already been pre-enriched with the required binders.

The naïve library system does not have this pre-enrichment but it does potentially have a great many more specificities. By creating a library from the naïve antibody repertoire, it should have a vast diversity of clones each expressing a different target specificity. In theory, a large enough library could negate the need for the production of the pre-immunised libraries. If the large naïve library contained so many specificities it is highly likely to have clones that can bind to almost any target, the only difficulty remaining would be the selection of the correct clone (Vaughan *et al.,* 1996).

These libraries can be expressed in a number of different systems. These include bacterial, yeast, insect and mammalian cells as well as in transgenic plants (Longstaff *et al.,* 1998). The choice of expression system will depend on the attributes that are required from the final antibody or antibody fragment. Some of these systems do not allow glycosylation of the proteins and this may affect the function of any complete antibody. The folding of the proteins may not also be optimal in some of the systems.

A bacterial expression system is utilised in this study due to its ease of use, its low cost and the simplicity of the required manipulations (Charlton *et al.,* 2001). The antibody fragments that will be developed are scFv's. These are fragments containing the variable heavy and light chains linked together by a short peptide (Figure 1.2). The bacterial system does not allow for glycosylation but this small antibody fragment does not require this modification as it is a binding fragment and is not required for any other function. It may be fused genetically to other proteins to allow for easy detection or purification but usually the addition of the

carbohydrate moiety is not required for the binding of the fragment to the target or other required functions (Muller *et al.,* 1999).

The cytosol of the bacterial cell does not encourage the correct folding of proteins due to its reducing nature and so many recombinant proteins produced there require re-folding before use (George, 1997). An alternative strategy would be to direct the protein to the periplasmic space of the cell using a genetic leader sequence. This has an oxidising environment and contains molecules (chaperonins) that aid in the re-folding of proteins. Using these methods it is possible to produce large quantities of functional proteins from bacterial cultures.

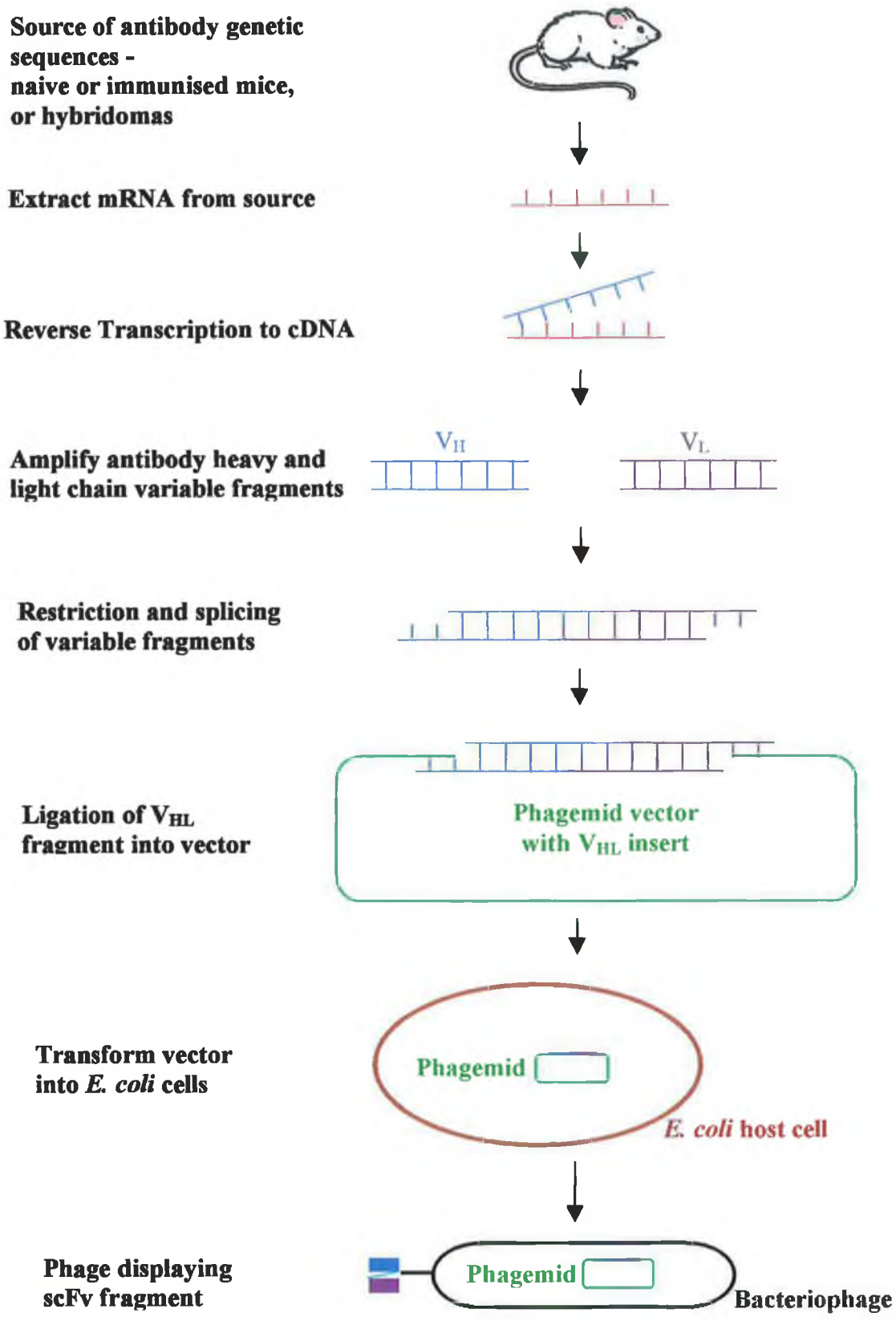


***Figure 1.5** : Schematic representation of the production of a phage display library.*

1.2.4.1 Phage Display Technology

This technology allows mimicking of key features of *in vivo* antibody production and the antigen-driven maturation processes by the expression of large libraries of antibody fragments on the surface of filamentous bacteriophage (Berdichevsky *et al.,* 1999). Three advances that have led to the development of phage display technology are

- the ability to produce functional antibody fragments in bacterial culture
- the isolation of the genes coding for the variable domains of both the heavy and light antibody chains
- and the ability to express antibody fragments as fusion proteins on the surface of filamentous bacteriophage (Chiswell and M^{c}Cafferty, 1992; Berdichevsky *et al.,* 1999).

This final point is the reason why the large libraries constructed through recombinant means can be easily screened. By expressing the antibody fragment as part of the surface protein pIII the phage particles themselves can be used as the immune reagent and subjected to immunoaffinity selection (M^{c}Cafferty *et al.,* 1990). The genes for the antibody fragment may be cloned into either a phage or a phagemid vector. The phage vectors have been in the main superseded by the phagemid vectors, the phage vectors being difficult to maintain and manipulate (George, 1997).

The phagemid vector is one that has both a phage and a bacterial origin of replication. This allows it to be maintained in bacterial cells such as *E. coli* and yet the proteins can be packaged and expressed by helper bacteriophage infecting the cell.

Once the library has been constructed and ligated into the phagemid vectors, it is then transformed into the host cells. These cells can be grown and stocks of the library are made. The size of the library is estimated by counting the number of host cells harbouring the phagemid. The cells can then be infected with helper phage, which provides the necessary proteins to produce phage particles containing the DNA from the phagemid. These phage particles express the fusion protein encoded on the phagemid on their surface and they are released into the supernatant of the bacterial culture.

The phage are collected and subjected to what is termed biopanning. This can take on many forms and it is discussed in detail in Section 6.1.5. It is a process whereby the phage are allowed to bind to a target antigen and the phage that bind are re-infected into bacterial cells and amplified (Figure 1.6). This is akin to the affinity maturation seen in the selection of antibodies *in vivo*. The biopanning can be repeated a number of times to enhance the population of binders.

Individual clones can then be isolated on agar plates. These can be grown under different conditions to induce the production of soluble antibody fragments. Some systems require the transferring the phagemid to a different bacterial strain or even splicing the antibody coding genes into a specialised expression vector to allow the soluble production of the antibody fragments. The route to soluble antibody production will depend on the system in use.

Once the proteins have been produced they must be purified and possibly concentrated. A number of different tags can be engineered into the phagemid vector to allow for easy purification and detection of the recombinant protein. These included the *c-myc* tag that is recognised by the monoclonal antibody 9E10 (Vaughan *et al.,* 1996), the FLAG tag, a polyhistidine label (Krebber *et al.,* 1997) and a cellulose binding domain (Berdichevsky *et al.,* 1999). The labels are all short polypeptide sequences that are detectable by commercially available antibodies allowing for immunodetection and affinity purification. The polyhistidine tag can also be used to purify the proteins by immobilised metal affinity chromatography (IMAC) (M^{c}Cafferty *et al.,* 1994; Casey *et al.,* 1995).

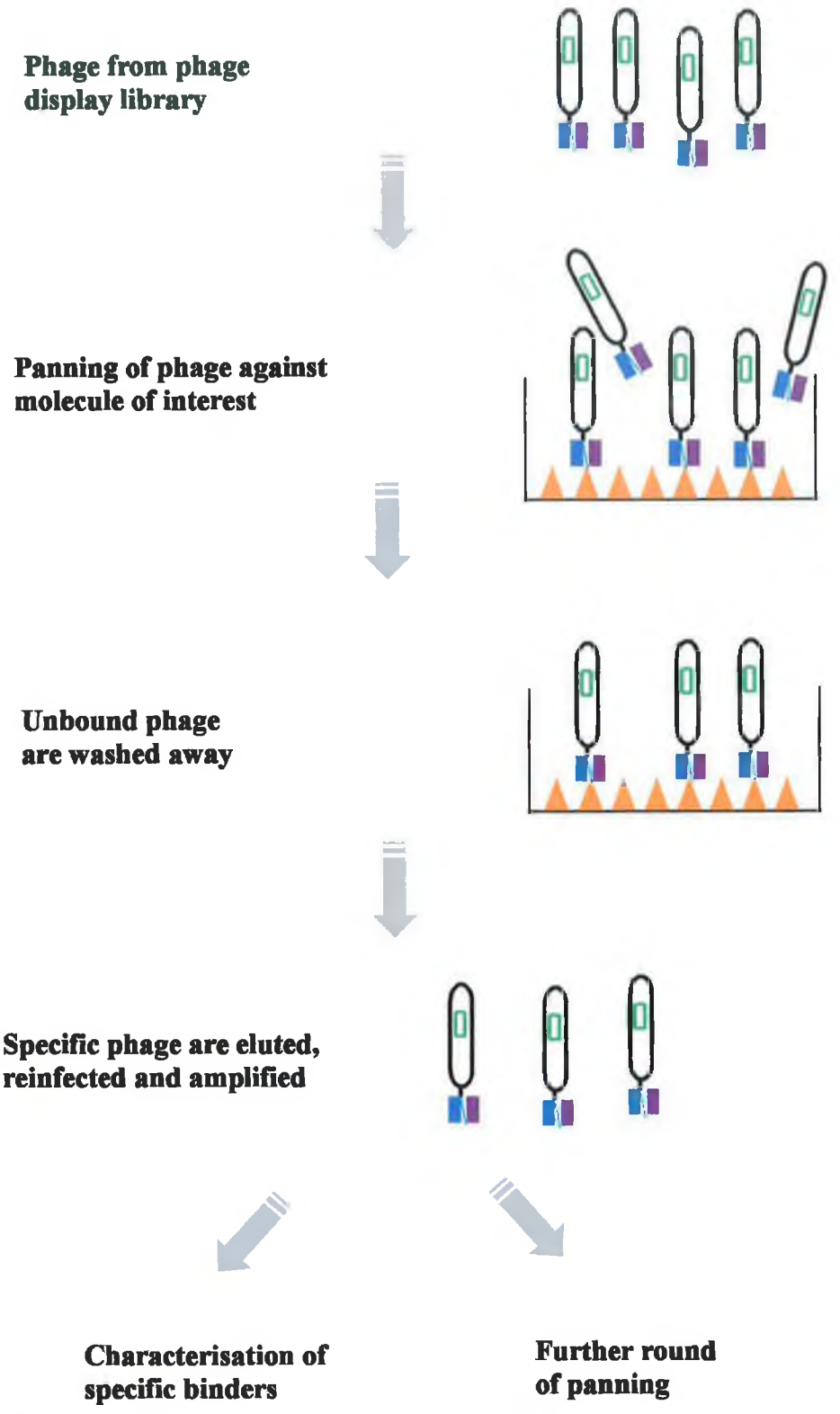


Figure 1.6 : Schematic representation of the biopanning process for the selection of phage displaying the antibody fragment, e.g. scFv, of the required specificity. Phage libraries can be subjected to numerous rounds of panning to select clones with specificity for the target anitgen. The specific phage are captured on a solid support and eluted from it once the non-specific phage have been washed away. The specific phage were then re-infected into E. coli cells for further rounds of selection.

1.3 The Hormones

Hormones, neurotransmitters and local mediators are the three functional groups of first biochemical messengers. The word hormone was introduced in 1905 by Starling and it comes from the Greek meaning to excite or arouse (Hardie, 1991). This group of biochemical messengers are probably the group most familiar to the general public through their varied and widespread use in medicine and also through their abuse in sports.

Hormones are produced and secreted by the cells of the endocrine system and travel in the bloodstream to their target cells (Hardie, 1991). This mode of action differentiates hormones from other classes of first messengers, such as neurotransmitters and local mediators. A neurotransmitter originates in the nerve terminal of a neurone and interacts directly with a target cell whereas a local mediator is produced by a cell and moves to a target cell via the extracellular fluid. Hormones are the only messengers that use the circulatory system to travel and interact with cells quite distant to their site of origin. Hormones are secreted by endocrine glands and these include the pituitary, thyroid, liver, pancreas, ovaries and testes. The hormones themselves are divided into subcategories, the steroid, thyroid and peptide hormones.

1.3.1 The Peptide Hormones

Peptide hormones can have one or two chains and anything from three to 199 amino acid residues. Table 1.1 shows the peptide hormones and describes their structure. The peptide hormones are first synthesized as large peptides called pro-hormones, which are subsequently cleaved to give the active form of the protein. The glycoproteins as shown in the Table 1.1 all have an identical α chains but the have very distinct β chains, which give the hormones their biological activity. The carbohydrate molecule helps both the folding of the two chains and the stabilisation of the molecule to increase its circulating half-life.

Table 1.1 : The peptide hormones, (adapted from Khanna and Waisman, 1988).

Hormone	Structure	Site of Origin
Adrenocorticotropin (ACTH)	139 amino acids (aa)	Anterior Pituitary
α-Melanocyte stimulating Hormone (MSH)	13 aa	
β-Endorphin	31aa	
Thyroid stimulating hormone (TSH)	Glycoprotein : α chain 92aa β chain 112aa	
Follicle stimulating hormone (FSH)	Glycoprotein : α chain 92aa β chain 118aa	
Luteinizing hormone (LH)	Glycoprotein : α chain 92aa β chain 115aa	Anterior Pituitary
Somatotropin (growth hormone)	191aa	
Prolactin	199aa	
Vasopressin	9aa	Posterior Pituitary
Oxytocin	9aa	
Thyrotropin releasing hormone	3aa	Hypothalamus
Somatostatin	14aa	
Insulin	51aa	Pancreas
Glucagon	29aa	
Parathyroid hormones	84aa	Parathyroid
Calcitonin	32aa	Thyroid
Gastrin	17 or 34aa	Gastro-intestinal tract
Cholecystokinin	33 or 39aa	
Secretin	28aa	
Chorionic gonadotropin (hCG)	Glycoprotein : α chain 92aa β chain 142aa	Placenta
Epidermal growth factor	53aa	Unknown
Nerve growth factor	118aa	Submaxillary glands

1.3.2 The Thyroid Hormones

The main hormones contained within the division of the thyroid hormones are thyroxine, (T_4, 3,5,3',5'-tetraiodothyronine), 3,5,3'-triiodothyronine, (T_3), 3,3,5'-triiodothyronine (rT_3, reverse T_3) and 3,3'-diiodothyronine, (3,3'-T_2). Of the compounds listed, T_4 and T_3 are active whereas rT_3 and 3,3'-T_2 are inactive, (Khanna and Waisman, 1988). The thyroid hormones have very wide and varied effects on the body including stimulation of carbohydrate, lipid and skin metabolism, changes in muscle and heart activity, and developmental regulation of the brain, muscle and bone. This variety of effects is produced by the fact that each cell reacts differently to the hormone stimulation depending on cell type and developmental stage. Even though the effects are diverse they all appear to be mediated by an interaction between the hormone and a nuclear receptor. The different consequences arise through the subsequent binding of the hormone-receptor complex to genes to activate or inactivate them. In different cell types different genes will be affected allowing the small number of thyroid hormones to have their multiple effects (Khanna and Waisman, 1988).

1.3.3 The Steroid Hormones

The third and final grouping of hormones are the steroids. They are the group involved in this research project. Table 1.2 outlines the main classes of steroid hormones. The steroid hormones share a basic sterane structure and the differences in the function of the molecules occurs with the functional groups linked to this 4-ring nucleus (Figure 1.7).

As can be seen from the Table 1.2 the hormones' effects can range from carbohydrate metabolism to maturation and they also affect a wide variety of different organs. All steroid hormones have a common parent molecule, cholesterol. Cholesterol is a 27-carbon molecule with the basic four-ring structure that all steroids possess and a long side chain at the 17 position. The structure of cholesterol and the steps involved in producing the other steroids is shown in Figure 1.8.

Steroid hormones themselves have a huge variety of effects and some of these have been manipulated using dosages of natural or synthetic analogues of the hormones. One of the most common uses of these hormones is in the

contraceptive pill. There are two types of pill, one is the progesterone only pill and the other is the combined pill. The combined pill contains both a progestin and an oestrogen. Two of the commonly used oestrogen analogues are mestranol and ethynyl estradiol, (EE). EE is also used as a growth promoter in animals and is one of the focus compounds of this project.

Table 1.2 : The origin, targets and functions of steroid hormones, (after Malkinson, 1975).

Hormone	Endocrine Gland	Target Tissue	Physiological Response
Glucocorticoid: cortisol	Adrenal Cortex	General	Metabolism of carbohydrates, proteins and lipids; mediation of inflammatory response
Mineralocorticoids: aldosterones, deoxycorticosterone	Adrenal Cortex	Kidney, parotid gland, sweat and salivary glands, gastrointestinal tract	Regulates transepithelial sodium transport
Oestrogens: estradiol, estrone	Ovary (follicle)	Breast, uterus, vagina Bone, brain	Maturation and normal cyclic function Development of secondary sex characteristics
Androgens: testosterone, dihydrotestosterone	Testis, adrenal cortex	Prostate gland, seminal vessicle Bone, brain, hair bulb	Maturation and normal function Development of secondary sex characteristics
Progestin: progesterone	Ovary (Corpus luteum)	Uterine endometrium	Preparation for zygote implantation

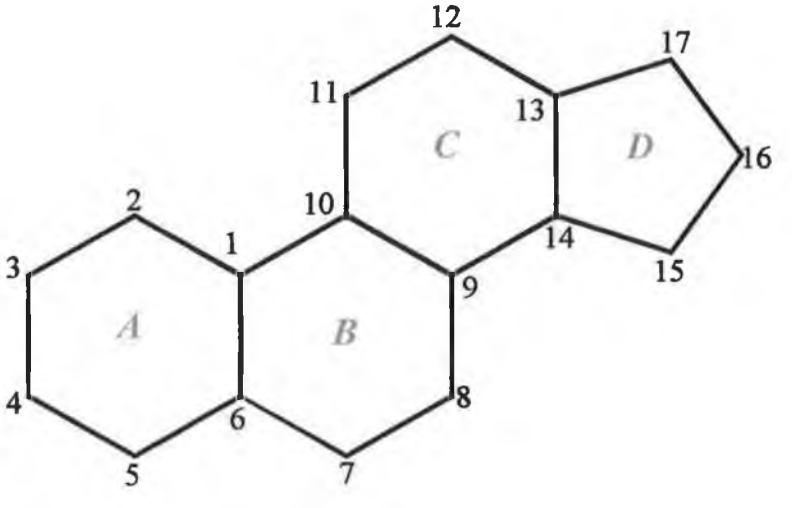


***Figure 1.7** : Sterane nucleus common to all steroids. The carbon molecules and rings are numbered according to standard steroidal nomenclature, (Power and Fottrell, 1993).*

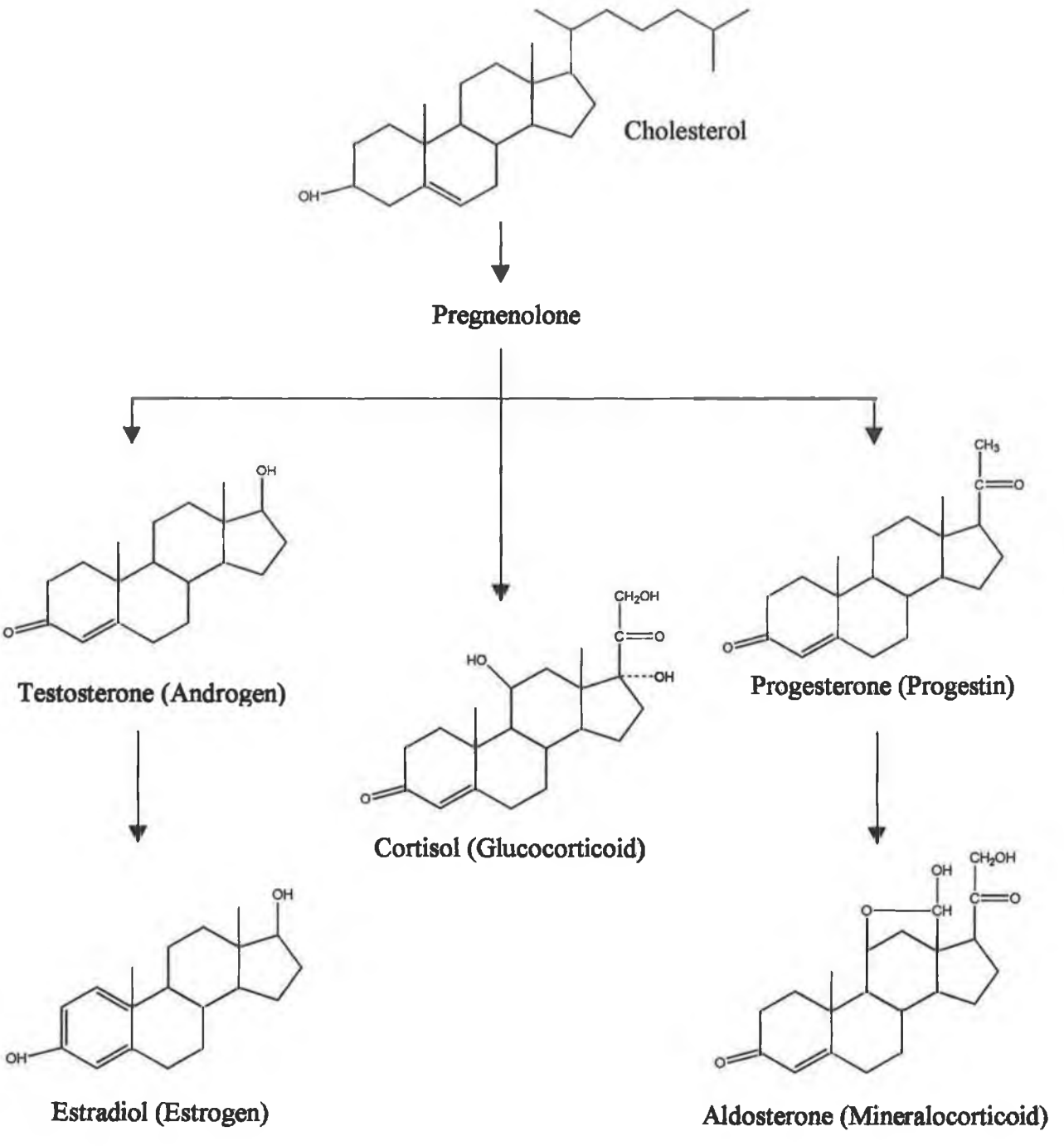


***Figure 1.8** : Routes of biosynthesis of some of the important steroid hormones from the parent compound cholesterol. (Each arrow may contain multiple steps.) (Adapted from Malkinson, 1975 and Paterson, 1983)*

1.3.3.1 Use of Steroid Hormones as Growth Promoters

The muscles of male animals are generally larger than the corresponding muscles in female animals and the inevitable question arose as to why this should occur. Many experiments have been carried out to determine the cause of these differences. It was found to involve the sex hormones and the associated sex organs (Lawrie, 1985). It was noticed that castrated animals had a reduced efficiency of weight gain when compared to whole animals. This implied that the sex organs had an effect on muscle growth through their biochemical messengers, the sex hormones.

The anabolic or protein-accreting effects of the gonadal hormones are eliminated during the process of castration. To investigate this effect the diets of castrated animals were supplemented with hormones and this counteracted the effects of the sex organ removal. This effect of extra weight gain is very desirable in meat-producing cattle as less feed is used and the animals can be slaughtered earlier leading to lower production costs. It has also been shown that the administration of both an estrogen and androgen is required for maximum growth response and that females respond better to the administration of androgens, showing a marked improvement in their rate of weight gain (Lawrie, 1985).

At first when the natural hormones were administered to the animals the beneficial effects were not the only changes noticed. The administering of these compounds, which are by definition linked to the sexual characteristics, also caused changes in the secondary sex characteristics. To avoid the side effects the steroids were chemically modified to produce analogues, which still possessed the desired metabolic functions but also minimised the side effects.

Growth promoters were seen as a way of increasing the meat producers' profit margin. They have been used since the 1970's when their anabolic effects were proven. It was suggested that the consumer also benefits from the use of the hormones. With the use of hormones the animal feed is converted to muscle at a higher rate and so the meat produced by these treated animals is leaner and this is in much demand by the public (Meyer, 2001). The saving to the producer is brought about by the ability to slaughter the animals earlier so saving on feeding and housing costs. The use of hormones as growth promoters is still widespread in the U.S. where the use of six steroids are permitted as growth promoters,

whereas all non-therapeutic uses of hormones in cattle has been banned completely in the European Union since the late 1980's, (EU Directive 81/602/EC). The EU is not convinced that the use of steroid growth promoters in meat-producing animals is safe for the consumer.

The method of administration of the commercial preparation is by means of an implanted pellet under the skin of the backside of the earflap. This implant slowly dissipates over time releasing hormones into the animals system. None of the hormones, except for one of the permitted US steroids, melengestrol, are given as feed supplements (Penner, 1992). The level of hormone does not remain constant but falls as the pellet gets older. The life of the pellet is calculated to be the length of time it remains excreting hormones at a level to have a beneficial effect on the feed conversion efficiency. Hormones are implanted in both sheep and cattle but swine are not normally subjected to this treatment (Penner, 1992).

1.3.3.2 The Need for Monitoring of Steroid Levels in Food

This question of the need to monitor levels of steroids in foods is a very complex and important one. The human endocrine system is a complex, very delicately balanced system. This is illustrated quite clearly from the effect that small amounts of hormones in the contraceptive pill have. These small amounts almost completely block (98%) a woman's ability to conceive by altering the normal functioning of the menstrual cycle. With this in mind the reasons for monitoring the levels of these endocrine-disrupting chemicals are clear. It is also possible for some of the steroid analogues to have other detrimental characteristics, for example DES and estradiol are listed as carcinogens (Opinion of SCVPH, 2002). The food we ingest should be tested to ensure that both the levels of any hormones, either legal or illegal, are not at levels that would cause health problems. Hormone measurements are also used to time the menstrual cycle of animals and any external hormones may interfere with the readings and so cause problems. However, breeding cattle should never be subjected to steroid growth promoters.

The use of these hormones in animals that are destined for slaughter is of concern if we do not know the consequences of the particular dosages and their respective residues. We may find the residues of these hormones and their metabolites in both the meat products and milk products derived from treated animals. When

tolerances of these compounds have been established it must proved extensively that these levels in ingested foods or that continual consumption of these tainted products could not cause any effect on the human system. To facilitate the enforcement of the EU directives banning the use of these hormones each member state must put in place proper testing regimes and reference laboratories. These requirements are set out in the directives 85/358/EEC, 86/469/EEC and 96/23/EC.

Hormones are still in use in some parts of the world as growth promoters and yet are banned within the EU. Some authorities, such as the American FDA, allow some growth promoters as they have concluded that the residue amounts in meat from these treatments would most likely not cause any effects. The American authorities allow the use of a small number of steroidal hormones as growth promoters, namely estradiol, testosterone, progesterone, trenbolone acetate and zeranol. The first three of these hormones are naturally occurring in animals and humans whereas the other two are synthetically produced and are never found naturally. The U.S. FDA have concluded that there would be no physiological effect that could be expected in people consuming the meat from treated animals (Penner, 1992). This conclusion stems from studies which have shown that in the case of the natural hormones the amount consumed would be much less that 1% of the amount that a pre-pubital child would produce in a day. The FDA has even gone as far as to say that even if the hormones were mis-used the levels would not rise above the safe limits (Penner, 1992). The synthetic hormones required much more extensive testing, as they are not already found in the body. It had to be shown that not only did the hormones have no toxicological effects on the animals but also that the residual amounts left in the meat would be below a very low limit e.g. 50ppb TBA and 20ppb zeranol.

The agencies that carry out the testing in the US are the Food Safety and Inspection Service and the US Department of Agriculture. They test for the compounds zeranol and DES (Penner, 1992). They do not test for the natural hormones for a number of reasons. First of all it would be impossible to distinguish between naturally occurring hormones and their metabolites and those introduced artificially into the animals system. They also believe that the levels of hormones present in the meat of these animals would not approach dangerous levels even in cases of extreme misuse. The testing for zeranol ensures

that any use of the growth promoters is within guidelines and the residues are below the tolerance limit of 20ppb. The use of DES as a growth promoter is banned globally as it has been proven to be unsafe even in small doses. Any residues of this chemical must be found and the tainted meat removed from the food chain.

The use of hormones as growth promoters has had a number of scientific reviews because of the potential hazards they could present to the consumer. The Codex Committee on Residues of Veterinary Drugs in Foods met in June 1987 and they concluded that the six hormones allowed in the US (progesterone, testosterone, estradiol, zeranol, melengestrol acetate and trenbolone acetate) when used properly were unlikely to constitute a human health hazard (Gandhi and Snedeker, 2000). This committee consisted of eleven experts from seven countries. An EU Scientific Committee on Veterinary Measures relating to public health has concluded that for the 6 hormones permitted as growth promoters in the US a risk to the consumer was present if they were ingested at high levels (Opinion of SCVPH, 2002). This report also stated that 17β-estradiol should be considered as a complete carcinogen and endocrine, developmental, immunological, neurobiological, immunotoxic, genotoxic and carcinogenic effects of all 6 hormones could be envisioned. This report was unable to set threshold limits for any of the hormones due to their intrinsic properties and other epidemiological findings.

The European ban led to a conflict between the EU and the US agricultural community. The European directive banned most of the American produced beef from being sold on the European market. In retaliation for this loss in revenue the USA added a 100% tariff on wide range of European agricultural produce including canned tomatoes, fruit juices and ham. This lead in turn to the EU threatening to impose tariffs on other US products, (nuts and fruits). The US believes that the EU fears about the safety of hormone-treated meat are unfounded and that the ban is a clear case of unfair trade. However, the EU is carrying out the wishes of their citizens and they are following the advice of their scientific committees. In the early 1980's DES was detected in baby food made from veal from treated animals. Pre-pubertal children are at the highest risk from these hormone residues as the amount of natural hormones present is quite low

and the discovery of this potent xenoestrogen in baby food was a cause of grave concern. This and other evidence encouraged the EU to impose its ban for the safety of the consumer.

1.4 Immunoassays

Immunoassays can be defined as any analytical technique that employs an antigen-antibody interaction. Two important characteristics of antibodies, their specificity and the strength of their binding, have enabled immunoassays to expand into many areas. Immunoassays have many formats and can be used to measure small drugs, macromolecules and proteins. One major division of immunoassays is based on whether or not the antibody-antigen complex has to be separated from unbound reagents. Homogeneous assays do not require a separation step whereas heterogeneous assays do (O'Sullivan *et al.,* 1979).

A number of different immunoassays formats will be discussed in this section including dot or western blots, agglutination assays, dip stick or lateral flow assays, immunomagnetic separation and immunofiltration. Other important assay formats, ELISA and biosensors, will also be discussed in detail in later chapters 3 and 4.

Historically, radioimmunoassays, introduced by Yalow and Berson (1959), were the most popular form of quantitative immunoassays. The more recent trend has moved from these radio-labelled tracers to detection systems using safer molecules such as enzymes. The radiolabelled molecules can have a limited shelf life, they are dangerous to prepare, waste disposal can be problematic, it can be difficult to automate the assays and detection equipment and isotopes are expensive (Landon *et al.,* 1975).

1.4.1 Enzyme-Linked ImmunoSorbent Assay (ELISA)

ELISA is a heterogeneous immunoassay that often utilises a 96 well plastic plate as its solid matrix. Either the antibody or the antigen is absorbed onto the surface of the wells and the binding is mediated by a variety of forces including, electrostatic, hydrophobic, non-covalent and van der Waal's forces. Once these proteins are bound the areas of the plastic that are not covered by the specific protein must be blocked. This step ensures that reagents added to the wells later

in the assay process do not bind non-specifically to the support. This would alter the outcome of the assay and produce results that do not reflect the contents of the samples being analysed. Blocking involves the addition of a non-specific protein solution to bind to the remaining sites on the plastic.

As the proteins are all bound to the surface of the plate washing steps become very simple. The inversion of the plate removes the reaction solutions and by filling the wells with a buffer and emptying them a number of times most unbound molecules can be eliminated (Delves, 1995).

The addition of the sample usually follows the blocking stage and, depending on the exact ELISA format in use, a competing antigen, second antigen-specific antibody or second antibody-specific antibody can be added. One of the components will be labelled with an enzyme such as alkaline phosphatase or β-galactosidase and the amount of these bound can be detected using a substrate. Colourimetric substrates are often used but chemiluminescent or fluorimetric reagents can also be utilised (Dankwardt, 2000).

Two of the most widely used ELISA methods are the sandwich and the competitive formats. In sandwich ELISAs the antigen is captured onto the solid surface by one specific antibody and then bound by a second antibody with detection label (Figure 1.9). The signal observed is directly proportional to the amount of antigen in the sample. This system can only be used with antigens that contain two distinct epitopes that can be bound by different antibodies. Most small molecules do not have two epitopes and so the competitive format is used. Competitive ELISAs can coat either antibody or antigen on the surface of the plate and labelled reagent and sample are added (Figure 3.2). The more antigen contained in the sample the less labelled reagent is bound and an inverse signal response occurs, as shown in Chapters 3 and 4.

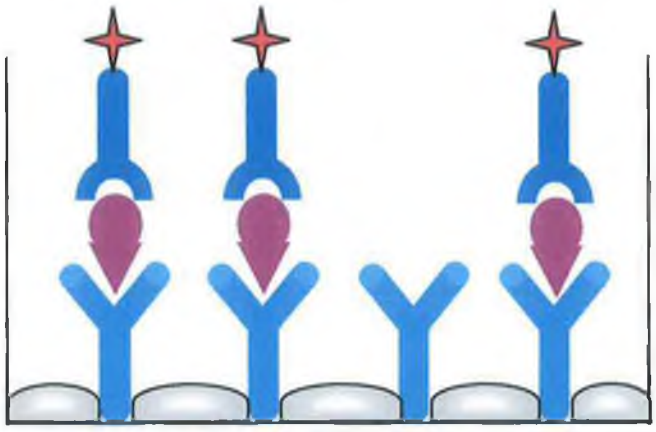


***Figure 1.9** : The binding sequence in a sandwich ELISA. The plate is coated with a specific antibody (light blue antibody) and then blocked (grey mounds). The antigen (purple teardrop) is then added and allowed to bind. The second specific labelled antibody (dark blue antibody with red enzyme) is added and allowed to bind to any antigen captured by the immobilised antibody. The amount of antigen present in the sample is directly proportional to the amount of enzyme signal.*

1.4.2 Biosensors

Biosensors can be defined as an analytical device that uses a biological or biologically derived sensing element integrated with a physiochemical transducer (Sternesjö *et al.,* 1995). A biosensor uses a biological component such as a ligand/receptor or an antibody/antigen-binding interaction to detect the presence of some analyte. The binding is converted to a measurable signal by a transducer and this is fed into a data acquisition device where the signal is analysed (Figure 1.10). There are many different types of biosensor, all using different sensing technologies, different transducer and different methods of signal measuring.

Biosensors can show results and binding events in 'real-time' and that can be a very useful research tool in the life sciences (Hock *et al.,* 1995). Biosensors are usually catagorised by their sensing method, e.g. optical, mass, piezoelectric or electrochemical (Malan, 1994; van Emon *et al.,* 1998). An optical biosensor was used in this work and it is discussed in detail in section 4.1.5.

Biosensors can operate on a batch system by analysing one sample at a time and either regenerating or replacing the sensor to perform another assay. They can also be used as a continuous or semi-continuous flow through system that would allow the constant monitoring of a sample. This will be of great use for effluent management, water treatment or in-process monitoring in manufacturing (Sherry, 1997)

***Figure 1.10** : Schematic representation of the principle of a biosensor. An event occurs that creates a change in the system, e.g. an antibody binding to an antigen. The signal is then fed to the transducer. A transducer is an instrument that changes a signal into a form compatible with a data processing device. The transducer senses that change and converts it into a signal. The altered signal is then passed to a data processing unit for handling, storage and display.*

1.4.3 Dot Blots and Western Blots

These are heterogeneous detection systems, which use a membrane as a solid matrix. These two assays use very similar detection techniques although they both use different methods to immobilise the primary immunoreagent. With dot blots a reagent is dotted onto a membrane in a particular spot either manually or using a dot blot apparatus (Ploum *et al.,* 1991) and is illustrated in Figure 1.11. This dot can be a pure analyte, a capture antibody or even a complex mixture of molecules. The molecules absorb onto the surface of the membrane and the rest of the surface is blocked with a non-specific protein solution. When carrying out a western blot, proteins sample are first subjected to electrophoresis and then the separated proteins are transferred to a membrane using an electric current (Towbin and Gordon, 1984). The resulting membrane contains all the proteins from the original sample that are now separated. This membrane is then blocked and the rest of the reaction is carried out.

A labelled antibody or antigen can be used to probe the membrane directly and after washing a substrate that promotes the deposition of an insoluble colour precipitate is added. This gives a localised colour where the label is bound.

1.4.4 Agglutination Assays

Agglutination assays are homogeneous and rely on the bivalency of antibodies to cross link particles and produce a precipitation effect. They have short incubation times and the assay protocols are quite simple (Wild and Davies, 1994). They can also be easier to automate as there is no requirement for a separation step.

In these assays the antibody (or antigen if antibody is being measured) is immobilised on the surface of a red blood cell (haemagglutination) or a latex bead, (latex agglutination). In the presence of the analyte the antibodies bind to it and the carrier beads or cells clump together (Figure 1.12). This clumping can be detected by light scattering or absorption methods.

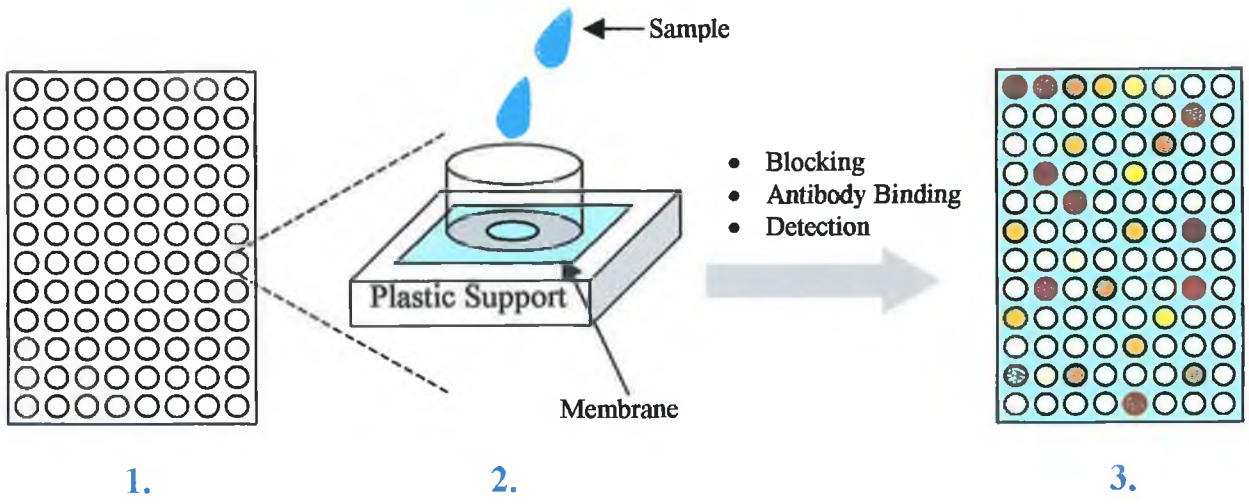


Figure 1.11 *: Arrangement of dot blot apparatus. 1. The sample block has a number of wells (e.g. 96) into which samples can be added. A membrane (e.g. nitrocellulose) is secured under wells on a plastic support. 2. The samples contact the membrane though a small gap in the bottom of the plastic wells. 3. The membrane is then removed from the platform and further reagents are added to the entire membrane, (blocking solution, labelled antibody, precipitating substrate). Each dot then develops a colour or not depending on the original sample added.*

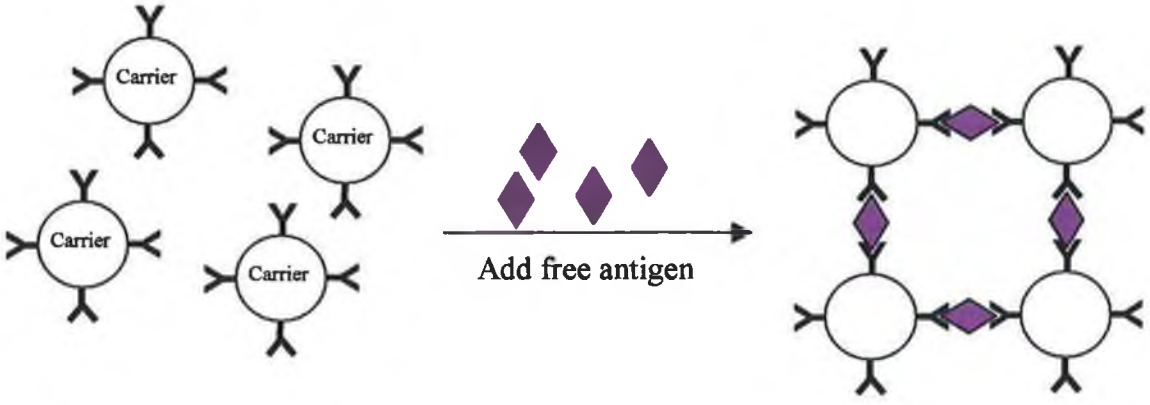


Figure 1.12 *: Agglutination reaction. The antibodies on the carrier molecules bind to epitopes on the antigen and other antibodies bind to other epitopes. This creates a cross-linking effect, which produces the insoluble aggregates seen. The bivalent nature of antibodies can also be used to bind two molecules to each antibody. This can form an aggregate if the target molecule has more than one recognised epitope.*

1.4.5 Lateral Flow Immunoassays

This is a very simple, rapid immunoassay system that uses a liquid flow to initiate the movements of all the components of the assay to an area where the reaction is to happen, Figure 1.13. These assays can take the form of competitive assays or of sandwich (double antibody) assays (Wennig *et al.,* 1998; Paek *et al.,* 2000).

In general, the sample is applied to a piece of absorbent material impregnated with one part of the immune reagents linked to a detection label (usually colloidal gold or a coloured latex bead). The sample and the reagent react forming a complex that moves along the test strip towards a wick at the other end. At a point along the strip an antibody or antigen is immobilised and the immune complex will bind to it, creating a visual colour signal (Price *et al.,* 1997). If the immune complex does not form then no colour will be seen and a negative result is recorded. A control line can also be included that will show colour when the assay is run correctly giving an internal quality check on the assay.

These assays are very useful in bedside, roadside, farm-based testing or any application that requires a quick qualitative result but they can lack the sensitivity needed in other more clinical applications (Ploum *et al.,* 1991). The usefulness of this assay format is exemplified by a urine test strip that can carry out 10 analysis on a single sample (Kutter, 2000).

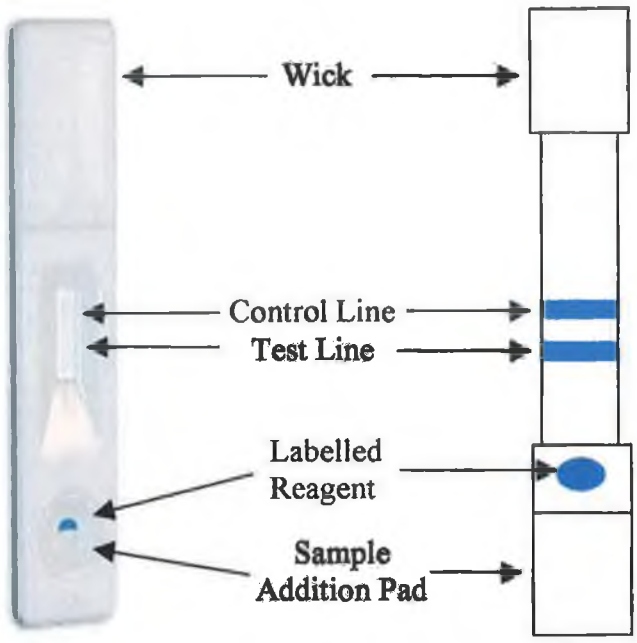


***Figure 1.13** : Dip stick or lateral flow assay device. The sample is added to the first absorbent pad of the assembly and it is pulled along the strip by capillary action. The sample rehydrates the labelled reagent and reacts with it. This mixture then moves along the nitrocellulose strip until it meets the immobilised reagents on the test and control line. These can be a capture antibody or antigen. The labelled reagent will bind to the control line and depending on the reaction (positive or negative) on the control line the contents of the sample can be deduced.*

1.4.6 Immunomagnetic Separation

Immunomagnetic separation uses the combination of the biorecognition of the antibody-antigen pair and magnetic beads to allow for a fast, accurate and gentle method of separation. The antibodies are attached to magnetised beads either directly or through Protein A or G. These can be added to samples to bind cell surface receptors, bacterial antigens, tumour markers or biologically active compounds. Once bound the magnetic beads are isolated using magnets and the rest of the sample is removed. The resulting preparations are relatively pure and can be used in further analysis leading to greater sensitivity and accuracy than many conventional assays. They can also be utilised in the screening of hybridomas to extract cells displaying the required antibody specificity (Hock *et al.,* 1995).

1.4.7 Immunofiltration

The assay systems described by immunofiltration or flow through systems are similar to those of ELISA and immunoblotting but use a membrane as the solid phase rather than solid plastic. In this case the antibody (or antigen) is absorbed onto a membrane and the samples are applied and they pass through the membrane. Any components not binding to the antibody will pass through and have no further part in the assay. The bound components can be detected by a precipitating or a soluble substrate depending on the assay system in use (Sibanda *et al.,* 1999).

Advantages to using this system include the ability to pass larger volumes through the membrane than are used in conventional ELISAs, thus giving a more sensitive assay. This system has great potential in the area of bacterial cell detection where a very small number of cells need to be detected.

1.5 Aims of Thesis

The aim of this work was to generate simple immunochemical detection methods for the analysis of three steroid hormone analogs that are in use as illegal growth promoters in meat. These methods will also be investigated for their suitability for use with a real sample matrix, such as bile.

The production and characterisation of polyclonal antibodies for the detection of trenbolone is described in chapter 3. The antibodies were applied to two ELISA formats and the resulting assays show good accuracy and precision for the detection of free trenbolone. The assay performance was also assessed using spiked trenbolone samples with bile as a sample matrix. The results show that these reagents provide a reliable and robust assay for the detection of trenbolone in buffer or bile matrices.

The development of assays for the detection of ethynyl estradiol in buffer and bile is described in chapter 4. Polyclonal antibodies were produced and applied to the two ELISA systems as previously described. In addition, a surface plasmon resonance-based immunoassay was developed. All assays were investigated for their performance characteristics.

The application of the polyclonal antibodies to a rapid ELISA format using a portable automated device is described in chapter 5. This enables the detection of steroids to be carried out in virtually any location by relatively unskilled operators.

The final results chapter (chapter 6) describes attempts to produce recombinant antibodies specific for the steroids under study. This would allow further maipulation of the assay formats and would generate an infinite supply of a single-chain variable fragment.

CHAPTER 2

MATERIALS AND METHODS

2.1 Equipment

Equipment	Supplier
BIAcore 3000 CM5 Sensor chips	BIAcore AB, Uppsala, Sweden.
Titretek Twinreader Plus Sterile universal containers Corning pH Meter 220 Memmert Static incubator	M.S.C., Damastown, Mulhuddert, Dublin 12, Ireland.
Grant Y6 Waterbath Milipore Filtration Device (De-gassing apparatus) Milli-Q Academic - Ultrapure Water System	A.G.B., Dublin Industrial Estate, Glasnevin, Dublin 9, Ireland.
Biometra T-Gradient Thermocycler (ramping rate 4°C sec^{-1})	Anachem, Anachem House, Luton, Bedfordshire, England.
UV-160A Spectrophotometer	Shimadzu Corporation, Kyoto, Japan.
Orbital incubator	Gallenkamp, Leicester, England.
Atto minigel AE 6100 Atto midigel AE600 Atto Dual minislab AE6450	Atto Corporation, Bunhyo-Kui, Tokyo 113, Japan.
Eppendorf Centrifuge 5810R	Eppendorf AG, 10 Signet Court, Swann Road, Cambridge, U.K.
Eppendorf Tubes	Sarstedt, Wexford, Ireland.
Hermle Centrifuge (Z 200 M/H)	Hermle Labourtechnik, 78564-Wehingen, Gosheimerstr. 56, Germany.
Image Master VDS	Pharmacia Biotech, San Francisco, California, U.S.A.
Nunc Maxisorp Plates Nunc Immunotubes Nunc Maxisorp Break-apart wells Reaction wells for Rapid Elisa Device	Nunc, Kamstrup DK, Roskilde, Denmark.
Sorvall Refrigerated Centrifuge	Du Pont Instruments, Newton, Connecticut, U.S.A.
Tomy Autoclave (SS325)	Mason Technology, Greenville Hall, 228 South Circular Road, Dublin 8, Ireland
Aquatron A400 Water Still for distilled water	Bibby Sterilin, Staffordshire, England.

All chemicals were supplied by Sigma-Aldrich Chemicals, Ireland unless stated otherwise. The steroids were also supplied by this company. The derivative EE-6-CMO was supplied by Steraloids, US.

2.2 Media Formulations

Media Formulations	Components
2X Tryptone Yeast Extract broth media (2X TY, 1L)	16g Tryptone (Oxoid) 10g Yeast Extract (Oxoid) 5g NaCl
2X TY agar (1L)	As 2X TY broth media with 15g Technical Agar (Oxoid)
SOC (1L) (Super optimal catabolites)	20g Tryptone 5g Yeast Extract 0.5g NaCl Sterilise by autoclaving Add 10 ml 1M $MgCl_2$ Add 10 ml 1M $MgSO_4$ Add 16 ml of 25% (w/v) Glucose
Top Agar (1L)	10g Tryptone 5g Yeast Extract 10g NaCl 7g Technical Agar
Minimal Agar (1L)	15g Agar 750 ml H_2O Sterilise by autoclaving Add 200 ml 5X M9 Salts 16 ml 25% (w/v) Glucose 1 ml 20% (w/v) $MgCl_2$ 0.5 ml 1% (w/v) Thiamine-HCl
5X M9 Salts (1L)	64g Na_2HPO_4 15g KH_2PO_4 5g NH_4Cl 2.5g NaCl Sterilise by autoclaving

2.3 Buffer Formulations

Buffer	Components
Phosphate buffered saline (PBS)	1 tablet from Oxoid dissolved in 100 ml of distilled H_2O gives a solution of Dulbecco's A PBS, pH 7.4, containing 0.15M NaCl 2.5mM KCl 10mM Na_2HPO_4 18mM NaH_2PO_4
Hepes Buffered Saline (HBS)	10mM Hepes 0.15M NaCl 3.4mM EDTA (disodium-2-hydrate) 0.05% (v/v) Tween 20 pH adjusted to 7.4 with 2M NaOH, filtered and degassed before use
Tris Acetate EDTA buffer (10X TAE Buffer, Sigma T9650)	0.04M Tris Acetate 1mM EDTA pH 8.3
Tris Buffer for rapid assay device (50X)	0.124M Tris 3.85M NaCl 0.117M Sodium Azide pH to 7.5 5% (v/v) Tween 20
TES (Tris EDTA Sucrose)	200mM Tris 0.5M Sucrose 0.5mM EDTA pH to 8 with HCl
Wash 1 for Ni-NTA column	50mM NaH_2PO_4 300mM NaCl 10mM Imidazole
Wash 2 for Ni-NTA column	50mM NaH_2PO_4 300mM NaCl
Elution Buffer for Ni-NTA column	50mM NaH_2PO_4 300mM NaCl 250mM Imidazole

2.4 Methods

2.4.1 Preparation of steroid derivatives

2.4.1.1 Derivatisation of trenbolone to trenbolone 17-hemisuccinate (TR-17-HS)

The method used was a modification of the procedure used by Jondorf (1980). Trenbolone (100 mg, Sigma-Aldrich Chemicals, Figure 3.1) was dissolved in 3 ml of dichloromethane and 2 ml of dry pyridine. Succinic anhydride (187 mg) was added and the reaction was left at room temperature for 48 h. The reaction was evaporated to dryness using a rotary vacuum evaporator with a heat gun, and the residue was dissolved in 10 ml of chloroform. The residue was washed twice with water (2 x 5 ml) to remove excess succinic anhydride and the chloroform was then removed under vacuum. The residue was re-dissolved in ethanol and stored in the dark at room temperature (Figure 3.3).

2.4.1.2 Derivatisation of diethylstilbestrol to monocarboxypropyl-diethylstilbestrol (DES-CP)

This reaction was carried out using a modification of the procedure published by Degand *at al. (*1989). DES (900 mg, Sigma-Aldrich Chemicals, Figure 6.1), NaH (90 mg) and bromobutrylethylester (600 mg) were placed in a round-bottomed flask and dissolved in dry dimethylformamide. The solution was allowed to react for 48 h in the dark and it was monitored by thin layer chromatography. The product was precipitated by pouring the mixture into a beaker containing 100 ml of ice cold water and 1.5 ml of HCl. The mixture was extracted with ethyl acetate and washed with water to remove any remaining DMF. The organic solution was evaporated to dryness on a rotary evaporator. The solution was purified using a silica column and ethylacetate:hexane (30:70) was used to elute the fraction required. This fraction was then evaporated to dryness.

The ethylenated product (50 mg) was mixed with 3g of KOH dissolved in 25 ml ethanol and refluxed for 1.5h. The resulting solution was neutralised and the precipitate was collected (Figure 6.2).

2.4.2 Preparation of drug-protein conjugates

2.4.2.1 Production of steroid-protein conjugates using EDC/NHS chemistry

The production of this conjugate was carried out with a modification of the carbodiimide procedure described by van Look *et al.* (1991). The steroid derivative (5 mg) was dissolved in 500µl of dioxane in a glass vial. Solid NHS was added to give a final molarity of 0.1M. EDC (5 mg) was dissolved in 250µl of distilled water and added to the steroid solution. This was incubated for 10 min, with stirring. A solution containing 10 mg of carrier molecule (BSA, THY or DEX (amino dextran: Molecular Probes, Oregon, USA)) in 700 µl of 0.05M phosphate buffer, pH 7.8, was added to the reaction mixture. The reaction was allowed to proceed for 75 min. The resultant mixture was dialysed for 48h against 4 changes of PBS at 4°C. The concentration of the protein conjugates was determined using a standard BCA protein microassay (Section 2.4.6.3) while the concentrations of the dextran conjugates was estimated.

2.4.2.2 Production of steroid-protein conjugates using mixed anhydride chemistry

This was prepared using a modification of the mixed anhydride procedure described by Nambara *et al.* (1982) based on the reaction elucidated by Erlanger *et al.* (1957). The steroid derivative (10 mg) was dissolved in 200 µl of DMF. Tributylamine (8 µl) and 2.5µl of butylchloroformate were added and the reaction was stirred on ice for 12 min. A solution containing 20 mg of OVA or BSA in 1 ml of water, 2.5 ml of DMF and 25 µl of 1N NaOH was cooled to 0°C and added to the activated hapten. The reaction was left at 4°C for at least 20h. The conjugate was then dialysed overnight against 5L of PBS to remove any unconjugated hapten. The concentration of this antibody was determined by BCA microassay (Section 2.4.6.3).

2.4.2.3 Production of steroid-horseradish peroxidase conjugates using mixed anhydride chemistry

This synthesis was carried out according to a modification of the procedure described by Meyer and Hoffmann (1987). The steroid derivative (1.5 mg) was dissolved in 100µl of DMF and 1µl of methylmorpholine was added. This

solution was cooled to -15°C and 1 µl of butylchloroformate was added. The solution was left to stir at -15°C for 3 min. This mixture was added slowly to a pre-cooled (0°C) solution of 11 mg of HRP in 100 µl water and 75 µl DMF. This was stirred at -15°C for 60 min followed by 0°C for 120 min. 1 mg of $NaHCO_3$ was then added and the products were dialysed overnight against PBS.

2.4.3 Characterisation of drug-protein conjugates

2.4.3.1. Ultraviolet spectroscopy

Conjugates were diluted to approximately 1 mg ml^{-1} and their UV spectra was analysed from 200-400nm. The UV spectrum of the unconjugated proteins and drugs were also recorded. The comparison of the spectra gave an indication of whether the conjugation was successful and the degree of substitution of the conjugates. This analysis was not possible for all conjugates as the spectrum of some of the drugs may have been masked by the spectra of the carrier proteins.

2.4.3.2 Immunisation

In cases where the UV spectra of the hapten was masked by the carrier protein, the success of the conjugation was assessed by immunising mice and determining if a specific immune response to the free steroid was induced. The mice were immunised with a mixture of the conjugate and Freund's Complete Adjuvant. The mice were boosted after two weeks, using Freund's Incomplete Adjuvant, and a tail bleed was performed 10 days later. The serum was tested for antibodies that recognised the target molecule by non-competitive ELISA and competitive ELISA.

2.4.4 Antibody Production

2.4.4.1 Immunisation procedure for the production of rabbit antiserum to EE-6-CMO-OVA and DES-CP-OVA

Two female New Zealand White rabbits were immunised sub-cutaneously for each drug with 1 ml of EE-6-CMO-OVA or DES-CP-OVA conjugate at a concentration of 0.5 mg ml^{-1} mixed with an equal volume of Freund's Complete Adjuvant. The rabbits were then boosted with the conjugate mixed with Freund's Incomplete Adjuvant at 2, 5, 11, 16 and 18 weeks following initial immunisation.

One rabbit was sacrificed at 7 weeks (EEAb2) and the other 3 at 20 weeks (EEAb1, DESAb1 and DESAb2).

2.4.4.2 Immunisation procedure for the production of rabbit antiserum to TR-17-HS-THY

Two female New Zealand White rabbits were immunised with 1 ml of TR-17-HS-THY conjugate at a concentration of 0.5 mg ml^{-1} mixed with an equal volume of Freund's Complete Adjuvant. The rabbits were then boosted with the conjugate mixed with Freund's Incomplete Adjuvant at 2, 4, and 6 weeks following initial immunisation. The rabbits were sacrificed at 7 weeks.

2.4.4.3 Immunisation procedure for the production of recombinant scFv libraries

Balb/c mice were immunised intra-peritoneally with a mixture of the conjugate (0.5 mg ml^{-1}) and Freund's Complete Adjuvant. The mice were boosted after 2, 4, 8 and 12 weeks, using Freund's Incomplete Adjuvant. A tail bleed was performed 7 days after the last booster injection and the titre and specificity of the serum was determined. If the titre was satisfactory the mice were sacrificed and the spleen was harvested (Section 2.4.10.1).

2.4.5 Preparation of Serum

2.4.5.1 Preparation of rabbit serum

The blood was allowed to clot at room temperature for 2-3 h at which point the clot was ringed, (separated from the side of the blood tube). The blood was left at 4°C overnight to allow the clot to contract. The blood was centrifuged at 4,000rpm for 10min and the serum was recovered and re-centrifuged to remove any remaining blood cells. The serum was stored at -20°C until required.

2.4.5.2 Preparation of mouse serum

The blood gathered from the tail bleed was centrifuged at 14,000rpm for 2min to separate the cells from the serum. The serum was removed to a fresh tube and stored at -20°C until required.

2.4.6 Antibody purification and characterisation

2.4.6.1 Saturated ammonium sulphate precipitation

The IgG fraction was first isolated from the serum by differential precipitation using saturated ammonium sulphate (SAS) according to the method of Hudson and Hay (1989). 10 ml of serum was placed in a beaker on ice and an equal volume of SAS was added dropwise with continuous gentle stirring. This was left on ice for one hour and then centrifuged at 3000rpm for 20min. The pellet was resuspended in 10 ml of 45% (v/v) SAS and centrifuged again. This step was repeated and the final pellet was dissolved in 5 ml of PBS and dialysed overnight at 4°C against 5L of PBS to remove any residual ammonium sulphate.

2.4.6.2 Protein G affinity chromatography

2.5 ml of the dialysate from the SAS precipitation was passed through a 1 ml protein G-sepharose column, which had been equilibrated with PBS. The antibody solution was passed through the column 3 times to allow for maximum binding. The column was washed with PBS until protein was no longer seen in the wash. The bound fraction was eluted with 0.1M glycine-HCl, pH 2.2, that was left in contact with the column for 10min, and 1 ml fractions were collected, which were immediately neutralised with 100 µl of 2M Tris-HCl, pH 8.6. The optical density of the fractions at 280nm was recorded and those that contained protein were pooled and dialysed overnight at 4°C against PBS. Sodium azide was added to a final concentration of 0.05% (w/v) as a preservative and the purified antibody was aliquotted into 0.5 ml fractions and stored at -20°C.

2.4.6.3 Bicinchoninic acid (BCA) protein assay (Pierce)

Standard protein solutions of either BSA or IgG were prepared with varying concentrations between 0-2 mg ml^{-1} depending on the type of protein to be analysed. 10 µl of the sample or the standard was placed in a well of a microtitre plate and 200 µl of BCA working solution (50 parts reagent A to 1 part reagent B) was added. The plate was gently shaken and incubated for 30min at 37°C. The absorbance of the wells was determined at 562nm on a Titretek Twinreader Plus

plate reader. A standard curve of protein concentration versus absorbance was constructed and the values of the unknown protein solutions were determined.

2.4.6.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out to assess the efficiency of the purification of the antibodies and the relative purity of the final product. The polyacrylamide gels (15% w/v), buffers and sample buffer were prepared as described in Table 2.1. The samples were diluted to approximately 1 mg ml^{-1} and mixed with the 5X sample buffer and, along with the protein markers, were boiled for 5min. The gels were run at 15mA per plate on an Atto AE-6450 mini-gel rig until the tracking dye moved to the bottom of the staking gel. At this point the current was increased to 20mA per plate. The gels were run until the dye had moved to the end of the resolving gel. The proteins on the gel were then visualised using Coomassie blue stain or the gel was used for western blot analysis.

2.4.6.5 Coomassie blue staining of a SDS-PAGE gel

Coomassie staining solution (0.2% (w/v) Coomassie blue R250 in 30:10:60 (v/v/v) methanol:acetic acid:water) was prepared and used to stain SDS-PAGE gels for 30min at room temperature with gentle shaking. The stain was then removed and the gels were destained with destaining solution (in 30:10:60 (v/v/v) methanol:acetic acid:water) overnight with gentle shaking. The banding patterns on the gels were recorded by electronic image scanning.

Table 2.1 : Components required for the construction, running and visualisation of SDS-PAGE gels.

Solution	Components
Electrophoresis Buffer	• 3 g Tris • 14.4 g Glycine • 1 g SDS in 1L
4X Resolving Gel Buffer (Stored at 4°C)	• 75 ml 2M Tris-HCl, pH 8.8 • 4 ml 10% (w/v) SDS • 21 ml distilled H_2O (d H_2O)
4X Stacking Gel Buffer	• 50 ml 1M Tris-HCl, pH 6.8 • 4 ml 10% (w/v) SDS • 46 ml dH_2O
30% (w/v) Acrylamide solution	• 29.2 g Acrylamide • 0.8 g bis-acrylamide • to 100 ml with dH_2O
15% (w/v) Resolving Gel (10 ml)	• 2.5 ml 4X resolving gel buffer • 5 ml 30% (w/v) acrylamide solution • 2.5 ml dH_2O • 100 µl 10% ammonium persulphate • 20 µl TEMED
Stacking gel (5 ml)	• 1.25 ml 4X stacking gel buffer • 0.833 ml 30% (w/v) acrylamide solution • 2.877 ml dH_2O • 50 µl 10% (w/v) ammonium persulphate • 10 µl TEMED
Sample buffer	• 60 mM Tris-HCl, pH 6.8 • 25% (v/v) glycerol • 2% (w/v) SDS • 14.4 mM 2-mercaptoethanol • 0.1% (w/v) bromophenol blue

2.4.7 ELISA Procedures

2.4.7.1 Preparation of steroid standards in buffer

A 5 mg ml^{-1} ethanolic stock of each of the steroids was prepared. This stock was diluted 1/50 with PBS (or HBS) to give a 100 µg ml^{-1} steroid solution in buffer containing 2% (v/v) EtOH. Subsequent dilutions were performed using PBS/2% (v/v) EtOH as the diluent to maintain the same buffer across the concentration range.

2.4.7.2 Preparation and use of bovine bile

Bovine bile was collected from Kepak, Co. Dublin, centrifuged and stored at -20°C until required. Samples were defrosted and filtered through a 0.45µm syringe filter before use. Standards were prepared by spiking the bile with the steroid from the ethanolic stock and using the bile to prepare a wide range of concentration standards.

2.4.7.3 Non-competitive ELISA for the determination of antibody titre and optimal steroid-HRP concentration for competitive ELISAs

A range of concentrations of anti-drug polyclonal antibody were prepared using 0.05M carbonate buffer, pH 9.6, and these were coated onto Nunc Maxi-sorp Immunoplates at 100µl/well and left overnight at 4°C. They were washed 3 times with PBST (PBS containing 0.05% (w/v) Tween 20) and 3 times with PBS. Each well was blocked with 150 µl of 2% (w/v) marvel solution in PBS and left to incubate at 37°C for 1h. The plates were washed again and various dilutions of the hormone-HRP conjugate were applied across the differing coated concentrations of antibody at 100µl/well. The plates were incubated at 37°C for 1h and washed. HRP enzymatic activity was detected using the O-Phenylenediamine Dihydrochloride (OPD) Fast Substrate system (Sigma) and the absorbance was read at 405 nm. 100 µl of OPD substrate was added to each well and left to develop in the dark for 30 min and read on a Titretek Twinreader Plus at 405 nm.

2.4.7.4 Competitive ELISA Procedure

Nunc Maxi-sorp Immunoplates were coated overnight at 4°C with anti-steroid polyclonal antibody at the optimal concentration in 0.05M carbonate buffer, pH 9.6, (100µl/well). The plates were washed and blocked as described in section 2.4.7.3. 50µl of a spiked sample was added to the wells followed by 50µl drug-HRP conjugate in PBS. The plate was left at 37°C for 1h. OPD Substrate was used, as above, to detect the amount of HRP enzyme present. Inter and intra-day assays were carried out using 5 replicates of each standard run on 5 different occasions (Section 2.4.7.7).

2.4.7.5 Non-competitive ELISA for the determination of antibody titre and optimal concentration of protein concentration for inhibitive ELISAs

A range of concentrations of hormone-BSA conjugates were prepared using PBS and these were coated onto Nunc Maxi-sorp Immunoplates at 100µl/well and left overnight at 4°C. The plates were washed and blocked as described in section 2.4.7.3. The plates were washed again and various dilutions of the polyclonal antibody were applied across the differing coated concentrations of steroid-BSA conjugates at 100µl/well. The plates were incubated at 37°C for 1h and washed. A commercial anti-rabbit antibody conjugated with HRP was then applied to each well, (100µl/well) at the recommended concentration and incubated for 1h at 37°C. The plates were washed again and 100 µl of OPD substrate was added. The plates were incubated in the dark for 30 min and read on a Titretek Twinreader Plus at 405 nm.

2.4.7.6 Inhibition ELISA Procedure

Nunc Maxi-sorp Immunoplates were coated with steroid-BSA conjugate solution in PBS and blocked as described previously. The samples (50µl/well) were added, followed by the anti-hormone polyclonal antibody and left for 1h at 37°C. 100µl of the secondary antibody (1/5000 dilution of commercial HRP-conjugated goat anti-rabbit IgG) was added to each well and left for 1h at 37°C. The HRP enzyme was detected using OPD and inter- and intradays assays were carried out (Section 2.4.7.7).

2.4.7.7 Data processing of ELISA results

Each concentration point was run with 5 replicates in each assay. The mean absorbance is plotted against the concentration value to produce the calibration curve. The percentage coefficient of variation (% CV) is calculated as the standard deviation of the 5 replicates divided by the mean. This value is a measure of the reproducibility and precision within each assay. To assess these parameters between assays the assays were run on 5 different occasions. The absorbance of each of the standards was normalised by dividing the mean absorbance value for each EE concentration by the mean value for that assay's zero concentration value, (A/A_0). The normalised absorbance values were compared across the 5 assays and the mean values were plotted against the free drug concentrations to produce an inter-assay calibration curve. The % CV was calculated to assess the inter-assay reproducibility and precision.

The results obtained from the immunoassays carried out showed good agreement with a 4-parameter curve-fitting model. The model is described below;

$$y = Rhi - \frac{Rhi - Rlo}{1 + \left(\frac{x}{A1}\right)^{A_2}}$$

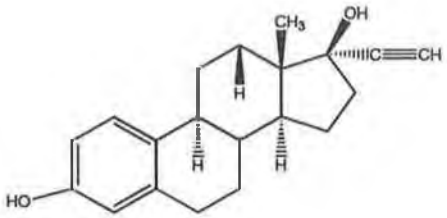
where y is the absorbance or normalised absorbance value, *Rhi* is the absorbance value as the concentration tends to infinity, *Rlo* is the absorbance value as the concentration tends to zero, x is the concentration value and A_1 and A_2 are fitting parameters.

This model was fitted to the data using BIAevaluation 3.1 software and the accuracy of the model curves was calculated by comparing the actual concentration values with those obtained by substituting the recorded absorbances into the model. The comparison is shown as a percentage accuracy where 100% is an ideal fit.

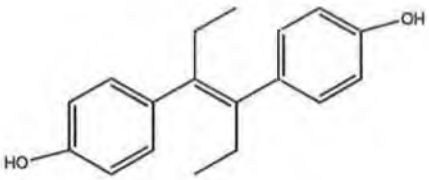
2.4.7.8 Cross reactivity studies

A standard curve of the drug the antibody was raised against was set up in PBS containing 2% (v/v) ethanol. Serial dilutions of the steroid analogues to be tested were prepared (Figure 2.1) and run in parallel with the samples of the specific steroid. The concentration that suppresses the zero signal by 50% is determined

for each steroid being tested. The concentration of the specific steroid that gives 50% signal in the assay is expressed as a percentage of the value calculated for each of the cross-reacting steroids.



Ethynyl Estradiol



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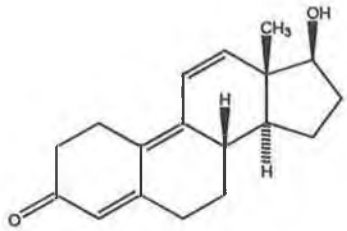

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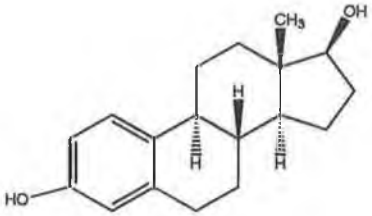
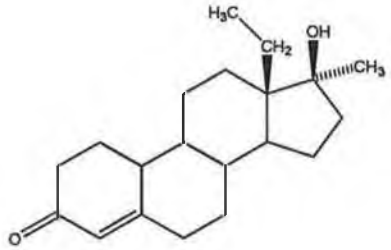
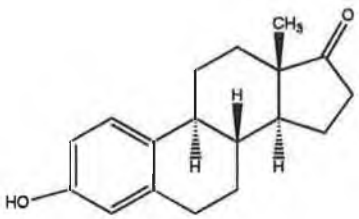


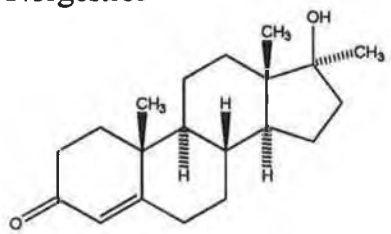
17β-estradiol



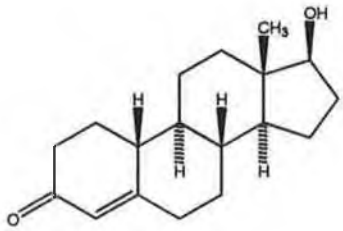
Norgestrol



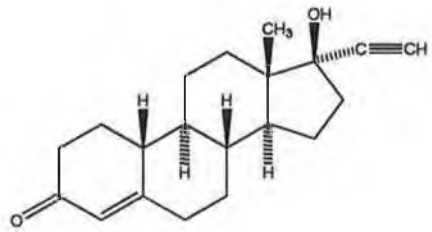
Estrone



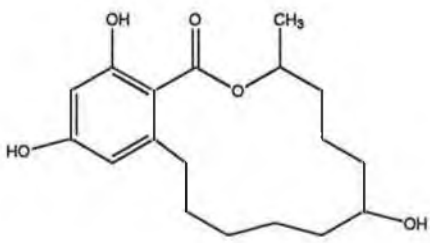
Methyl Testosterone



19-Nortestosterone



Norethisterone



Zeranol

***Figure 2.1** : Structures of the steroids used for the cross reactivity studies.*

2.4.8 Rapid ELISA Protocols

2.4.8.1 Non-competitive ELISA using the rapid ELISA protocol

The wells of a 96 well plate were coated with anti-steroid specific antibody (TRAb1 and DESAb1) and blocked as described in section 2.4.7.1. Various dilutions of steroid-HRP conjugate were prepared and added across the coated concentrations. The conjugate was left in the wells for 5min at 37°C and the plates were washed using two PBST washes followed by two washes in PBS. OPD substrate was added to the wells and the absorbance read at 5min and 20min.

2.4.8.2 Competitive ELISA using the rapid ELISA protocol

The plates were coated with steroid-specific antibody diluted in carbonate buffer, with every fifth well left uncoated, and blocked as previously described, section 2.4.7.1. The steroid standards were prepared and 50 µl was added to the wells followed by 50 µl of the optimised steroid-HRP conjugate dilution. The plate was incubated for 5min at 37°C with three 10 second shakings and washed. OPD substrate was added to detect the bound HRP and the absorbance read after 5 and 20min at 37°C.

2.4.8.3 Assay protocol using the rapid assay device

The reaction wells were coated with 100 µl of the optimised anti-steroid antibody dilution in 0.05M carbonate buffer, pH 9.6, overnight at 4°C. The wells were washed (3 times with PBST and 3 times with PBS) and 250 µl of 2% (w/v) MPBS was used to block each well. This was left at 37°C for 1h. The wells were washed again and left dry at room temperature or with PBS at 4°C until needed. The wells were allowed to come to room temperature before use. Each run with the machine used 4 wells coated with the antibody and one well that had been coated with buffer only. The containers used by the machine are listed below and the positions are illustrated in Figures 5.1 and 5.2.

- Position 1 Eppendorf with 700 µl of sample
- Position 2 Eppendorf with 1 ml of TMB substrate
- Position 3 Eppendorf, empty (for waste)

- Position 4-8 Nunc breakapart wells with 100 µl of conjugate solution
- Position 9-12 Reaction wells coated with antibody
- Position 13 Uncoated reaction well
- Buffer reservoir Tris Buffer

The device aliquots the sample into the 5 conjugate-carrying wells (4-8) and the samples are mixed briefly. The machine transfers the samples from each of the mixing wells to the corresponding reaction well, (4→9, 5→10, 6→11, 7→12, 8→13). The reaction wells are incubated at 35°C with mixing for 4min. The wells are aspirated and washed 3 times with a tris buffer. TMB is added to each of the reaction wells and a transmission reading is taken immediately. The substrate is allowed to develop for 5min with shaking and the transmission is read again. The ratio of the second reading to the first is used as the analytical value and it is normalised against the value obtained in the uncoated control well.

2.4.9 BIAcore Methods

2.4.9.1 Preconcentration Studies

A number of aliquots of 10mM sodium acetate with a pH range between 3.8 and 5 were prepared. The drug derivative to be immobilised on the BIAcore CM5 sensor chip was dissolved in each of these various solutions at a concentration of 50 $\mu g\ ml^{-1}$ and passed across the surface of the chip. The solution that gave the largest change in response units was deemed to be the optimal pH for the immobilisation of the conjugate to the dextran chip surface.

2.4.9.2 Covalent linking of drug conjugate to BIAcore CM5 chip

The conjugate was dissolved in 10mM sodium acetate at the optimised pH and the required concentration (usually 50 $\mu g\ ml^{-1}$). Equal amounts of EDC (0.4M) and NHS (0.1M) were mixed and passed over the chip surface to activate the binding sites of the dextran for 7 min at 5 $\mu l\ min^{-1}$. The conjugate dissolved in acetate buffer was then passed over the surface at a slower flow rate (2 $\mu l\ min^{-1}$) for 20 min. The unused binding sites were then capped by passing 1M ethanolamine, pH 8.5 over the surface for 7min. This procedure removed any remaining active sites on the chip surface.

2.4.9.3 Regeneration Studies

The optimal solutions for the removal of antibody from the conjugate immobilised on the chip surface and the stability of the conjugate with repeated exposure to that regeneration solution were determined. The antibody was passed over the surface and allowed to bind under the same conditions as it would be used in an assay. The regeneration solution was then passed over the surface and this cycle is repeated until the surface no longer shows an ability to bind the antibody at the required level.

2.4.9.4 Inhibition BIAcore assay

Samples were prepared by diluting EE in HBS/bile (1:1) and then mixing the standard with the appropriate dilution of antibody, diluted in HBS. This was incubated at 37°C for 1h. The samples were then transferred to the autosampler of the biosensor. The assay was controlled by a computer program generated

using the BIAcore software. Flow rates on the BIAcore® 3000 were set at 5µl min^{-1}. The samples were passed over the conjugate-immobilised surface of the chip followed by the appropriate regeneration solution. Each standard was injected 3 times and the standards were analysed in random order.

The first antibody, EEAb1, was used at a concentration of 40µg ml^{-1} and the sample/antibody mixture was passed over the chip surface for 4min. The regeneration protocol was a two minute pulse of 1M ethanolamine, pH 13. EEAb2, was used at a concentration of 34.2µg ml^{-1} and 20µl of the sample was passed over the surface. This antibody was regenerated using a two minute pulse of 30mM HCl followed by a further two minutes of 30mM NaOH containing 10% (v/v) acetonitrile. If the assay was run at room temperature the acetonitrile evaporated from the sodium hydroxide solution. To maintain the acetonitrile in the regeneration solution the rack bases of the BIAcore® 3000 were kept at 10°C. Intra and inter-day assays were carried out using 3 replicates of each point on the standard curve run on 3 different occasions.

2.4.10 Recombinant Methods for scFv Library Production

2.4.10.1 Extraction of mRNA from spleen of an immunised mouse

The mouse was sacrificed by cervical dislocation and the spleen was extracted and weighed using aseptic technique. 1 ml of Trizol reagent was added for each 50 mg of tissue and it was then homogenised. The mixture was left at room temperature for 5 min. 200 µl of chloroform was added for every 1 ml of Trizol used and shaken vigorously by hand for 15s followed by a 3 min room temperature (RT) incubation. The solution was then centrifuged at 4°C for 15 min, at 14,000rpm. The upper aqueous phase was removed to a clean tube and 500 µl of isopropyl alcohol was added to it and incubated at RT for 10 min and re-centrifuged for 10 min. The pellet was washed in 1 ml of 75% (v/v) ethanol and centrifuged at 4°C for 5 min at 10,000rpm. The pellet was dried briefly at 37°C and finally dissolved in 50 µl of "RNase-free" water at 4°C overnight.

2.4.10.2 Reverse transcription of mouse spleen mRNA

A Promega Reverse Transcription System Kit was used to carry out the production of cDNA from the isolated mRNA. The components were assembled as described below and incubated for 10min at RT followed by 90min at 42°C.

Component	Stock Concentration	Concentration in 20µl
10X Buffer	10X	1X
$MgCl_2$	25 mM	5 mM
dNTP	10 mM	1 mM
RNase Inhibitor	40 U μl^{-1}	1 U μl^{-1}
Random Hexamer Primers	0.5 µg μl^{-1}	0.01 µg μl^{-1}
AMU RT	20 U μl^{-1}	0.75 U μl^{-1}
mRNA		7-10 µg per reaction
Ultrapure H_2O		to 20 µl

2.4.10.3 Amplification of the variable fragments of antibody heavy and light chains using polymerase chain reaction

2.4.10.3.1 PCR primers

The primers used to isolate and amplify the variable heavy and light chain antibody fragments are listed below. All the light chain primers were used for the light chains and similarily for the heavy chains.

The codes used for the bases are as follows:

A: Adenosine

C: Cytosine

G: Guanine

T: Thymidine

R: A or G

Y: C or T

M: A or C

K: G or T

S: C or G

W: A or T

H: A or C or T

B: C or G or T

V: A or C or G

D: A or G or T

Variable Light chain back primers

LB1 5' gccatggcggactacaaaGAYATCCAGCTGACTCAGCC 3'

LB2 5' gccatggcggactacaaaGAYATTGTTCTCWCCCAGTC 3'

LB3 5' gccatggcggactacaaaGAYATTGTGMTMACTCAGTC 3'

LB4 5' gccatggcggactacaaaGAYATTGTGYTRACACAGTC 3'

LB5 5' gccatggcggactacaaaGAYATTGTRATGACMCAGTC 3'

LB6 5' gccatggcggactacaaaGAYATTMAGATRAMCCAGTC 3'

LB7 5' gccatggcggactacaaaGAYATTCAGATGAYDCAGTC 3'

LB8 5' gccatggcggactacaaaGAYATYCAGATGACACAGAC 3'

LB9 5' gccatggcggactacaaaGAYATTGTTCTCAWCCAGTC 3'

LB10 5' gccatggcggactacaaaGAYATTGWGCTSACCCAATC 3'

LB11 5' gccatggcggactacaaaGAYATTSTRATGACCCARTC 3'

LB12 5' gccatggcggactacaaaGAYRTTKTGATGACCCARAC 3'

LB13 5' gccatggcggactacaaaGAYATTGTGATGACBCAGKC 3'

LB14 5' gccatggcggactacaaaGAYATTGTGATAACYCAGGA 3'

LB15 5' gccatggcggactacaaaGAYATTGTGATGACCCAGWT 3'

LB16 5' gccatggcggactacaaaGAYATTGTGATGACACAACC 3'

LB17 5' gccatggcggactacaaaGAYATTTTGCTGACTCAGTC 3'

LBλ 5' gccatggcggactacaaaGATGCTGTTGTGACTCAGGAATC 3'

Variable Light chain forward primers

LF1 5' ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTGATTTCCAGCTTGG 3'

LF2 5' ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTTATTTCCAGCTTGG 3'

LF4 5' ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTTATTTCCAACTTTG 3'

LF5 5' ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTCAGCTCCAGCTTGG 3'

LFλ 5' ggagccgccgccgcc(agaaccaccaccacc)$_2$ACCTAGGACAGTCAGTTTGG 3'

Variable Heavy Chain back primers

HB1 5' ggcggcggcggctccggtggtggtggatccGAKGTRMAGCTTCAGGAGTC 3'

HB2 5' ggcggcggcggctccggtggtggtggatccGAGGTBCAGCTBCAGCAGTC 3'

HB3 5' ggcggcggcggctccggtggtggtggatccCAGGTGCAGCTGAAGSASTC 3'

HB4 5' ggcggcggcggctccggtggtggtggatccGAGGTCCARCTGCAACARTC 3'

HB5 5' ggcggcggcggctccggtggtggtggatccCAGGTYCAGCTBCAGCARTC 3'

HB6 5' ggcggcggcggctccggtggtggtggatccCAGGTYCARCTGCAGCAGTC 3'

HB7 5' ggcggcggcggctccggtggtggtggatccCAGGTCCACGTGAAGCAGTC 3'

HB8 5' ggcggcggcggctccggtggtggtggatccGAGGTGAASSTGGTGGAATC 3'

HB9 5' ggcggcggcggctccggtggtggtggatccGAVGTGAWGYTGGTGGAGTC 3'

HB10 5' ggcggcggcggctccggtggtggtggatccGAGGTGCAGSKGGTGGAGTC 3'

HB11 5' ggcggcggcggctccggtggtggtggatccGAKGTGCAMCTGGTGGAGTC 3'

HB12 5' ggcggcggcggctccggtggtggtggatccGAGGTGAAGCTGATGGARTC 3'

HB13 5' ggcggcggcggctccggtggtggtggatccGAGGTGCARCTTGTTGAGTC 3'

HB14 5' ggcggcggcggctccggtggtggtggatccGARGTRAAGCTTCTCGAGTC 3'

HB15 5' ggcggcggcggctccggtggtggtggatccGAAGTGAARSTTGAGGAGTC 3'

HB16 5' ggcggcggcggctccggtggtggtggatccCAGGTTACTCTRAAAGWGTSTG 3'

HB17 5' ggcggcggcggctccggtggtggtggatccCAGGTCCAACTVCAGCARCC 3'

HB18 5' ggcggcggcggctccggtggtggtggatccGATGTGAACTTGGAAGTGTC 3'

HB19 5' ggcggcggcggctccggtggtggtggatccGAGGTGAAGGTCATCGAGTC 3'

Variable Heavy chain forward primers

HF1 5' ggaattcggcccccgaggcCGAGGAAACGGTGACCGTGGT 3'

HF2 5' ggaattcggcccccgaggcCGAGGAGACTGTGAGAGTGGT 3'

HF3 5' ggaattcggcccccgaggcCGCAGAGACAGTGACCAGAGT 3'

HF4 5' ggaattcggcccccgaggcCGAGGAGACGGTGACTGAGGT 3'

2.4.10.3.2 Components and conditions for PCR amplification of V_H and V_L gene sequences

The PCR's were set up as follows using cDNA and the appropriate primers for either light or heavy chain amplification.

Component	Stock Concentration	Concentration in 50µl
Back Primers	Varied	0.025nmol per reaction
Forward Primers	Varied	0.025nmol per reaction
dNTP	10mM	1mM
10X Buffer (incl. $MgCl_2$)	10X	1X
Template cDNA		2 µl per reaction
Ultrapure H_2O		to 50 µl
Taq polymerase	1 U μl^{-1}	5 U per reaction

The PCR was carried out using the following conditions.

Step 1: 94°C X 5min

Step 2: 72°C X Hold (Add Taq enzyme for a 'Hot Start')

Step 3: 94°C X 1min

Step 4: 63°C X 30sec

Step 5: 58°C X 50sec

Step 6: 72°C X 1min

Repeat steps 3-6 for 8 cycles

Step 7: 94°C X 1min

Step 8: 63°C X 30sec

Step 9: 72°C X 1min

Repeat steps 7-9 for 30 cycles

Step 10: 4°C X Hold

2.4.10.4 Purification of amplified V_H and V_L fragments from PCR products

PCR purification was carried out using the Wizard PCR prep DNA purification kit (Promega). An Atto AE-6100 gel electrophoresis system was used. The PCR product was run on a 1-2% (w/v) low melt agarose gel (Promega) containing 0.5 µg ml^{-1} ethidium bromide for approximately 2h at 50V. The 400bp fragment was

excised from the gel using a clean scalpel blade and placed in a sterile microfuge tube. The gel was melted in a 70°C water bath and 1 ml of purification resin was added. The tube was mixed gently for 20s and the slurry was loaded into the barrel of a 5 ml syringe attached to a minicolumn. The slurry was pushed into the column followed by 2 ml of 80% (v/v) isopropanol to wash the resin. The column was centrifuged for 2min at 14,000rpm to remove any remaining alcohol from the resin. 50 µl of sterile ultrapure water was added to the column and it was placed in a sterile microfuge tube and centrifuged for 20s to elute the DNA from the resin. The purified DNA was stored at -20°C until required.

2.4.10.5 Quantification of purified PCR products

The purified PCR products were electrophoresed on a 1% (w/v) agarose gel along with quantitative Molecular Weight Markers (Promega, 100bp Molecular Weight Markers). The 500bp fragment of this marker contains 150 ng of DNA and 50 ng of the other bands when 5 µl is loaded onto the gel. Using these quantities as references, densitometry was carried out to determine the concentration of the purified PCR products.

2.4.10.6 Splice by overlap extension (SOE) PCR for the combining of purified V_H and V_L fragments to V_{HL} construct

Stock concentrations of V_H and V_L PCR products at 10 ngμl^{-1} were made for use in the SOE-PCR. This step joins the two 400bp products into a single 800bp fragment and then amplifies this construct.

2.4.10.6.1 SOE Primers

Sc back 5' ttactcgcggcccagccggccatggcggactacaaaG 3'

Sc forward 5' ggaattcggcccccgag 3'

2.4.10.6.2 Components and conditions for the SOE-PCR combining of V_H and V_L PCR products

Component	Stock Concentration	Concentration in 50µl
10X Buffer (incl. $MgCl_2$)	10X	1X
dNTP	10mM	0.4mM
Ultrapure H_2O		to 50µl incl. all components
V_H	10 ng μl^{-1}	0.2 ng μl^{-1}
V_L	10 ng μl^{-1}	0.2 ng μl^{-1}
scfor	0.2 nmol μl^{-1}	0.05 nmol per reaction
scback	0.2 nmol μl^{-1}	0.05 nmol per reaction
Taq polymerase	1 U μl^{-1}	5 U per reaction

The PCR was carried out using the following conditions.

Step 1: 94°C X 1min

Step 2: 45°C X 50sec

Step 3: 72°C X 1min

Repeat steps 1-3 for 7 cycles

Step 4: 72°C X Hold

Add scfor and scback

Step 5: 94°C X 1min

Step 6: 68°C X 30sec

Step 7: 72°C X 1min

Repeat steps 5-7 for 25 cycles

Step 8: 4°C X Hold

2.4.10.7 Preparation and purification of pAK100 vector using Wizard miniprep system (Promega)

A single colony of *E. coli* XL1-Blue cells containing pAK100 (all pAK vectors were kindly donated by Prof. Andreas Pluckthun, University of Zurich, Switzerland, Figure 2.2) was inoculated into 5 ml of 2xTY media containing 25 µg ml^{-1} chloramphenicol and 30 µg ml^{-1} tetracycline and left to incubate overnight at 37°C. The bacterial cells were pelleted by centrifugation for 5 min at 4,000rpm and the supernatant was discarded. The pellet was completely

resuspended in 250 µl of cell resuspension solution and an equal volume of cell lysis solution was added. The solution was mixed by inversion and incubated until the solution cleared, (3-5min). 10 µl of alkaline protease solution was added to the solution followed by a 5min incubation and 350 µl of neutralisation solution. The solution was then centrifuged at 14,000rpm for 10 min. The clear solution was transferred to a spin column and centrifuged at 14,000rpm for 1 min. The column was firstly washed with 750 µl of column wash solution followed by a wash using 250 µl of the wash solution. The DNA was eluted from the spin column using 100 µl of "nuclease-free" water by centrifugation at 14,000rpm for 1 min and stored at -20°C until required.

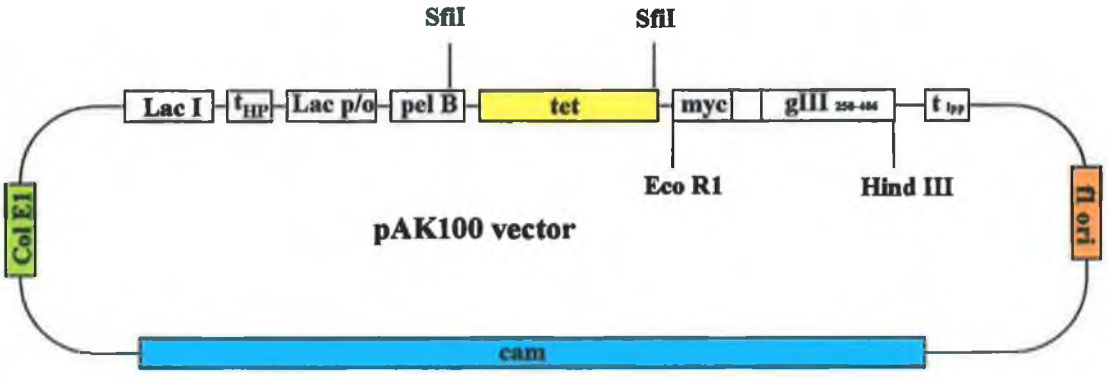


***Figure 2.2** : The pAK100 phagemid vector is illustrated. Replication in* E. coli *is via the Col E1 ori. Resistance is via the cam (chloamphenicol) and tet (tetracycline) resistance genes. For phage packaging, pAK100 carries the intrinsic region from f1 phage. Directional insertion of light and heavy chain genes ligated to produce SOE product is via the SfiI restriction enzyme sites, which are downstream from the Lac promoter/operator and the pelB leader sequence. Downstream of the SOE insertion position is the gIII, for phage surface expression of scFv fragments.*

2.4.10.8 Digestion of SOE-PCR products and pAK100 using the restriction enzyme Sfi1

S*fi*1 is a restriction enzyme derived from *Streptomyces fimbriatus* that recognises a 4bp sequence on either side of the position where it cuts DNA.

The advantage of using this enzyme is the fact that it will only cut if it has two sites to restrict simultaneously and it will not self ligate due to the random bases contained in the actual restriction site.

2.4.10.8.1 Components and conditions for Sfi1 digestion of V_{HL} constructs and plasmid vectors

Component	Stock Concentration	Concentration in 20µl
NEB Buffer 2	10X	1X
BSA	100X	1X
S*fi*1	20U μl^{-1}	20U per reaction

The purified DNA samples were then added. For the V_{HL}, the rest of the reaction volume, usually 20µl, was made with the purified product of the SOE-PCR. The pAK100 plasmid DNA, 10 µl, was restricted in a volume of 50 µl with the rest of the volume being sterile ultrapure water.

The reactions were incubated overnight at 50°C and visualised on a 2% (w/v) agarose gel which allowed the small change in number of base pairs in the V_{HL} construct to be seen.

2.4.10.9 Ligation of Sfi1 restricted V_{HL} construct into the Sfi1 restricted PAK100 plasmid

The ligation reaction was set up using a 2 fold excess of vector over insert. The concentration of the purified, restricted pAK100 vector and V_{HL} was estimated and the amounts required for the ligation were calculated using the following formula.

$$\frac{\text{vector (ng) x insert (kb)}}{\text{vector (kb)}} \times \text{required molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{insert (ng)}$$

2.4.10.9.1 Components and conditions for ligation of Sfi1 digested V_{HL} constructs and plasmid vectors

Component	Stock Concentration	Concentration in 15µl
T_4 ligase (Boerinnger Mannheim)	1U μl^{-1}	3U per reaction
T_4 ligase buffer	5X	1X
Insert	Varied	x
Vector	Varied	x
Ultrapure water		to 20µl

The reaction was incubated at 15°C overnight and visualised on a 1% (w/v) agarose gel.

2.4.10.10 Transformation of E. coli XL1-Blue supercompetant cells with pAK100 vector containing the V_{HL} construct

Escherichia coli XL1-Blue supercompetant cells (Stratagene) were thawed on ice along with the supplied β-mercaptoethanol. 100 µl of cells were aliquoted into chilled universals, one tube each for the transformation and one for the control transformation. 1.7 µl of β-mercaptoethanol was added to each 100 µl of cells and they were incubated on ice for 10 min with a gentle swirling every 2 min. 15 µl of the ligation mixture produced in section 2.4.10.9.1 was added to the tube and left on ice for 30min, followed by a heat pulse at 42°C of 50s and a further ice incubation of 2min. At this stage 900 µl of pre-heated SOC medium was added to each tube and incubated at 37°C for 1h. The cells were then plated and titred on 2xTY agar supplemented with 1% (w/v) glucose, 25 µg ml^{-1} chloramphenicol and 50 µg ml^{-1} tetracycline. The control reaction was plated as per the manufacturer's instructions. The stock plates were scraped and the cells were mixed with sterile 80% (v/v) glycerol to a final concentration of 15% (v/v), flash frozen and stored at -80°C until required.

2.4.10.11 Rescue of phagemid particles; Krebber protocol

50 ml of 2xTY media supplemented with 1% (w/v) glucose and 25 µg ml^{-1} chloramphenicol was in inoculated with 100 µl of the glycerol library stock

(section 2.4.10.10) and incubated at 37°C with shaking until the optical density at 600nm ($O.D._{600nm}$) reached 0.5. The culture was then allowed to sit at 37°C for 10min. VCSM13 helper phage (approximately 10^{11}cfu) and 25 µl of 1M isopropyl-β-D-thiogalactopyranoside (IPTG) were added to the culture and incubated at 37°C for 15min. The culture was then diluted into 100 ml of 2xTY media supplemented with 1% (v/v) glucose, 25 µg ml^{-1} chloramphenicol and 0.5mM IPTG. The culture was then shaken for 2h at 26°C. At this point the 30 µl ml^{-1} of kanamycin was added and the culture was returned to 26°C and left overnight with shaking. The phage particles were present in the culture supernatant.

2.4.10.12 PEG/NaCl precipitation and concentration of phage particles

The culture supernatant from section 2.4.10.11 was transferred into a sterile Sorvall tube and centrifuged at 10,000rpm and 4°C for 10min. 30 ml of PEG/NaCl was added to the supernatant, mixed and incubated at 4°C for at least 1h. The mixture was centrifuged at 10,000rpm and 4°C for 30min and the pellet was resuspended in 40 ml of sterile ultrapure (u.p.) water and 8 ml of PEG/NaCl was added and left on ice for 20min. The mixture was centrifuged at 10,000rpm and 4°C for 20min. The supernatant was removed and the pellet was recentrifuged for 10min. All remaining liquid was removed and the pellet was suspended in 2 ml of sterile PBS. This was centrifuged at 4,000rpm and 4°C for 10min to remove any bacterial debris. This was stored at 4°C and used within a week.

2.4.10.13 Titre of phage particles

A single colony of *E. coli* XL1-Blue cells was inoculated into 4 ml of 2xTY with 50 µg ml^{-1} tetracycline and left to grow overnight at 37°C with shaking. The culture was diluted 1/100 (50 µl in 5 ml) into the same media and incubated until the $O.D._{600nm}$ reached 0.5. At this point the culture was left at 37°C for 15min without shaking. Serial dilutions of the phage particles were made using this culture, (10^{-1}-10^{-10}) and left to infect the cells for 30min at 37°C without shaking. 100 µl of each dilution was then spread plated on 2xTY agar containing 1%

(w/v) glucose and 25 μg ml^{-1} chloramphenicol and incubated overnight at 37°C and the colony forming units were counted.

2.4.10.14 Biopanning of phagemid library using immunotubes

Nunc immunotubes were coated with 500 μl of a drug conjugate at an appropriate dilution in PBS at 4°C overnight. The tubes were washed 4 times with PBST and 4 times with PBS. The tubes were then blocked completely with 4.5 ml of PBS containing 3% (w/v) marvel milk powder and incubated at 37°C for 1h. The tubes were washed as before and 200 μl of the eluted phage was added along with 300 μl of PBS containing 3% (w/v) milk and left to bind at 37°C for at least 1h. The tubes were washed and the bound phage were eluted by adding 800 μl of 0.1M glycine/HCl, pH 2.2, and incubating for 10min. The solution was then neutralised with 50 μl of 2M Tris/HCl, pH 8.6. 20 μl of the eluted phage was used to titre the phage (Section 2.4.10.13) and the rest of the phage were re-infected into XL1-Blue *E. coli* cells (Section 2.4.10.15).

2.4.10.15 Re-infection of XL1-Blue E. coli cells with phage eluted after biopanning

A single colony of *E. coli* XL1-Blue cells was inoculated into 4 ml of 2xTY with 50 μg ml^{-1} tetracycline and left to grow overnight at 37°C with shaking. The culture was diluted 1/100 (50 μl in 5 ml) into the same media and incubated until the $O.D._{600nm}$ reached 0.4. At this point the culture was left at 37°C for 10min without shaking. 700 μl of the eluted phage from section 2.4.10.14 was added to the cells and incubated at 37°C for 30min to allow for infection. The cells were centrifuged and the pellet was resuspended in 600 μl of 2xTY media. The cells were then plated onto 2xTY/1% (w/v) glucose/25 μg ml^{-1} chloramphenicol agar and incubated overnight at 37°C. The resulting colonies were scraped from the plates into 2xTY media, supplemented with 80% (v/v) glycerol to a final concentration of 15% and titred.

2.4.10.16 Rescue of phagemid particles in 96 well plate format for the purposes of screening

96 individual colonies, from the plates used to titre the phage eluted from the immunotube, were each inoculated into 150 µl of 2xTY media supplemented with 1% (w/v) glucose and 25 µg ml^{-1} chloramphenicol in a sterile 96 well plate and incubated overnight at 37°C with shaking. This was termed the masterplate. A replica plate was prepared using 180 µl per well of the above media into which 20 µl from the masterplate was added. 30 µl of 80% (v/v) glycerol was added to the masterplate and this was stored at -20°C. The replica plate was incubated at 37°C until the culture had become turbid (4-5h) and 25 µl of 2xTY media containing 1% (w/v) glucose, 25 µg ml^{-1} chloramphenicol, 4.5 mM IPTG and 5 x 10^9 VCSM13 helper phage per ml was added to each well. The plate was incubated at 37°C for 30min without shaking, followed by 1h with agitation. The cells were pelleted by centrifugation and the media in the plates was replaced with 2xTY containing 1% (w/v) glucose, 25 µg ml^{-1} chloramphenicol, 1.5mM IPTG and 30 µg ml^{-1} kanamycin. The plates were left to produce phage particles overnight at 26°C. The plates were centrifuged to pellet the cells and the supernatant was used in further analysis (Section 2.4.10.17).

2.4.10.17 Non-competitive ELISA for the screening of phage particles for the presence of antigen-specific binders

Nunc immunoplates were coated with a drug conjugate at a concentration between 2-20 µg ml^{-1} in PBS and incubated overnight at 4°C. The plates were washed and blocked as previously described in section 2.4.7.1. Following washing, 50 µl of supernatant produced according to section 2.4.10.16 was added to the corresponding well of the ELISA plate along with 50 µl of PBS/4% (w/v) milk and incubated at 37°C for 1h. Commercial anti-fd bacteriophage polyclonal antibody was used at a dilution of 1/1000 in PBS/2% (w/v) milk, 100 µl per well, and incubated for 1h at 37°C. This antibody was then detected with a HRP-conjugated goat anti-rabbit IgG antibody, 1/5000 dilution in PBS/2% (w/v) milk, 100 µl per well, 1h at 37°C. The HRP label was detected using OPD substrate and the absorbance read at 405nm after 30min incubation at room temperature. The reaction may also have been stopped using 25 µl of 2M H_2SO_4 and read at 492 nm.

2.4.11 Recombinant Antibody Procedures for the naive human Library

The methods are as described for the Krebber system with the following exceptions.

- The host *E. coli* strain was TG1 and the helper phage was M13K07 (Strategene).
- The growth media was 2xTY supplemented with 2% (w/v) glucose.
- The phagemid vector carried ampicillin resistance gene. This was used as the selection antibiotic.
- The helper phage used were M13 KO7.
- TG1 stocks were maintained on minimal agar.
- PEG/NaCl precipitation was only used for the first round of panning after that the culture supernatants were used.

2.4.11.1 Production and Purification of soluble scFv fragments with Immobilised Metal Affinity Chromatography (IMAC)

10 ml of 2xTY containing 2% (w/v) glucose and 100 μg ml^{-1} ampicillin was inoculated with TG1 cells harbouring the specific phagemid and grown overnight at 30°C. This was diluted into 500 ml of 2xTY containing 0.1% (w/v) glucose and 100 μg ml^{-1} ampicillin and left to grow till the OD_{600} reached 0.9. 500 μl of 1M IPTG was added and the culture was left for 4h. The culture was centrifuged and the pellet was resuspended gently in 10 ml of cold tris-EDTA-sucrose (TES). This was transferred to a cool universal tube and 15 ml of 20% (v/v) TES was added and left to incubate on ice for 30min. The solution was centrifuged at 14,000rpm at 4°C for 10min to pellet the cell debris.

1.6 ml of Ni-NTA metal affinity slurry (800 μl resin, Qiagen) was washed in wash buffer 2 and 50 μl of 1M $MgCl_2$ and 200 μl of protease inhibitor cocktail (Roche) was added. The supernatant from the TES isolation above was added to the resin and the mixture was allowed to interact overnight at 4°C with gentle rotation. The resin was pelleted by centrifugation at 2,000rpm for 5min at 4°C and washed twice in 50 ml of wash buffer 2. The pellet was resuspended in 3 ml of wash buffer 1 and loaded into a plastic support column. The column was washed twice with 600 μl of wash buffer 2. 200 μl of the elution buffer was added to the column and the flow through was collected. 500 μl aliquots of the elution buffer were passed through the column. To remove imidazole from the solutions containing the scFv the fractions were passed through a NAP-5 column pre-equilibrated with 10 ml PBS. The majority of the scFv was seen in the first two fractions (1 ml) from the Ni-NTA column.

CHAPTER 3

PRODUCTION AND CHARACTERISATION OF ANTI-TRENBOLONE POLYCLONAL ANTIBODIES

3.1 Introduction

3.1.1 Trenbolone

17β - Trenbolone (TR, Figure 3.1) is a powerful synthetic steroidal androgen with a molecular weight of 270.4g that is used as a growth promoter in cattle. It has been used to improve the feed conversion rate and carcass characteristics of cattle destined for the human food chain (Phillips and Harwood, 1982). It can be used in on its own but when used in concert with an estrogenic compound the best results are obtained (O' Keefe, 1984).

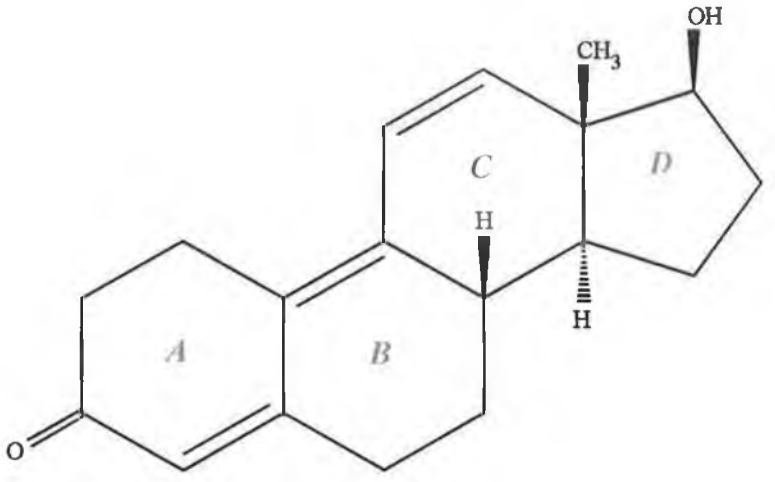


***Figure 3.1** : Structure of the androgenic steroid, 17β- trenbolone, (17β – hydroxyestra - 4, 9, 11 - triene - 3 - one).*

Trenbolone acetate (TBA) is rapidly hydrolysed to 17β-trenbolone after administration to cattle (Schiffer *et al.*, 2001). This metabolite, 17β-trenbolone is the active form of trenbolone acetate and is the main metabolite found in muscle and fat tissues of implanted cows (van Ginkel *et al.*, 1988). 17α-trenbolone is the other major metabolite and it is the main form found in liver and kidney tissues (van der Merwe and Pieterse, 1994). Both 17β-trenbolone and 17α-trenbolone have been found to be devoid of genotoxic activity and are not thought tot be initiators of cancer (Richold, 1988)

Like all steroids TR and TBA have been banned as growth promoters for use in cattle rearing within the European Union (van Ginkel *et al.*, 1988). The EU is not convinced of the safety of treating animals used for meat production with steroids to increase their weight gain. Insufficient information is available on the

effects of these steroids on the consumer when they are ingested in meat products. Commercial growth-promoting treatments consisting of estrogens and androgens are available for sale and use in others parts of the world (Lange *et al.,* 2001). Trenbolone acetate is one of the six FDA permitted compounds used within the USA as a growth promoter. Meat coming from outside the EU must be tested for this compound, or its metabolites, and removal of such treated meat from the food chain in Europe is necessary. Each country is required to have a testing system in place to enforce the EU ban on the use of these growth promoters and so prevent the use of these substances and their subsequent incorporation into the food chain (Sawaya *et al.,* 1998b).

3.1.2. Detection of TR

Over the course of the past 30 years many assay systems have been developed for the detection of trenbolone acetate and its metabolites. Hoffmann and Oettel (1976), described a radioimmunoassay for the detection of trenbolone and trenbolone acetate in bovine tissue and plasma. A lengthy procedure was used to extract the free steroids in tissue to a buffer matrix. Any conjugated steroids remaining in the sample were subjected to an enzymatic deconjugation. The assay had a sensitivity of 40ρg for the detection of trenbolone and approximately 70ρg for the detection of trenbolone acetate in fat. TBA can only be found in the implantation site, and in the fat of the animal, making it a difficult compound to measure. TR was detected in the plasma at low nanogram levels up to 67 days after administration and, in general, the conjugated TR amounted to approximately 20% of the free TR. The analysis of 100 samples would require the input of one technician for 5 days. This testing level is far too low for any effective screening method.

In 1987 Meyer and Hoffmann described a microtitre plate-based enzyme immunoassay for the detection of trenbolone. Their paper described the pre-treatment of the biological matrices, bile, urine, muscle, liver and faeces to extract trenbolone to be detected using the assay. Some of these extraction steps, while isolating the trenbolone, required considerable time to complete with some procedures using an 18h incubation in conjunction with organic extraction methods. The assay itself was very sensitive with an absolute detection limit of

1pg. However, the assay did require approximately 23h of incubations, so that each sample needed 3 days to complete the analysis from collection to result.
van Ginkel *et al.* (1988) described an assay system that combined an immunoaffinity clean-up step with HPLC-TLC detection. This system analyses bovine urine for the presence of the metabolites of TBA. The samples were deconjugated enzymatically and passed through an anti-trenbolone immunoaffinity column. The TR-containing fractions were then extracted and applied to a HPLC column. The resulting fraction from the column containing the TR was then subjected to TLC and developed. This is a lengthy and complicated procedure that requires much sophisticated equipment and trained personnel. It does, however, have a detection limit of 1-2 ng ml^{-1} by on-line HPLC monitoring at 350 nm but the off-line TLC detection showed greater sensitivity with a detection limit of 0.5 ng ml^{-1}.

3.1.3 Acceptable residue limits for TR

The use of all steroid hormones in cattle is illegal within the EU so a confirmed positive result indicates that the animal was dosed. These drugs have a high potency and so are administered at low levels (Rodriguez *et al.,* 1994). The present maximum residue level accepted by the FAO/WHO for TBA is 2 ng g^{-1} (Sawaya *et al.,* 1998a). Enzyme immunoassays have the capability to detect these low levels. They also have the advantage of being able to test samples in large batches. The HPLC-TLC method in section 3.1.2 can analyse 40 samples in 4h. ELISAs, depending on the exact format, can test up to 48 samples and standards, in duplicate, in as little as 1.5h. The type of analysis that is required must also be considered. There are conditions where a result for a single sample would be required as opposed to multiple sample analyses. The type of result required, the time allowed, the equipment required and the conditions under which the assay must be performed should all be considered when a choice of assay system is to be made (Turkes *et al.,* 1981).

3.1.4 Choice of matrix

Due to the numerous and diverse matrices that can be used to detect non-endogenous steroids within a bovine system each assay must be validated to

ensure that any positive results are as a result of the detection of the target analyte. It is important that the false negative rate due to interference effects from the matrix in use is low and so any assay must be validated in the sample matrix to be used (Cooper *et al.,* 1998). This is especially important when the test matrix is to be used with little or no pre-treatment. Administered steroids have been found to persist longer in bile than in urine, thus implying that bile be chosen as the superior matrix for analysis (M^{c}Evoy *et al.*, 1998).

Bile and faeces were determined to be the most appropriate matrices for the detection of trenbolone (Hewitt *et al.,* 1993). Bile is also a liquid and so unlike solid matrices such as faeces, hair and eyeballs, which can retain high levels of steroid for analysis, it can be handled easily and quickly. This allows it to be used rapidly within any of the assay formats described in this chapter.

3.1.4 Enzyme-Linked ImmunoSorbent Assay (ELISA) Technology

Enzyme-linked immunosorbent assay was the form of enzymatic immunoassay used in this study. This format used a solid phase, an antibody and enzymes to create an assay system that can be used to detect amounts of free drug. The advantages of the microplate system are its simplicity, speed, ease of use and it can analyse up to 96 individual wells at the one time (van Weemen *et al.,* 1979). Larger plate formats have also been developed allowing for even greater numbers of samples to be analysed (Roda *et al.,* 2000). Two formats of heterogeneous competitive ELISAs have been developed. They have been designated a competitive assay and an inhibition format for ease of identification, (Figure 3.2).

The solid phase used is a 96 well immunoplate and, in all cases, a horse radish peroxidase (HRP) label is used for detection. Both of the formats are shown in Figure 3.2 and the exact protocol is described in section 2.4.7. The inhibitive assay begins with the coating of the drug on the wells of the plate. The adsorption of the small drug molecule, such as a steroid, can be very problematic and so a conjugate is used. The larger protein carrier facilitates a much more stable and even coating of the wells. The conjugates are absorbed to the wells during an incubation stage and any remaining uncoated sites on the plastic wells are blocked by a protein solution to prevent non-specific binding.

The free drug solution (the sample or standard) and the specific antibody are added to the wells and allowed to reach binding equilibrium, (usually this will occur quickly and 1h at 37°C is sufficient time). In some assays the specific antibody may be labelled with an enzyme but if it is not a labelled secondary antibody that will recognise the idiotype of the specific antibody can be used. In either case the enzyme label is detected using a substrate that will change colour in the presence of the enzyme. The colour is then read using a spectrophotometric plate reader. The wells are washed to remove any unbound or loosely bound material between each of the steps described. This ensures that only the molecules bound to the plate remain in the well and are involved in further binding or detection steps. The unbound fractions are removed and can not cause any interference.

The alternative format, designated competitive in this thesis, is also shown in Figure 3.2. Here the specific antibody is coated to the wells of the plate and blocked as described above. The free drug is then added along with a HRP-labelled drug conjugate. The two drugs then compete for binding to the immobilised antibody, equilibrium is reached and the unbound is removed. The amount of bound HRP is measured.

The HRP enzyme is detected using O-phenylenediamine dihydrochloride (OPD) substrate. This changes from a clear liquid to a yellow/orange colour in the presence of HRP. This colour can be read at either 450nm or 405nm as it contains two absorbance peaks. For the inhibitive and competitive ELISAs the substrate absorbance was read at 405nm. In both of these assay formats the amount of free drug present is inversely proportional to the absorbance. The maximum level of absorbance is seen at zero free drug concentration and it drops as the concentration of the sample or standard rises.

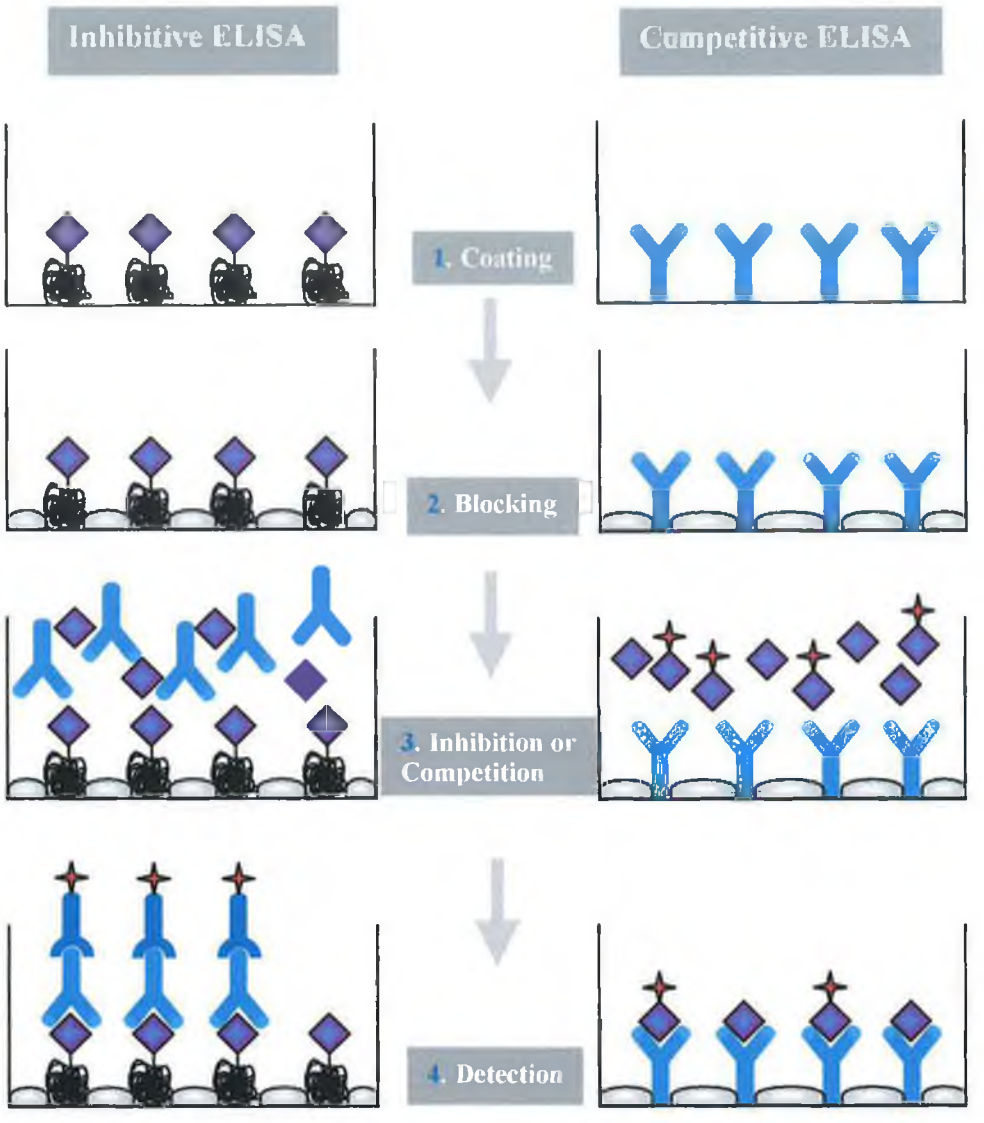


***Figure 3.2** : Schematic representation of the inhibitive and competitive ELISA formats. (1) represents the coating of either the drug conjugate or the specific antibody to the plate. (2) shows the blocking of the free areas of the plastic of the immunoplates. (3) shows the reaction between the immobilised reagent, the sample and the competing substance, (labelled drug or specific antibody), while (4) shows the method of detection for each of the assay types.*

Drug-protein conjugate

Free drug, (sample or standard)

Blocking protein

Drug enzyme conjugate

Enzyme-labelled anti-specific antibody

Specific antibody

3.2 Results

3.2.1 Production and conjugation of trenbolone hapten

The reaction to convert trenbolone (TR) to the derivative TR-17-hemisuccinate (TR-17-HS, Figure 3.3) was carried out as described in section 2.4.1.1. The success of the reaction was confirmed by nominal mass spectrometry. This showed the presence of derivative with a weight of 368 daltons, (See appendix B). This weight corresponds to that of an alkalinated form of TR-17-HS. This derivative was used to produce ovalbumin (OVA), bovine serum albumin (BSA), thyroglobulin (THY) and horse radish peroxidase (HRP) conjugates of trenbolone using both mixed anhydride and the NHS ester chemistries for use as immunogens and assay reagents. The chemistries used for each conjugate are listed below,

Conjugate	Linking Chemistry
TR-17-HS-OVA	Mixed anhydride
TR-17-HS-BSA	Mixed anhydride
TR-17-HS-HRP	Mixed anhydride
TR-17-HS-THY	EDC/NHS

All the conjugates were investigated spectrophotometrically and the presence of a peak at about 350nm was taken to confirm the presence of the hapten bound to the proteins (Jansen *et al.*, 1985). The proteins alone do not show this peak and therefore if it present after the dialysis of the conjugate it must indicate the successful conjugation. Dialysis should remove all the unbound trenbolone derivative as it is to small to be retained by the dialysis tubing, MW cut-off 10kDa.

To test the suitability of the conjugates as immunogens the OVA and THY conjugates were used to immunise mice. 10 days after the immunisation the mice were bled. The sera were tested against a number of conjugates and carrier proteins. The results showed that the OVA conjugates were not particularly good immunogens but the THY conjugates gave a much higher response and also one that was directed against the hapten and not the carrier protein, (Figure 3.4 and 3.5). The anti-TR-17-HS-THY sera did not recognise the TR-17-HS-OVA

conjugate although it recognised the other conjugates tested to some degree. This implies that the conjugation of TR to OVA was not as successful as shown by the spectrophotometer analysis. The background response is high in this assay and that obscures some of the other more specific responses. This problem may be overcome by continuing the immunisation protocol. TR-17-HS-THY was used in all subsequent immunisations.

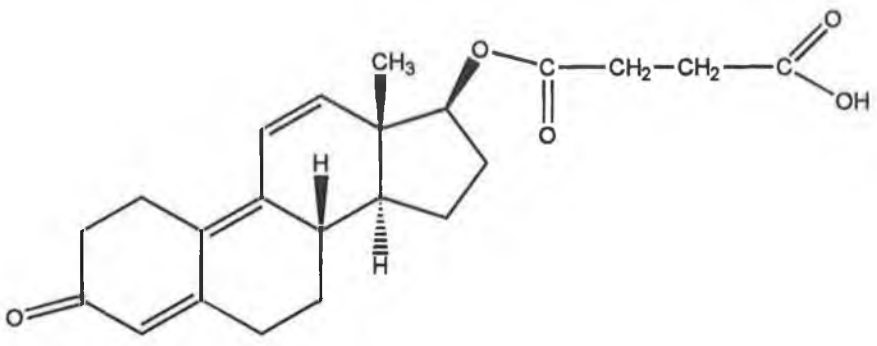


***Figure 3.3** : Structure of trenbolone-17-hemisuccinate, the derivative used to link carriers to trenbolone.*

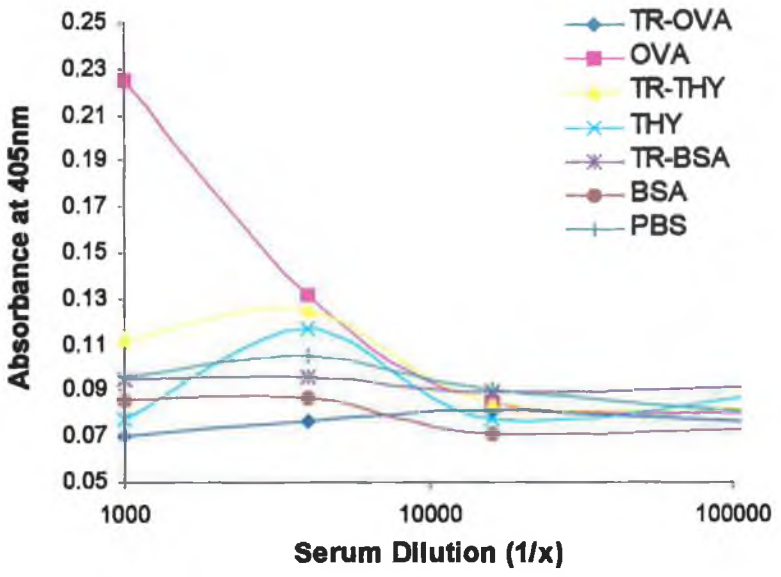


***Figure 3.4** : Specificity of anti-TR-17-HS-OVA sera against various TR conjugates and carrier proteins. The antibodies show recognition of OVA and a slight response to THY. No specific response is seen towards TR.*

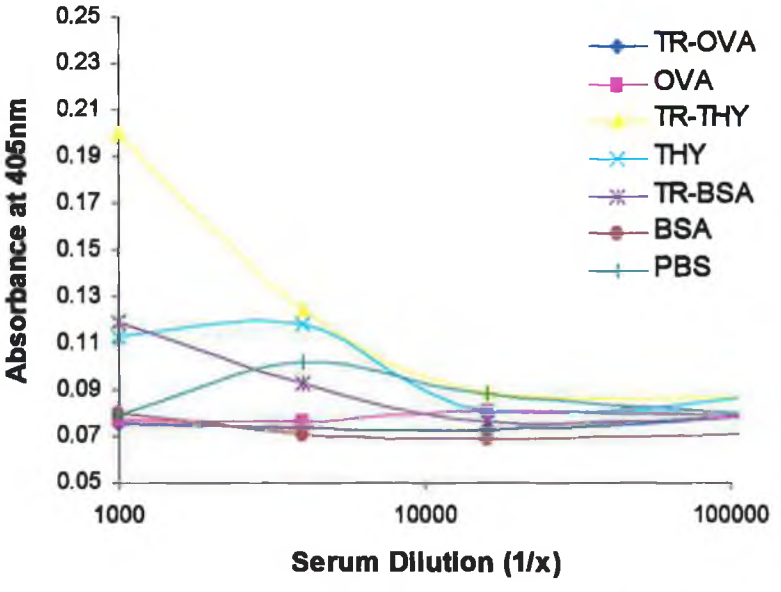


***Figure 3.5** : Serum dilution of anti-TR-17-HS-THY antibody and it's specificity against various TR conjugates and carrier proteins. A response is seen against TR-THY and TR-BSA. There is also recognition of the THY carrier protein but it is much below the level seen for the conjugate.*

3.2.2 Polyclonal antibody production and characterisation

The immunisation schedule was followed as outlined in section 2.4.4.2. The two New Zealand White female rabbits were immunised and boosted with 1 ml of THY conjugate and the serum was collected at the completion of the protocol. The immunoglobulin fraction of the serum was isolated using SAS precipitation followed by protein G affinity chromatography. The absorbance of the eluted samples at 280nm was measured and used to estimate their protein content. Figure 3.6 shows the typical elution profile from the protein G column. As can be seen the majority of the bound protein eluted in fractions 1 - 8 and these fractions were pooled, dialysed and used as the reagents TRAb1 and TRAb2 depending on the rabbit of origin. TRAb1 was determined to have an IgG concentration of 4.76 mg ml^{-1} by BCA assay and TRAb2 had a concentration of 6.59 mg ml^{-1}.

The purity of the final samples was determined by running the crude serum, the SAS-purified and the affinity-purified fractions on SDS-PAGE. Each of the samples was diluted to approximately 1 mg ml^{-1} before loading onto the gel. SDS-PAGE is a technique that dissociates any protein chains that are held together by disulphide bridges and it also coats the proteins with a uniform negative charge so that proteins can be analysed with respect to their component chains and their molecular weight. The results can be seen in Figure 3.7. The heavy and light chains of the antibody are visible at approximately 50 and 25kDa, respectively.

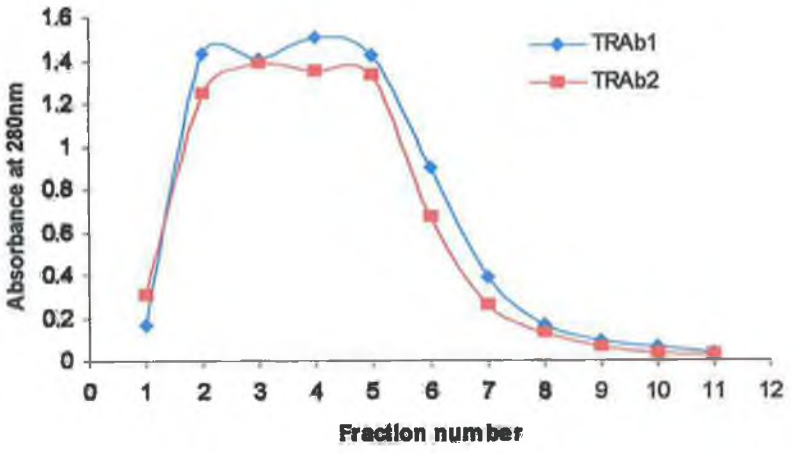


***Figure 3.6** : Isolation of immunoglobulin fraction of TRAb1 and TRAb2 from a 1 ml Protein G affinity column using 0.1M glycine-HCl, pH 2.2. 1 ml fractions were collected and fractions 1 - 8 contained measuable amounts of protein.*

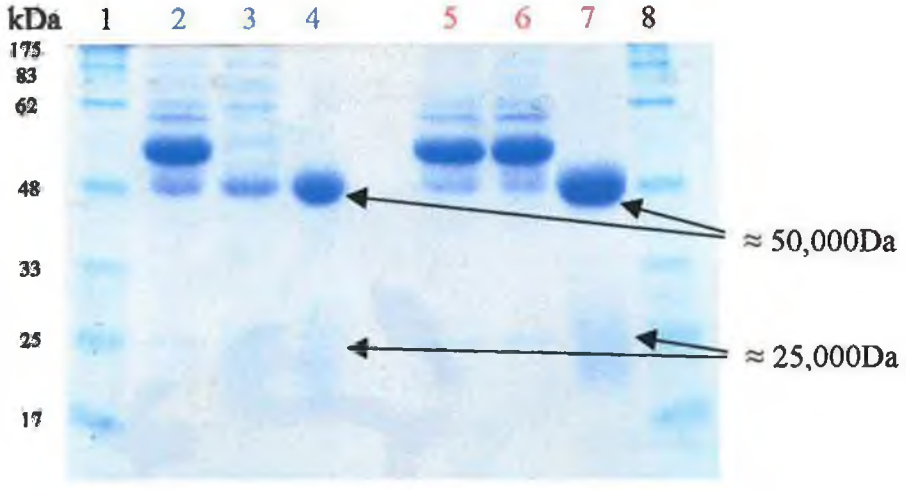


***Figure 3.7** : Coomassie stained SDS-PAGE gel with protein markers in lane 1 and 8. Lane 2: Serum from rabbit 1, lane 3: dialysed SAS cut of rabbit 1 serum, lane 4: protein G affinity-purified IgG fraction (TRAb1) and lane 5: serum from rabbit 2, lane 7: dialysed SAS cut of rabbit 2 serum, lane 8: protein G affinity-purified IgG fraction (TRAb2).*

3.2.3 Optimisation of parameters for the inhibitive and competitive ELISAs for the detection of free TR

The optimisation of the use of these antibodies and conjugates in ELISA systems was carried out. Competitive and inhibitive formats were developed. These are described in section 3.1.4.

The non-competitive checkerboard ELISAs were carried out as described in sections 2.4.7 to determine the optimal coating concentration either of TR-17-HS-BSA conjugate or anti-TR antibody, depending on the assay format, and the optimal concentration for the competing solution. A range of different coating concentrations was run with differing amounts of competing reagent. The lowest coating concentration that showed a steep dose response curve with strong measurable signals in the assay was chosen as the optimum. The optimal antibody dilution, (inhibitive ELISA) or HRP-conjugate dilution (competitive ELISA) was also elucidated from the results of these assays. Using the dose response curve generated using the optimal coating concentration, the amount of either antibody or TR-HRP conjugate that gave approximately 70% of the maximum signal of that curve were used in further assays. An example of the results of these assays is shown in Figure 3.8. The coating and competing concentrations used in the ELISAs are listed in Table 3.1.

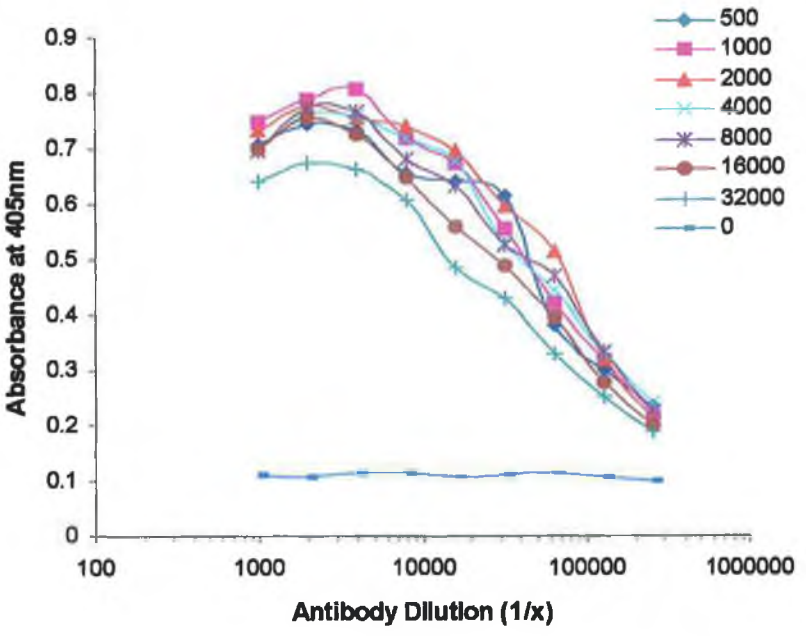


***Figure 3.8** : Non-competitive checkerboard ELISA for the determination of optimum TR-HS-BSA conjugate coating concentration and optimum TRAb2 antibody dilution to be used in the inhibitive ELISA.*

Table 3.1 : Optimal coating and competing concentrations for competitive and inhibitive ELISAs using both anti-TR polyclonal antibodies.

Assay	Coating Solution	Competing Solution
TRAb1 Inhibitive	0.64 µg ml^{-1} TR-HS-BSA	0.79 µg ml^{-1} TRAb1
TRAb1 Competitive	4.76 µg ml^{-1} TRAb1	0.10 µg ml^{-1} TR-HS-HRP
TRAb2 Inhibitive	1.29 µg ml^{-1} TR-HS-BSA	1.98 µg ml^{-1} TRAb2
TRAb2 Competitive	6.59 µg ml^{-1} TRAb2	0.10 µg ml^{-1} TR-HS-HRP

3.2.3.1 Range of detection of free TR in competitive and inhibitive ELISAs using TRAb1 and TRAb2 polyclonal antibodies

A wide range of concentration standards for TR (0.38 - 100,000 ng ml^{-1}) was prepared in PBS/2% (v/v) EtOH. These were run in the optimised ELISAs using the parameters described in section 3.2.3. From the resulting dose response curve a detection range was selected that included the most sensitive part of the curve, the pseudo-linear portion and approximately two points either side of this section. The ranges chosen were 0.76 - 781 ng ml^{-1} for the competitive assay and a range of 3.05 - 3,125 ng ml^{-1} for the inhibitive assay. The results for the competitive format are shown, as an example of these assays, in Figure 3.9. This section of the dose response curve gives a good approximation to the 4-parameter curve-fitting model.

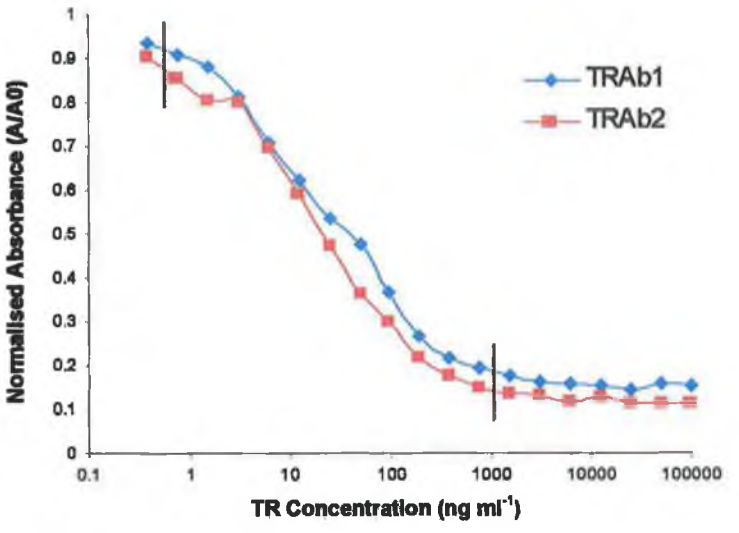


***Figure 3.9** : Determination of range of detection of TR using either TRAb1 or TRAb2 in the competitive ELISA format. Standards ranging from 0.38 - 100,000 ng ml^{-1} were run in the ELISA using the optimised parameters for the assay. Black lines bound the pseudo-linear portion, chosen as the range for the ELISA, on the graph.*

3.2.4 Competitive and Inhibitive ELISAs using pab TRAb1 and TRAb2 for the detection of TR in PBS/2% (v/v) EtOH

A range of standards of TR was prepared as described in section 3.2.3.1. The standards were added to a TR-17-HS-BSA-coated plate at 50 µl per well together with anti-TR pab (TRAb1 or TRAb2) at the required dilution with the inhibitive assay format. After the incubation and washing steps had been completed HRP-labelled anti-rabbit pab was added to the plates. In the competitive ELISA format the standards and the appropriate TR-17-HS-HRP dilution were added to an anti-TR pab-coated plate. After washing, the bound HRP conjugate was detected in both assays as specified previously, (section 2.4.7).

For intra-day assay variation studies each concentration standard was assayed 5 times on a single day and the mean absorbance was plotted against the TR concentration. Figures 3.10, 3.11, 3.14 and 3.15 show the intra-day assay results for the two assay formats using both antibodies. The accompanying tables 3.2, 3.3, 3.6 and 3.7 show the means, the standard deviations, the coefficients of variation (C.V.'s) and the percentage accuracy of the standard curve using 4-parameter curve fitting.

The inter-day assay variation was determined by carrying out the assay over 5 separate days. The absorbance of each of the standards was normalised by dividing the mean absorbance value for each TR concentration by the mean value for the day's zero concentration value, (A/A_0). The A/A_0 value was compared across the 5 days. Figures 3.12, 3.13, 3.16 and 3.17 show the inter-day assays for both assay formats using both antibodies. The accompanying tables 3.4, 3.5, 3.8 and 3.9 show the means, the standard deviations, the coefficients of variation (C.V.'s) and the percentage accuracy of the standard curve using 4-parameter curve fitting.

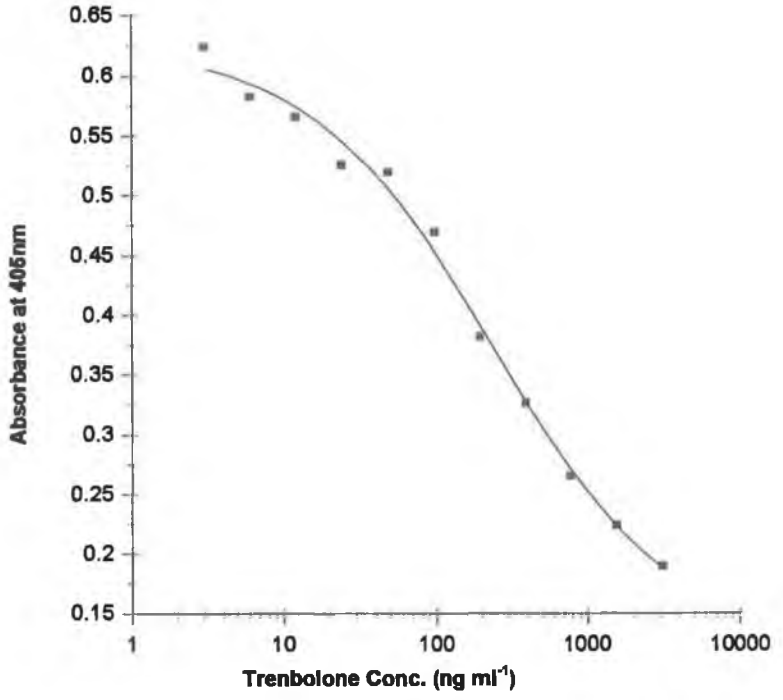


***Figure 3.10** : Intra-day studies on inhibitive ELISAs using the anti-TR Pab, TRAb1 for the detection of free TR in PBS/2% (v/v) EtOH. The inhibition assay uses TR-17-HS-BSA conjugate coated at 0.644 μg ml^{-1} and the antibody at a dilution of 0.793 μg ml^{-1}.*

Table 3.2 : Intra-day CVs and accuracies for TRAb1 inhibition ELISA in PBS. Five sets of eleven standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Trenbolone Concentration (ng ml^{-1})	Calculated Mean ± SD, (Abs)	Coefficients of variation (CVs), (%)	Accuracy, (%)
3125.00	0.190 ± 0.007	3.57	94.35
1562.50	0.224 ± 0.006	2.67	97.66
781.25	0.266 ± 0.010	3.71	106.24
390.63	0.327 ± 0.009	2.77	101.62
195.31	0.382 ± 0.015	3.96	110.49
97.66	0.470 ± 0.008	1.68	80.86
48.83	0.520 ± 0.016	3.14	78.97
24.41	0.526 ± 0.012	2.29	142.85
12.21	0.566 ± 0.004	0.76	124.48
6.10	0.583 ± 0.023	3.95	149.65
3.05	0.624 ± 0.020	3.26	2.89

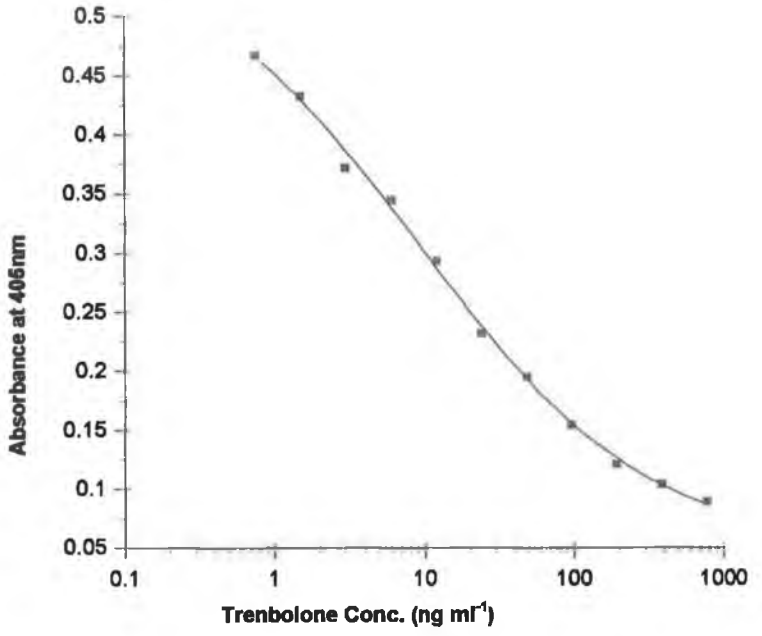


***Figure 3.11** : Intra-day studies on competitive ELISAs using the anti-TR Pab, TRAb1 for the detection of free TR in PBS/2% (v/v) EtOH. The plates were coated with 4.76 μg ml^{-1} solution of TRAb1 and the conjugate TR-17-HS-HRP was used at 0.10 μg ml^{-1}.*

Table 3.3 : Intra-day CVs and accuracies for TRAb1 competition ELISA in PBS/2% (v/v) EtOH. Five sets of eleven standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Trenbolone Concentration (ng ml^{-1})	Calculated Mean ± SD, (Abs)	Coefficients of variation (CVs), (%)	Accuracy, (%)
781.25	0.089 ± 0.002	2.56	88.78
390.63	0.104 ± 0.003	2.63	97.28
195.31	0.121 ± 0.010	7.91	113.83
97.66	0.154 ± 0.007	4.73	101.97
48.83	0.195 ± 0.006	2.92	96.09
24.41	0.232 ± 0.007	2.94	107.30
12.21	0.293 ± 0.004	1.27	90.99
6.10	0.344 ± 0.009	2.51	90.57
3.05	0.372 ± 0.007	1.87	122.66
1.53	0.433 ± 0.005	1.19	94.08
0.76	0.467 ± 0.017	3.67	95.50

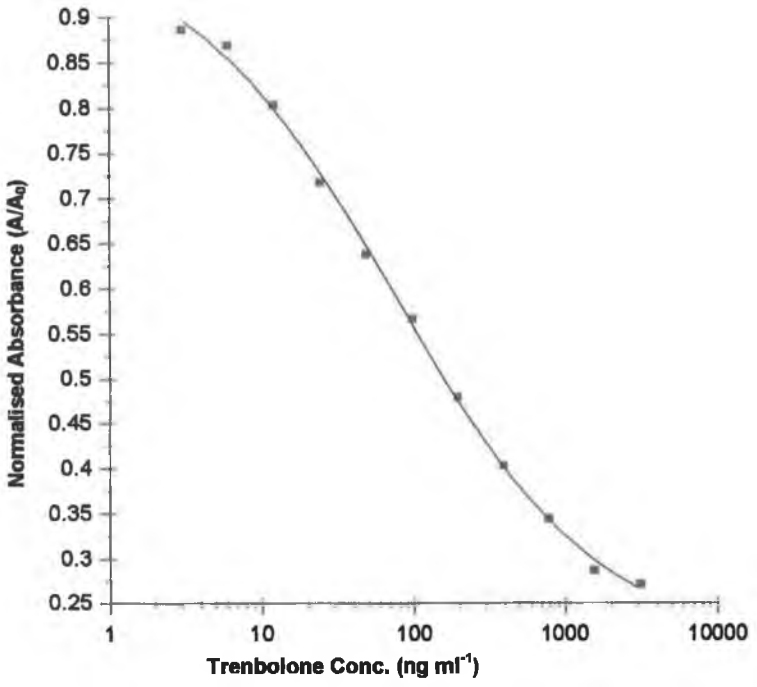


***Figure 3.12** : Inter-day studies on inhibitive ELISAs using the anti-TR Pab, TRAb1 for the detection of free TR in PBS/2% (v/v) EtOH. The inhibition assay uses TR-17-HS-BSA conjugate coated at 0.644 μg ml^{-1} and the antibody at a dilution of 0.793 μg ml^{-1}.*

Table 3.4 : Inter-day CVs and accuracies for TRAb1 inhibition ELISA in PBS. Five sets of eleven standards were analysed over five different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Trenbolone Concentration (ng ml^{-1})	Calculated Mean ± SD, (A/A_0)	Coefficients of variation (CVs), (%)	Accuracy, (%)
3125.00	0.271 ± 0.023	8.57	85.75
1562.50	0.287 ± 0.008	2.71	121.08
781.25	0.344 ± 0.025	7.16	98.38
390.63	0.403 ± 0.034	8.54	99.04
195.31	0.480 ± 0.045	9.47	96.80
97.66	0.567 ± 0.040	7.10	94.59
48.83	0.639 ± 0.031	4.84	106.43
24.41	0.718 ± 0.053	7.40	109.57
12.21	0.803 ± 0.018	2.28	95.09
6.10	0.870 ± 0.042	4.87	80.42
3.05	0.887 ± 0.067	7.51	120.67

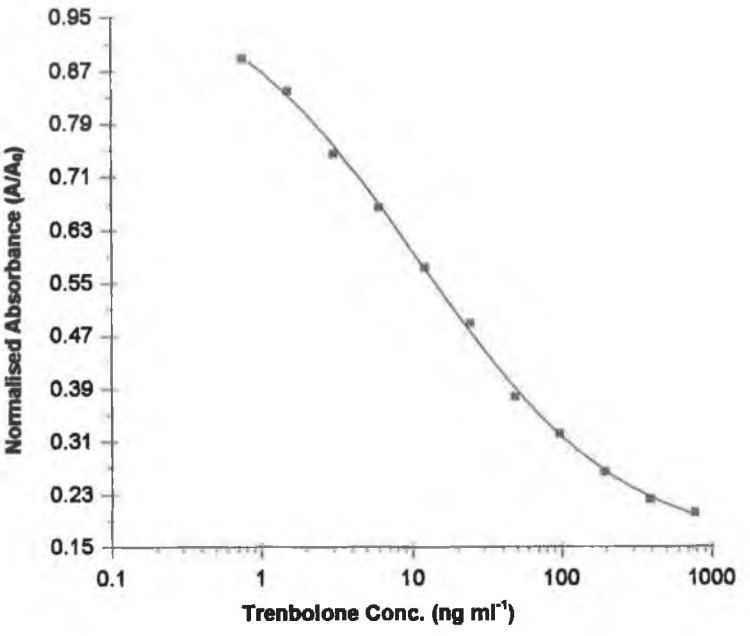


***Figure 3.13** : Inter-day studies on competitive ELISAs using the anti-TR Pab, TRAb1 for the detection of free TR in PBS/2% (v/v) EtOH. The plates were coated with 4.76 μg ml^{-1} solution of TRAb1 and the conjugate TR-17-HS-HRP was used at 0.10 μg ml^{-1}.*

Table 3.5 : Inter-day CVs and accuracies for TRAb1 competition ELISA in PBS/2% (v/v) EtOH. Five sets of eleven standards were analysed over five different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Trenbolone Concentration (ng ml^{-1})	Calculated Mean ± SD, (A/A_0)	Coefficients of variation (CVs), (%)	Accuracy, (%)
781.25	0.203 ± 0.025	12.32	88.94
390.63	0.224 ± 0.022	9.76	105.39
195.31	0.264 ± 0.026	9.67	102.92
97.66	0.322 ± 0.020	6.15	97.83
48.83	0.378 ± 0.031	8.21	112.29
24.41	0.489 ± 0.028	5.82	91.07
12.21	0.574 ± 0.018	3.11	99.14
6.10	0.665 ± 0.024	3.61	103.26
3.05	0.746 ± 0.020	2.62	110.91
1.53	0.841 ± 0.013	1.54	93.65
0.76	0.889 ± 0.039	4.40	105.56

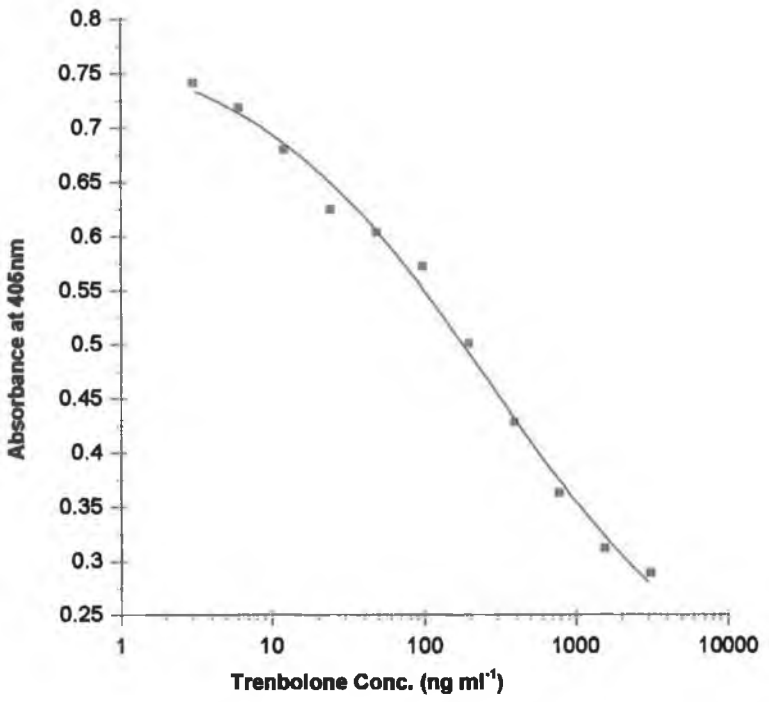


***Figure 3.14** : Intra-day studies on inhibitive ELISAs using the anti-TR Pab, TRAb2 for the detection of free TR in PBS/2% (v/v) EtOH. The inhibition assay uses TR-17-HS-BSA conjugate coated at 1.289 μg ml^{-1} and the antibody at a dilution of 1.983 μg ml^{-1}.*

Table 3.6 : Intra-day CVs and accuracies for TRAb2 inhibition ELISA in PBS. Five sets of eleven standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Trenbolone Concentration (ng ml^{-1})	Calculated Mean ± SD, (Abs)	Coefficients of variation (CVs), (%)	Accuracy, (%)
3125.00	0.289 ± 0.010	3.59	82.41
1562.50	0.312 ± 0.007	2.26	115.88
781.25	0.364 ± 0.013	3.63	114.45
390.63	0.429 ± 0.008	1.91	103.52
195.31	0.502 ± 0.017	3.43	90.05
97.66	0.573 ± 0.022	3.87	75.99
48.83	0.604 ± 0.028	4.59	100.44
24.41	0.626 ± 0.003	0.46	145.58
12.21	0.681 ± 0.016	2.41	110.79
6.10	0.719 ± 0.020	2.71	85.50
3.05	0.742 ± 0.006	0.86	74.42

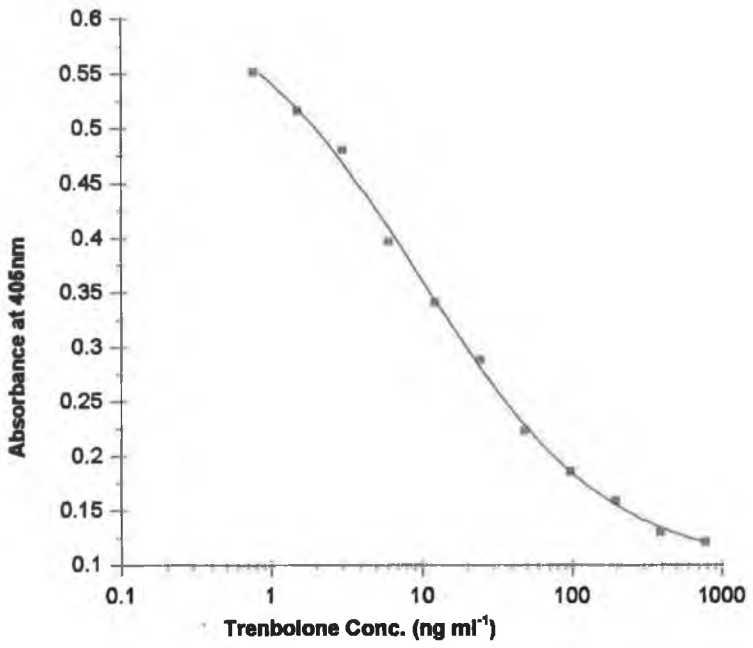


***Figure 3.15** : Intra-day studies on competitive ELISAs using the anti-TR Pab, TRAb2 for the detection of free TR in PBS/2% (v/v) EtOH. The plates were coated with 6.59 μg ml^{-1} solution of TRAb2 and the conjugate TR-17-HS-HRP was used at 0.10 μg ml^{-1}.*

Table 3.7 : Intra-day CVs and accuracies for TRAb2 competition ELISA in PBS/2% (v/v) EtOH. Five sets of eleven standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Trenbolone Concentration (ng ml^{-1})	Calculated Mean ± SD. (Abs)	Coefficients of variation (CVs), (%)	Accuracy, (%)
781.25	0.122 ± 0.005	4.44	96.69
390.63	0.131 ± 0.004	2.69	120.47
195.31	0.159 ± 0.005	3.00	90.15
97.66	0.187 ± 0.004	2.35	98.23
48.83	0.224 ± 0.005	2.16	105.81
24.41	0.289 ± 0.007	2.25	92.27
12.21	0.342 ± 0.003	0.96	102.79
6.10	0.397 ± 0.006	1.46	113.80
3.05	0.481 ± 0.009	1.79	85.12
1.53	0.517 ± 0.010	1.90	101.22
0.76	0.552 ± 0.012	2.14	106.56

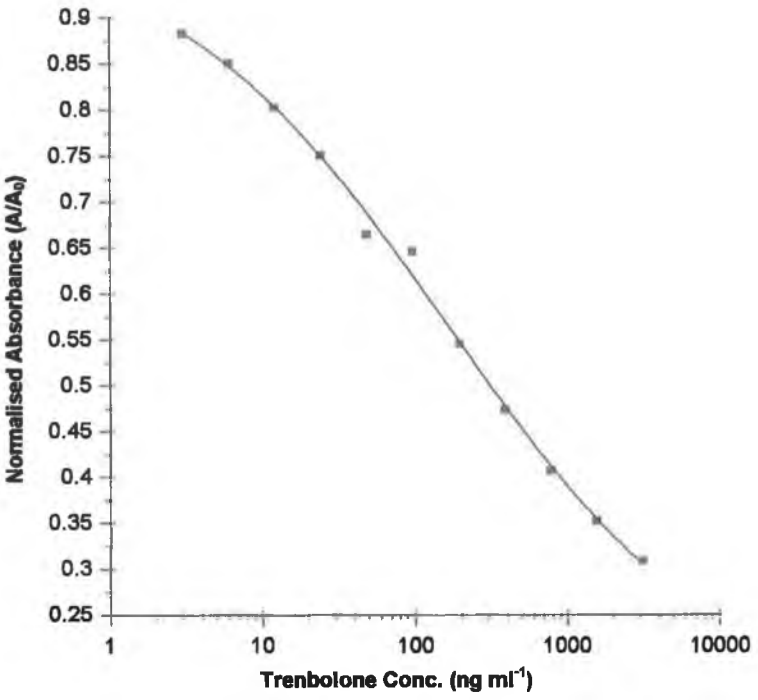


***Figure 3.16** : Inter-day studies on inhibitive ELISAs using the anti-TR Pab, TRAb2 for the detection of free TR in PBS/2% (v/v) EtOH. The inhibition assay uses TR-17-HS-BSA conjugate coated at 1.289 μg ml^{-1} and the antibody at a dilution of 1.983 μg ml^{-1}.*

Table 3.8 : Inter-day CVs and accuracies for TRAb2 inhibition ELISA in PBS. Five sets of eleven standards were analysed over five different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Trenbolone Concentration (ng ml^{-1})	Calculated Mean ± SD, (A/A_0)	Coefficients of variation (CVs), (%)	Accuracy, (%)
3125.00	0.309 ± 0.043	14.04	95.17
1562.50	0.353 ± 0.048	13.67	102.05
781.25	0.408 ± 0.047	11.53	105.27
390.63	0.474 ± 0.048	10.09	103.41
195.31	0.546 ± 0.057	10.49	102.46
97.66	0.647 ± 0.046	7.12	76.40
48.83	0.665 ± 0.065	9.80	127.04
24.41	0.751 ± 0.063	8.45	98.62
12.21	0.802 ± 0.052	6.47	102.25
6.10	0.851 ± 0.052	6.14	95.02
3.05	0.883 ± 0.052	5.94	101.47

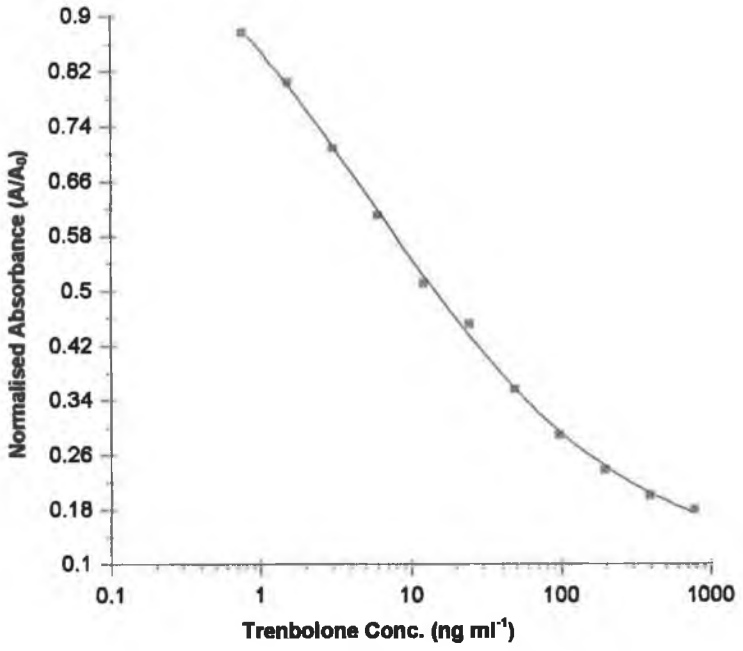


***Figure 3.17** : Inter-day studies on competitive ELISAs using the anti-TR Pab, TRAb2 for the detection of free TR in PBS/2% (v/v) EtOH. The plates were coated with 6.59 μg ml^{-1} solution of TRAb2 and the conjugate TR-17-HS-HRP was used at 0.10 μg ml^{-1}.*

Table 3.9 : Inter-day CVs and accuracies for TRAb2 competition ELISA in PBS/2% (v/v) EtOH. Five sets of eleven standards were analysed over five different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Trenbolone Concentration (ng ml^{-1})	Calculated Mean ± SD, (A/A_0)	Coefficients of variation (CVs), (%)	Accuracy, (%)
781.25	0.181 ± 0.015	8.38	87.71
390.63	0.202 ± 0.011	5.67	106.18
195.31	0.240 ± 0.012	5.18	106.25
97.66	0.290 ± 0.004	1.42	104.50
48.83	0.356 ± 0.009	2.42	100.66
24.41	0.452 ± 0.016	3.60	85.12
12.21	0.513 ± 0.028	5.37	105.79
6.10	0.611 ± 0.030	4.98	101.88
3.05	0.709 ± 0.033	4.60	98.43
1.53	0.805 ± 0.026	3.18	93.27
0.76	0.877 ± 0.028	3.17	98.30

3.2.5 Cross Reactivities of antibodies, TRAb1 and TRAb2

Both antibodies were tested for their ability to recognise other related steroid compounds. The assays were carried out as described in section 2.4.7.8. Table 3.10 shows the percentage cross reactivities that were found.

Table 3.10 : Cross reactivity of the polyclonal antisera to TR and other structurally related steroid hormones and cross-reacting compounds in inhibition ELISAs. [a]Percentage cross reactivity was calculated as (IC_{50}[EE]/IC_{50}[test compound])*100.

Compounds Tested	TRAb1	TRAb2
	% Cross Reactivity[a]	
Trenbolone	100.00	100.00
EE	<0.10	<0.10
Diethylstilbestrol	<0.10	<0.10
α-Estradiol	<0.10	<0.10
β-Estradiol	<0.50	<0.50
Estrone	<0.50	<0.50
Norgestrol	1.00	<0.50
19-Nortestosterone	4.00	2.00
Methyl Testosterone	<0.50	<0.50
Zeranol	<0.10	<0.10
Norethisterone	1.67	0.67

3.2.6 Detection of TR in Spiked Bile Samples

Both ELISA formats were used to detect free TR in spiked bile samples. The bile was prepared as described in section 2.4.7.2 and the TR standards were prepared using the bile as the diluent. The spiked bile was diluted with an equal volume of PBS before being applied to the assay plate. In the competitive assay using bile as a diluent, the TR-17-HS-HRP conjugate was used at a 1/10000 (0.5 $\mu g\ ml^{-1}$) instead of 1/50000 (0.1 $\mu g\ ml^{-1}$). The signals measured when the lower concentration of the HRP conjugate was used were very low and so to enhance the measurable signals the amount of TR-HRP conjugate used was increased. Figure 3.18 shows the different curves obtained when the two concentrations were used. The steeper dose response curve seen with the higher concentration of TR-HRP will give a more accurate assay and so it was chosen as the better dilution to use in the assay. The rest of the ELISA protocols were as previously described in Table 3.1.

Intra-day and inter-day assay variations were investigated as described for the ELISAs using PBS/2% (v/v) EtOH as diluent. Figures 3.19, 3.20, 3.23 and 3.24 show the intra-assay variations along with tables 3.11, 3.12, 3.15 and 3.16 The inter-assay variations are shown in Figures 3.21, 3.22, 3.25 and 3.26 and the accompanying tables 3.13, 3.14, 3.17 and 3.18.

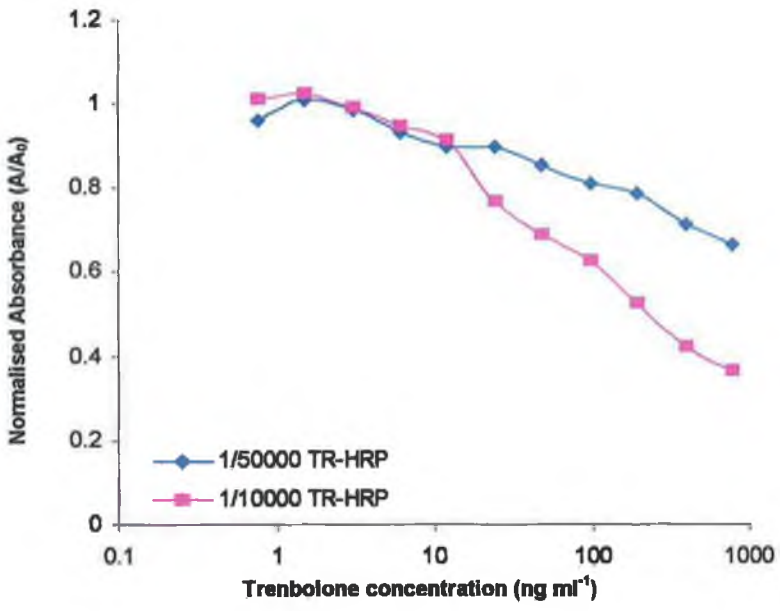


***Figure 3.18** : Effect of raising the amount of competing TR-HRP conjugate when bile is used as the assay diluent. The greater concentration of conjugate (1/10000, 0.5 μg ml^{-1}) shows a steeper curve and so a greater change in absorbance with changes in concentration.*

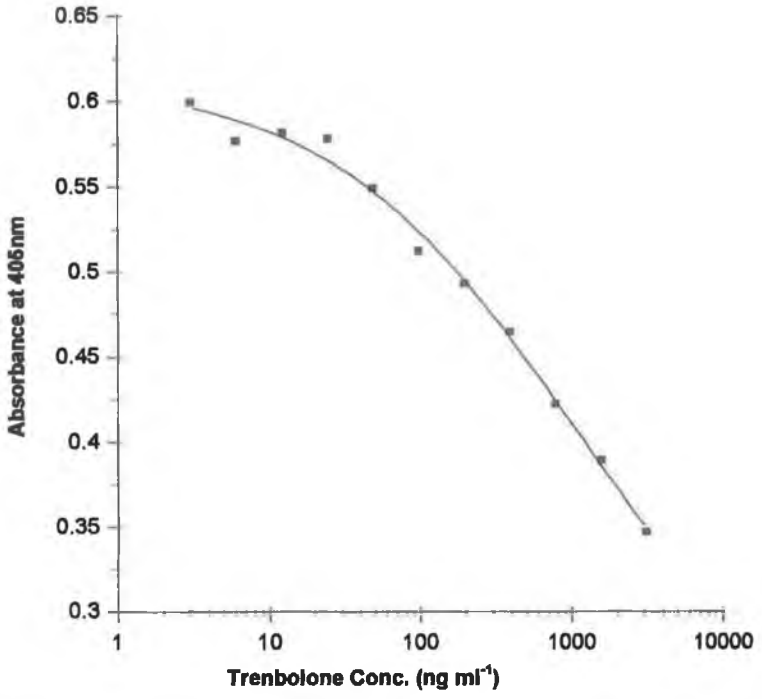


***Figure 3.19** : Intra-day studies on inhibitive ELISAs using the anti-TR Pab, TRAb1 for the detection of free TR in bile. The inhibition assay uses TR-17-HS-BSA conjugate coated at 0.644 μg ml^{-1} and the antibody at a dilution of 0.793 μg ml^{-1}.*

Table 3.11 : Intra-day CVs and accuracies for TRAb1 inhibition ELISA in bile. Five sets of eleven standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Trenbolone Concentration (ng ml^{-1})	Calculated Mean ± SD, (Abs)	Coefficients of variation (CVs), (%)	Accuracy, (%)
3125.00	0.347 ± 0.019	5.38	103.50
1562.50	0.389 ± 0.012	3.14	93.64
781.25	0.423 ± 0.017	4.04	102.31
390.63	0.465 ± 0.011	2.30	92.14
195.31	0.493 ± 0.018	3.70	103.01
97.66	0.512 ± 0.017	3.31	132.98
48.83	0.549 ± 0.016	2.95	93.19
24.41	0.578 ± 0.014	2.43	52.36
12.21	0.582 ± 0.016	2.73	85.36
6.10	0.577 ± 0.025	4.30	224.14
3.05	0.600 ± 0.026	4.35	67.81

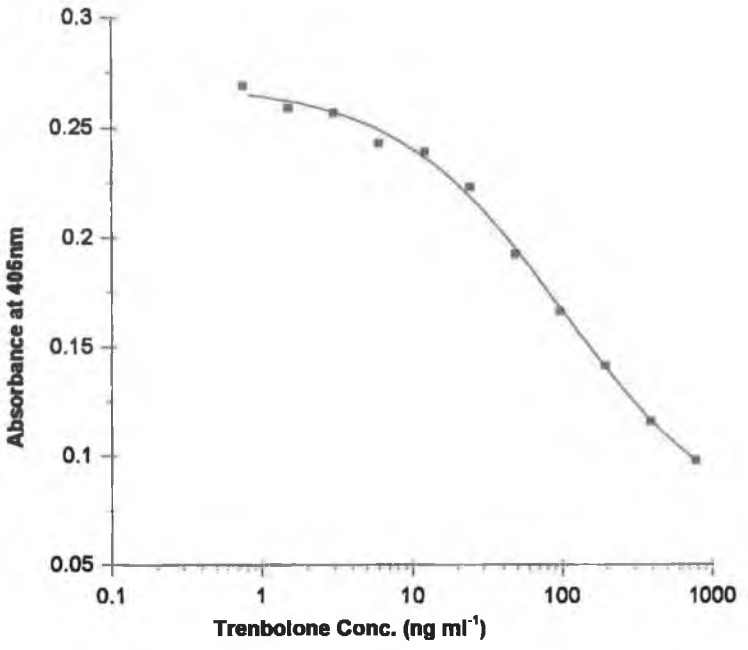


***Figure 3.20** : Intra-day studies on competitive ELISAs using the anti-TR Pab, TRAb1 for the detection of free TR in bile. The plates were coated with 4.76 μg ml^{-1} solution of TRAb1 and the conjugate TR-17-HS-HRP was used at 0.5μg ml^{-1}.*

Table 3.12 : Intra-day CVs and accuracies for TRAb1 competition ELISA in bile. Five sets of eleven standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Trenbolone Concentration (ng ml^{-1})	Calculated Mean ± SD, (Abs)	Coefficients of variation (CVs), (%)	Accuracy, (%)
781.25	0.098 ± 0.003	3.10	98.49
390.63	0.116 ± 0.004	3.20	100.51
195.31	0.141 ± 0.004	2.98	96.51
97.66	0.166 ± 0.003	1.59	102.91
48.83	0.192 ± 0.005	2.40	105.67
24.41	0.223 ± 0.004	1.75	83.84
12.21	0.239 ± 0.006	2.42	86.84
6.10	0.243 ± 0.007	2.77	139.22
3.05	0.256 ± 0.002	0.59	99.09
1.53	0.259 ± 0.005	1.79	144.81
0.76	0.269 ± 0.006	2.38	0.00

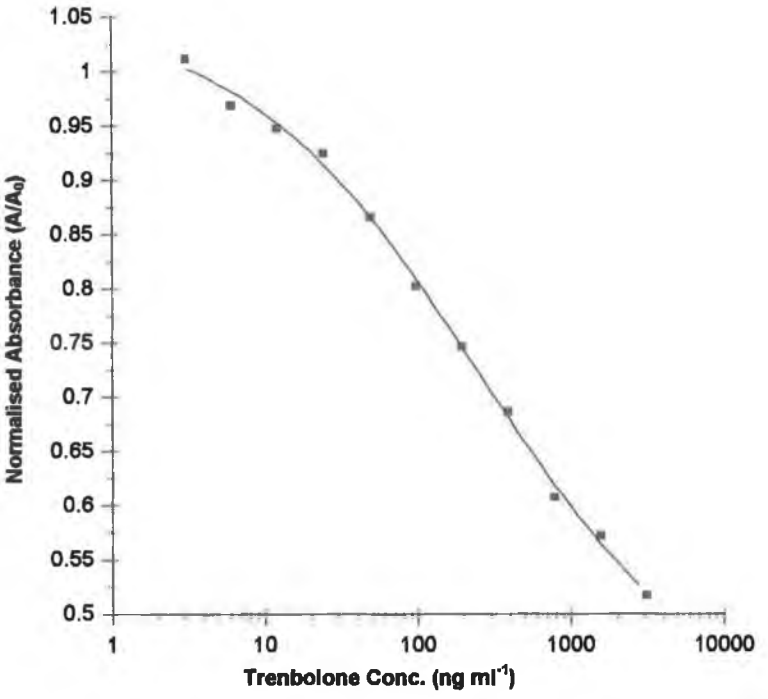


***Figure 3.21** : Inter-day studies on inhibitive ELISAs using the anti-TR Pab, TRAb1 for the detection of free TR in bile. The inhibition assay uses TR-17-HS-BSA conjugate coated at 0.644 μg ml^{-1} and the antibody at a dilution of 0.793 μg ml^{-1}.*

Table 3.13 : Inter-day CVs and accuracies for TRAb1 inhibition ELISA in bile. Five sets of eleven standards were analysed over five different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Trenbolone Concentration (ng ml^{-1})	Calculated Mean ± SD, (A/A_0)	Coefficients of variation (CVs), (%)	Accuracy, (%)
3125.00	0.518 ± 0.097	18.72	102.32
1562.50	0.572 ± 0.071	12.38	89.16
781.25	0.608 ± 0.109	17.85	112.95
390.63	0.687 ± 0.109	15.80	91.96
195.31	0.747 ± 0.108	14.51	96.84
97.66	0.803 ± 0.126	15.68	104.36
48.83	0.866 ± 0.115	13.32	96.29
24.41	0.925 ± 0.092	9.96	78.90
12.21	0.947 ± 0.107	11.27	103.74
6.10	0.969 ± 0.078	8.03	129.23
3.05	1.012 ± 0.066	6.51	59.87

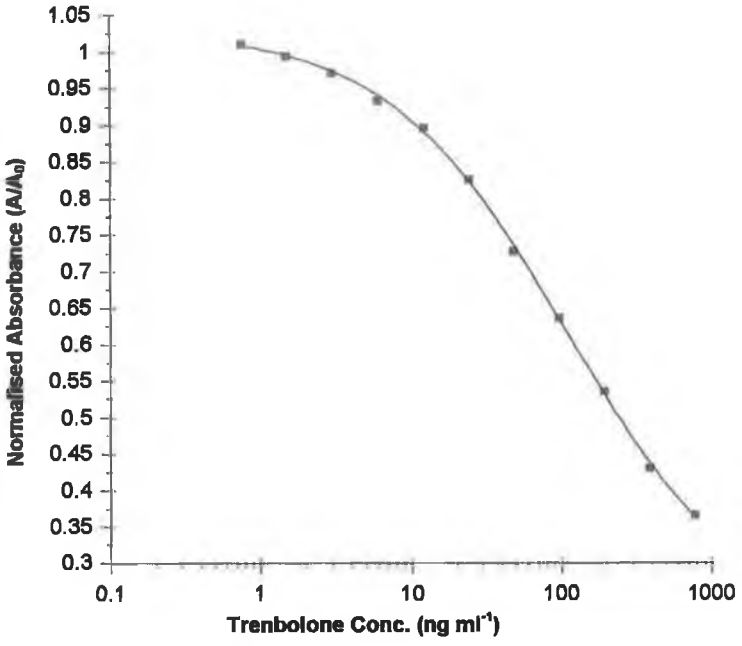


***Figure 3.22** : Inter-day studies on competitive ELISAs using the anti-TR Pab, TRAb1 for the detection of free TR in bile. The plates were coated with 4.76 μg ml^{-1} solution of TRAb1 and the conjugate TR-17-HS-HRP was used at 0.5μg ml^{-1}.*

Table 3.14 : Inter-day CVs and accuracies for TRAb1 competition ELISA in bile. Five sets of eleven standards were analysed over five different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Trenbolone Concentration (ng ml^{-1})	Calculated Mean ± SD, (A/A_0)	Coefficients of variation (CVs), (%)	Accuracy, (%)
781.25	0.365 ± 0.021	5.65	97.31
390.63	0.430 ± 0.016	3.77	106.48
195.31	0.536 ± 0.017	3.24	96.76
97.66	0.636 ± 0.024	3.69	98.44
48.83	0.727 ± 0.029	3.97	105.66
24.41	0.826 ± 0.046	5.52	96.69
12.21	0.897 ± 0.044	4.89	92.77
6.10	0.934 ± 0.037	3.92	110.24
3.05	0.972 ± 0.027	2.74	102.62
1.53	0.995 ± 0.037	3.70	98.43
0.76	1.011 ± 0.020	1.98	80.52

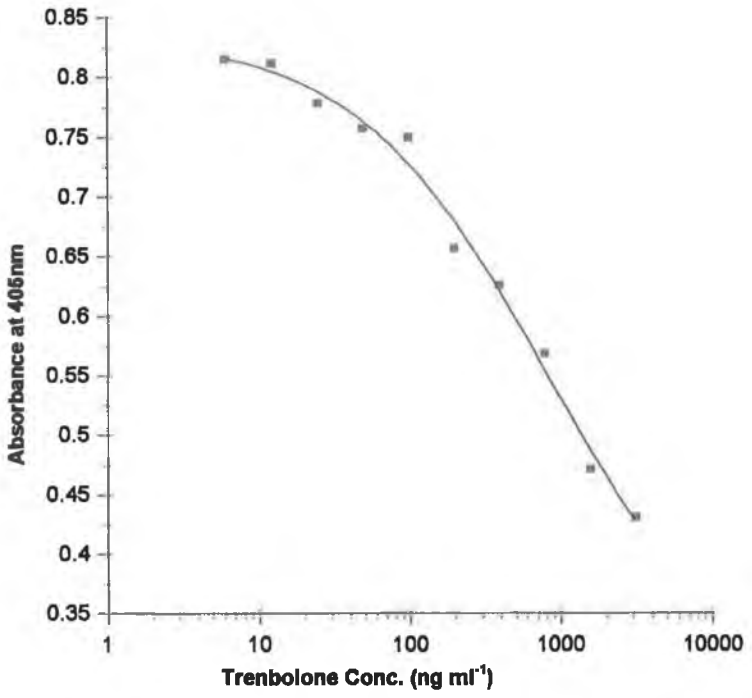


***Figure 3.23** : Intra-day studies on inhibitive ELISAs using the anti-TR Pab, TRAb2 for the detection of free TR in bile. The inhibition assay uses TR-17-HS-BSA conjugate coated at 1.289 μg ml^{-1} and the antibody at a dilution of 1.983 μg ml^{-1}.*

Table 3.15 : Intra-day CVs and accuracies for TRAb2 inhibition ELISA in bile. Five sets of eleven standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Trenbolone Concentration (ng ml^{-1})	Calculated Mean ± SD, (Abs)	Coefficients of variation (CVs), (%)	Accuracy, (%)
3125.00	0.431 ± 0.017	3.99	94.13
1562.50	0.472 ± 0.022	4.66	118.16
781.25	0.569 ± 0.017	2.99	86.47
390.63	0.626 ± 0.006	0.92	94.49
195.31	0.657 ± 0.017	2.66	132.96
97.66	0.750 ± 0.013	1.78	66.24
48.83	0.758 ± 0.019	2.49	112.42
24.41	0.779 ± 0.025	3.21	132.88
12.21	0.812 ± 0.011	1.35	65.25
6.10	0.815 ± 0.010	1.23	105.33

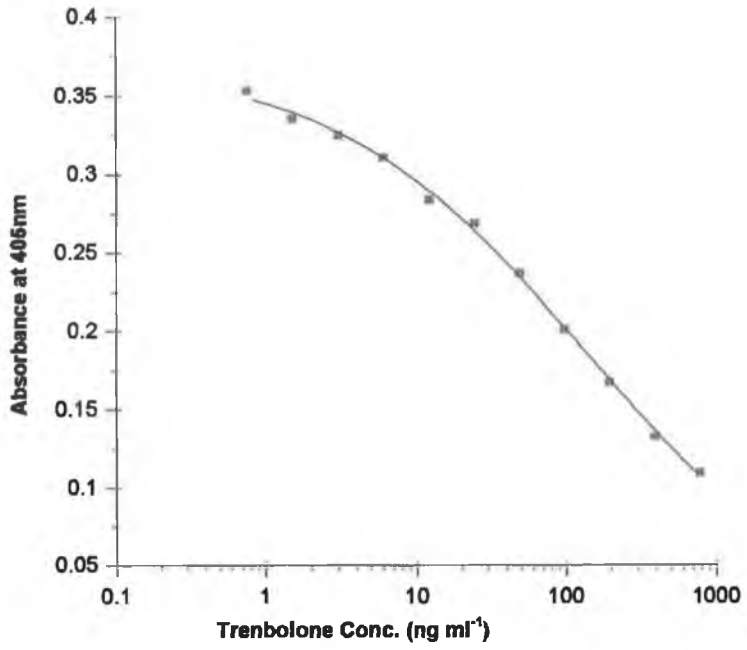


***Figure 3.24** : Intra-day studies on competitive ELISAs using the anti-TR Pab, TRAb2 for the detection of free TR in bile. The plates were coated with 6.59 μg ml^{-1} solution of TRAb2 and the conjugate TR-17-HS-HRP was used at 0.5μg ml^{-1}.*

Table 3.16 : Intra-day CVs and accuracies for TRAb2 competition ELISA in bile. Five sets of eleven standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Trenbolone Concentration (ng ml^{-1})	Calculated Mean ± SD, (Abs)	Coefficients of variation (CVs), (%)	Accuracy, (%)
781.25	0.110 ± 0.002	1.38	93.41
390.63	0.133 ± 0.004	2.64	108.52
195.31	0.167 ± 0.004	2.18	102.43
97.66	0.202 ± 0.005	2.26	100.77
48.83	0.237 ± 0.006	2.48	93.97
24.41	0.269 ± 0.006	2.22	88.56
12.21	0.284 ± 0.006	2.12	116.16
6.10	0.311 ± 0.010	3.17	96.69
3.05	0.325 ± 0.011	3.25	109.00
1.53	0.336 ± 0.012	3.47	127.76
0.76	0.353 ± 0.009	2.52	66.44

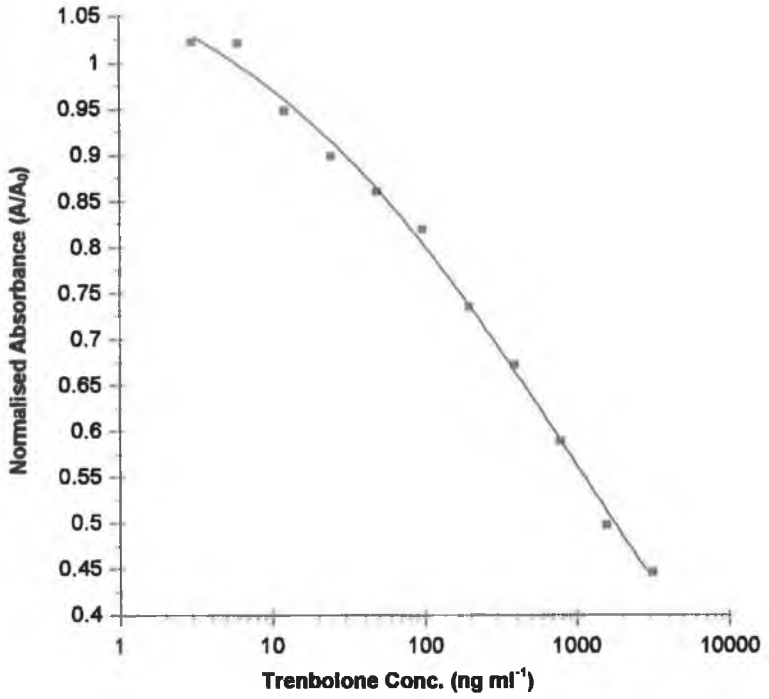


***Figure 3.25** : Inter-day studies on inhibitive ELISAs using the anti-TR Pab, TRAb2 for the detection of free TR in bile. The inhibition assay uses TR-17-HS-BSA conjugate coated at 1.289 μg ml^{-1} and the antibody at a dilution of 1.983 μg ml^{-1}.*

Table 3.17 : Inter-day CVs and accuracies for TRAb2 inhibition ELISA in bile. Five sets of eleven standards were analysed over five different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Trenbolone Concentration (ng ml^{-1})	Calculated Mean ± SD, (A/A_0)	Coefficients of variation (CVs), (%)	Accuracy, (%)
3125.00	0.447 ± 0.051	11.32	91.55
1562.50	0.498 ± 0.050	9.98	114.17
781.25	0.590 ± 0.079	13.43	99.35
390.63	0.672 ± 0.089	13.29	92.86
195.31	0.735 ± 0.086	11.64	100.50
97.66	0.819 ± 0.095	11.65	81.88
48.83	0.860 ± 0.107	12.42	100.95
24.41	0.899 ± 0.086	9.55	121.20
12.21	0.947 ± 0.072	7.58	117.74
6.10	1.021 ± 0.066	6.43	55.94
3.05	1.021 ± 0.090	8.85	111.39

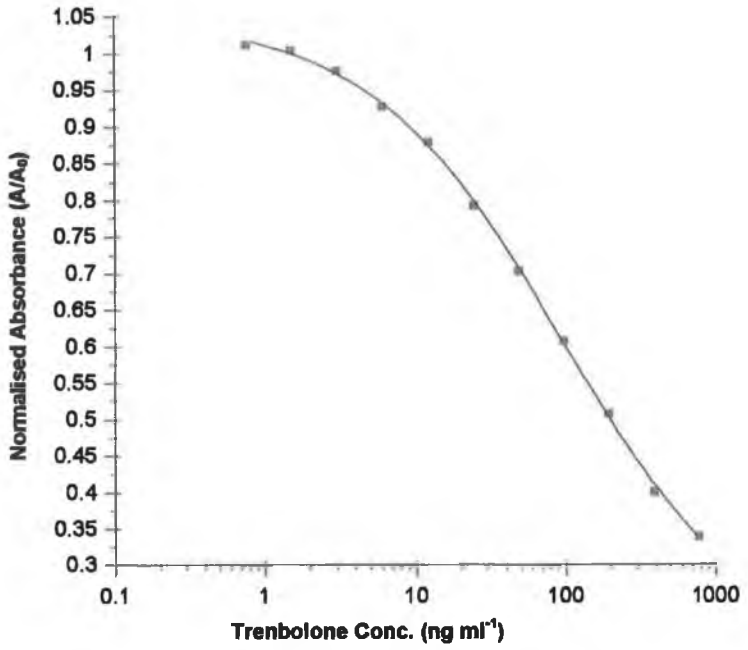


***Figure 3.26** : Inter-day studies on competitive ELISAs using the anti-TR Pab, TRAb2 for the detection of free TR in bile. The plates were coated with 6.59 μg ml^{-1} solution of TRAb2 and the conjugate TR-17-HS-HRP was used at 0.5μg ml^{-1}.*

Table 3.18 : Inter-day CVs and accuracies for TRAb2 competition ELISA in bile. Five sets of eleven standards were analysed over five different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Trenbolone Concentration (ng ml^{-1})	Calculated Mean ± SD. (A/A_0)	Coefficients of variation (CVs), (%)	Accuracy, (%)
781.25	0.340 ± 0.024	7.02	96.37
390.63	0.400 ± 0.018	4.52	108.82
195.31	0.507 ± 0.028	5.57	95.89
97.66	0.608 ± 0.022	3.59	97.13
48.83	0.702 ± 0.021	3.01	102.88
24.41	0.793 ± 0.017	2.14	105.28
12.21	0.879 ± 0.042	4.79	95.70
6.10	0.928 ± 0.025	2.70	106.51
3.05	0.976 ± 0.035	3.53	93.61
1.53	1.005 ± 0.031	3.12	87.46
0.76	1.012 ± 0.018	1.73	133.01

3.3 Discussion

Trenbolone has no chemical side groups through which it could be easily conjugated. To facilitate the production of conjugates for use as both immunogens and as ELISA assay reagents it was decided to produce the trenbolone-17-hemisuccinate (TR-17-HS) derivative of trenbolone. The two major metabolites of trenbolone acetate are 17β- and 17α- trenbolone, differing in the orientation about the 17-position only. To facilitate the production of an antibody that would bind to both epimers the conjugate was constructed through this position.

This hapten has been used previously to produce polyclonal antibodies specific for trenbolone (van Ginkel *et al.*, 1988). Trenbolone was reacted with succinic anhydride using pyridine as a catalyst. After the product was extracted and the excess succinic anhydride was removed the product was sent for analysis by nominal mass spectrometry. This confirmed the presence of a molecule of weight 368AMU, corresponding to an alkalinated derivative of TR-17-HS, (appendix B).

This derivative was then used to produce conjugates for use as both immunogens and as assays reagents. The majority of the conjugates were produced using mixed anhydride chemistry. However, the THY conjugate was produced using the NHS ester method. Both of these procedures are described in sections 2.4.2. The spectrum of each of the conjugates was analysed. TR shows a peak at 348 nm and the presence of this peak in the post-dialysed conjugate implies the presence of bound TR on the carrier protein. During dialysis if the TR was not bound to the protein it would have been free to pass through the dialysis membrane, which had a molecular weight cut off point of 10,000Da. Each of the TR-17-HS conjugates showed the presence of the peak at 348nm, which is characteristic of this derivative and not of the carrier proteins.

After initial immunisation experiments it was decided to use TR-17-HS-THY as the conjugate for antibody production. The other potential candidate, an OVA conjugate, was shown not to induce specific antibody production in mice. The sera produced from the immunisation using the THY conjugate was able to recognise TR but it did not, however, show specificity for the OVA conjugate.

This OVA conjugate was not used in ELISA and fresh conjugates were made for use in the recombinant antibody techniques, (Chapter 5).

New Zealand White rabbits were immunised with the TR-17-HS-THY conjugate and the resulting polyclonal antibodies were purified and characterised as described in section 2.4.6. The initial purification was carried out by saturated ammonium sulphate precipitation and the resulting mixture was subjected to protein G affinity chromatography for further purification. Figure 3.6 shows the elution profile from the protein G affinity column when low pH elution was used. The fractions were analysed for their protein content by their absorbance reading at 280nm. All the fractions with significant amounts of protein, above 0.15 absorbance units, were pooled and dialysed.

The success of the purification is shown by the purity of the final sample. The SDS-PAGE, (Figure 3.7), shows the contents of this sample and that of the crude serum and the SAS purified fraction. Only two bands, corresponding to the dissociated heavy and light chains of the antibodies, are visible in the purified fraction whereas the other samples show many other proteins.

The antibodies were applied to inhibitive and competitive ELISA formats, for the detection of free TR in both PBS/2% (v/v) EtOH and bile. The competitive assay uses a competition between free drug in a sample and labelled drug for binding to an immobilised specific antibody. The inhibitive format utilises the inhibiting effect of free drug in a sample to prevent the binding of added specific antibody to the immobilised drug conjugate on the plate surface. In both assays the measured signal is inversely proportional to the amount of free drug present in the sample.

To allow these assays to function effectively it is imperative that the parameters for the assays are optimised. The assays were optimised using non-competitive ELISAs; i.e. no free drug was used. From these assays the optimal dilutions of the immunoreagents were chosen for use in the ELISAs, (Table 3.1).

Using these conditions a wide range of concentrations of free TR in PBS/2% (v/v) EtOH, 0.38 - 100,000 ng ml^{-1} were assayed. In these model assays both antibodies showed competition within this range (Figure 3.9). For the competitive ELISA the antibodies showed a detection range of 0.76 - 781 ng ml^{-1}, whereas the inhibition ELISAs showed a higher range of 3.05 - 3,125 ng ml^{-1}. These results were similar regardless of which antibody was used. This implies

that the sensitivities of the antibodies are similar. The optimal ranges for the assays were used to carry out intra and inter-assay analysis of the ELISAs.

The results of this analysis for the detection of free TR in PBS is shown in Section 3.2.4. When using PBS as a diluent both formats and antibodies showed good reproducibility and in most cases excellent agreement with the 4-parameter curve-fitting model. Table 3.19 is a summary of the coefficients of variation found when PBS was used as a diluent.

Table 3.19 : Ranges of % CVs of the standards in ELISAs for the detection of free TR in PBS/2% (v/v) EtOH for the interval of 3.05 - 3,125 ng ml^{-1} in the inhibitive and 0.76 - 781 ng ml^{-1} in the competitive assay formats.

	Inhibitive		Competitive	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
TRAb1	0.76 - 3.96%	2.28 - 9.47%	1.19 - 7.91%	1.54 -12.32%
TRAb2	0.46 - 4.59%	5.94 -14.04%	0.96 - 4.44%	1.42 - 8.38%

These ranges show both the intra-assay and inter-assay reproducibility of the assays. They show that the assays can be used reliably to quantify the amount of free TR contained within a sample. The two assays that have ranges that are over 10% at their upper limit may not be as precise as the others described here. To avoid these errors it may be advantageous to use TRAb1 in the inhibitive assay while using TRAb2 in the competitive ELISA. However, all of the % CVs are below 15%, which is within the tolerated limits for assays of this type (Findlay *et al.*, 2000).

The accuracy of the 4-parameter model to fit the measured data of the ELISA was also investigated. The accuracy of the fit can be determined by comparing the actual TR concentration in the sample with the concentration values obtained when the mean absorbance for each standard was substituted into the curve equation. The acceptable range for the accuracy has been reported to be 80 - 120% for many assays although some workers have suggested a less stringent criteria of a 25% deviation from the norm for antibody based assays (Findlay *et al.*, 2000). Table 3.20 summarises the accuracy ranges for the ELISAs using both antibodies to detect the presence of TR in PBS/2% (v/v) EtOH. The difference in the accuracy levels of the two assay formats is immediately noticeable. The

competitive assay fits the 4-parameter model more closely than the inhibitive assay with all of the accuracy ranges falling well within the 75-125% interval.

Table 3.20 : Accuracy ranges of the standards in ELISAs for the detection of free TR in PBS/2% (v/v) EtOH for the interval of 3.05 - 3,125 ng ml^{-1} in the inhibitive assay and 0.76 - 781 ng ml^{-1} in the competitive.

	Inhibitive		Competitive	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
TRAb1	2.89 - 149.65%	80.42 - 121.08%	88.73 - 122.66%	88.94 - 112.29%
TRAb2	74.42 - 145.58%	76.40 - 127.04%	85.12 - 120.47%	85.12 - 106.25%

The competitive ELISA is 4-fold more sensitive than the corresponding inhibitive ELISA is. This is the same regardless of which antibody was used. This is different to the trend seen with the anti-EE antibodies in Chapter 4. Each of those antibodies shows a distinct detection range that does not alter significantly when the format is changed. Those antibodies were produced with different numbers of booster injections. However, the anti-TR antibodies were produced using identical protocols. This may contribute to the finding of similar sensitivities within assay formats as both animals were exposed to the antigen for the same period.

Neither antibody showed any significant recognition of the estradiols that were tested. This was expected as the immunogen was linked at the 17 position allowing the A-ring more access to the immune system. The A-ring of this compound and the estradiols is distinctly different. The compounds that do cross react with the compounds, i.e. norgestrol, 19-nortestosterone, and norethisterone; all have similar constructs about the A-ring. This implies that the antibodies are directed towards this area of the molecule. These compounds do inhibit the binding of the specific antibody and conjugate in the assay yet they do not do so at high levels. The low level of cross-reaction seen with these antibodies indicates they can be used to develop specific assays for the detection of TR without significant interference from related compounds that may be present in the sample.

The assays were then tested in a real biological matrix. The model system described above worked very well and was able to detect low nanogram amounts of TR but to be of a practical use the assay must be able to deal with the

detection of free TR in a biological matrix. The use of bile as the sample matrix has been discussed previously (section 3.1.4). It was chosen as it is an easy matrix to work with, being a liquid, and it retains steroid residues for a longer period than other matrices. The bile is simply diluted before its application to the assay. This is ample pre-treatment for the determination of TR concentration in spiked samples. For real samples it may be necessary to deconjugate the samples prior to application to the ELISA (Meyer and Hoffmann, 1987). This frees any and all TR that may have become conjugated by the natural processes of the animal's metabolism.

The assay reagents were applied to samples using bile/PBS as a standard diluent. In the competitive ELISAs the amount of labelled drug was seen to be insufficient at the level it was used at for the PBS assays as the signal became quite low and the dose response curve became shallow. To counter these issues the amount of TR-17-HS-HRP was increased from 0.1 $\mu g\ ml^{-1}$ to 0.5 $\mu g\ ml^{-1}$. The resulting dose response curve is steeper and shows a more accurate assay. The need for more conjugate may indicate that something in the bile has a high affinity for the TR, or that the enzyme is being denatured or becomes inactive by its incubation with the bile. The range of detection of the assays does remain constant when this alteration is made indicating that some of the conjugate is being bound by interfering elements contained in the bile fluid.

The assays have been shown to be reproducible and accurate when bile is used as the sample matrix. The coefficients of variation of the bile assays are all of similar magnitude to the corresponding assays using PBS as the sample diluent, (Sections 3.2.4 and 3.2.6). None of the % CVs exceed the suggested limits of 10% within assays and under 20% between assays.

Table 3.21 : Ranges of % CVs of the standards in ELISAs for the detection of free TR in bovine bile for the interval of 3.05 - 3,125 ng ml^{-1} in the inhibitive assay and 0.76 - 781 ng ml^{-1} in the competitive.

	Inhibitive		Competitive	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
TRAb1	2.30 - 5.38%	6.51 - 18.72%	0.59 - 3.20%	1.98 - 5.65%
TRAb2	0.92 - 4.66%	6.43 - 13.43%	1.38 - 3.47%	1.73 - 7.02%

When bile is used as a diluent the competitive assay retains its reproducibility better than the inhibitive format. One possible reason for this could be the use of an extra antibody, increasing the amount of errors brought to the system. But these errors are not seen in the previous model ELISAs and the bile has no contact with this secondary antibody. These data indicate that the format itself is less reproducible when bile is used as the matrix, that is the antibody - drug binding reaction is more susceptible in this format.

Using bile as a diluent reduces the accuracy of both the inhibitive and competitive assays (Table 3.22). To avoid these inaccuracies it may be necessary to raise the lower limit of the detection range. This will remove standards that are tending towards the asymptotes of the curve model. In this area of the curve small errors in measurement can cause large changes in calculated concentration values leading to very inaccurate determination of the concentration of a sample.

Table 3.22 : Accuracy ranges of the standards in ELISAs for the detection of free TR in bile for the interval of 3.05 - 3,125 ng ml^{-1} in the inhibitive assay and 0.76 - 781 ng ml^{-1} in the competitive.

	Inhibitive		Competitive	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
TRAb1	52.36 - 224.14%	59.87 - 129.23%	0 - 144.81%	80.52 - 110.24%
TRAb2	65.25 - 132.96%	55.94 - 121.20%	66.44 - 127.76%	87.46 - 133.01%

As seen in the PBS assays the accuracies of the competitive assays are superior to those of the inhibitive assays. By limiting the range of detection more accurate assays may be shown. The shorter detection ranges and the accuracy ranges that they represent for the assay that will benefit from a limiting of their detection range are listed in Table 3.23.

Table 3.23 : Detection and accuracy ranges for optimum accuracy of the 4-parameter model fit.

Assay	Intra-/Inter-assay	Detection range	Accuracy Range
TRAb1 Inhibitive	Inter-assay	12 - 3,125 ng ml^{-1}	78.90 - 112.95%
TRAb1 competitive	Inter-assay	3 - 781 ng ml^{-1}	83.84 - 139.22%
TRAb2 competitive	Inter-assay	1.53 - 781 ng ml^{-1}	87.46 - 108.82%

Most of these assays are now within ±25% of the normal value. Unfortunately to achieve assays of these accuracy levels it was required to lower their sensitivity. In combination the % CV and the accuracy levels indicate the usefulness of these assays for the detection of free TR in bovine bile samples.

The assays described in this chapter are for the detection of TR in bovine bile compare well to the published assays for TR. The competitive assays described can quantify a sample in less than 2 hours with minimal sample pre-treatment. They can detect as low as 0.76 ng ml^{-1} in a single sample, which is less than the maximum residue limit for this steroid of 2 ng ml^{-1}, and it needs only 50 µl of bile to complete the assay.

The radioimmunoassay for the detection of TR and TBA in bovine tissue and urine described by Hoffman and Oettel (1976) was able to detect very low amounts (40ρg and 70ρg, respectively) of both steroids. Bile was not tested in this study but the sample pre-treatment was more involved that described here. Even though this assay is more sensitive than the ELISAs described in this chapter for the detection of TR residues, it uses radioisotopes as the labels in the assay. The advantages of using enzyme-labels in place of radioactive labels are listed in section 1.4 and include safer handling and easier disposal. This assay was also carried out in single tubes and not on a 96 well format, which allows for up to 96 assays to be carried out simultaneously. The plate format is more suitable for use as a screening assay as it can handle more samples and it is easier to automate.

The system described by Meyer and Hoffmann (1987) is an assay similar to the competitive ELISAs in this chapter. This assay requires the incubation of the sample along with an alkaline phosphatase-TR tracer and a hormone-specific rabbit antibody in an anti-rabbit IgG-coated well. This incubation step alone takes 18h to complete, followed by 2h for the substrate to develop. The purification procedures used for the different sample matrices are also very time-consuming. The preparation of bile samples involves an overnight hydrolysis step. The amount of conjugated TR has been estimated at only 20% of the total TR present and so this step, which adds a day to the analysis time of a sample, may be superfluous. The assays presented in this chapter provide analysis of bile

samples to a level below that of the maximum residue permitted and it does so in less than 2h giving it many advantages over the this enzyme immunoassay.
Another method for the quantification of trenbolone used specific antibodies for the isolation of 17α-trenbolone and 17β-trenbolone before their separation and detection by HPLC-TLC (van Ginkel *et al.,* 1988). This assay can detect both isoforms of the drug but it requires specialised and expensive equipment. The assay is not as easy to use or as rapid as the analysis of samples using the ELISAs procedures developed here and the limit of detection of this assay was comparable, 0.78 ng ml^{-1} to 0.5 ng ml^{-1} with the off-line TLC. The ELISA also has a higher throughput with a single technician able to assay hundreds of samples per day. The immunoaffinity column clean up followed by HPLC-TLC can analyse up to 20 samples per day. A screening method must have a high throughput capability and the ELISA can provide this. A single technician can analyse many hundreds of samples each day with the ELISA system.
The applications of the assays developed here are numerous. They are very useful as screening methods when a great number of samples must be analysed quickly. These samples may originate from farm inspections or from slaughter houses. An abattoir is the area that the assays are most suitable. An employee with minimal training can analyse hundreds of samples in a small lab with only basic equipment. The capital outlay required for the introduction of this type of testing system is quite low compared to other systems such as the HPLC system described above or even a biosensor-based assay. The assay reagents could also be adapted for use with other sample matrices such as serum or urine to allow for easier sample collection from live animals. If single samples needed to be analysed individually, for example on-site at a farm, it may be possible to utilise a rapid ELISA system to provide quick on the spot results, (see Chapter 5).

In conclusion, trenbolone was derivatised to trenbolone-17-hemisuccinate using succinic anhydride and pyridine as a catalyst. This derivative was conjugated to BSA, OVA, THY and HRP. The THY conjugate was used to immunise rabbits and produce polyclonal antibodies. The polyclonal antibodies were purified and applied to two ELISA formats, competitive and inhibitive. Assays were developed for the detection of free TR in both PBS/2% (v/v) EtOH and bovine bile. These assays were analysed for their reproducibility and their accuracy

when fitted to the 4-parameter model. The cross-reactivity of the antibodies with related steroids was also investigated. The two antibodies produced had very low cross reactivity to most of the compounds tested with only slight recognition of nortestosterone, norethisterone and norgestrol. All assays were capable of detection of low nanogram quantities of free trenbolone in both PBS/2% (v/v) EtOH and bile and the assay compares well and, in some cases, out-performs previously described detection methods.

CHAPTER 4

PRODUCTION AND CHARACTERISATION OF ANTI- ETHYNYL ESTRADIOL (EE) POLYCLONAL ANTIBODIES AND THE DEVELOPMENT OF A BIACORE-BASED ASSAY FOR THE DETECTION OF EE

4.1 Introduction

4.1.1 Ethynyl Estradiol Structure and Function

Ethynyl estradiol (EE) is a very potent synthetic xenobiotic estrogenic compound. It is an analogue of the natural estrogen, 17-β estradiol, which has been ethynylated at the 17 position of the steroid nucleus (Figure 4.1). It has been used extensively as the main estrogenic component of the oral contraceptive pill. This was its first major use and it has continued unabated since the boom of the combined oral contraceptive pill that occurred in the 1970's (Agasan *et al.*, 1994). Today, this is still its most common usage.

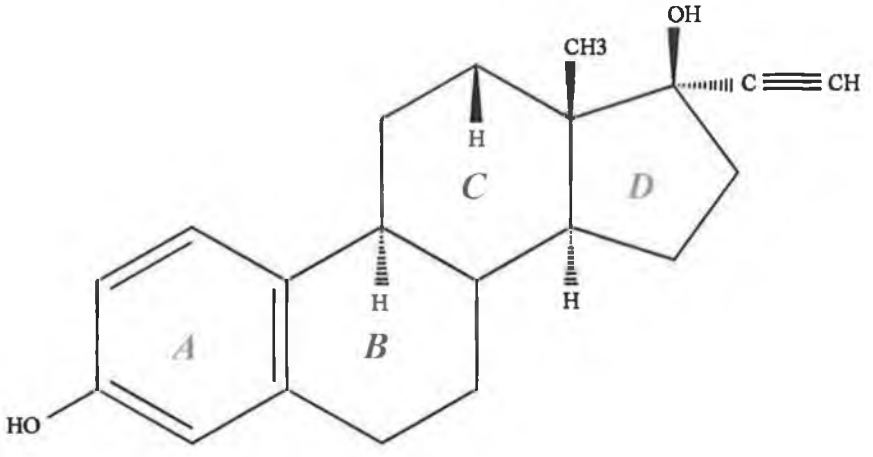


***Figure 4.1** : Chemical structure of ethynyl estradiol, (17-α ethynylestra - 1,3,5 (10) - triene - 3, 17 β - diol). Relative molecular weight of 296.4. The four rings are labelled A, B, C and D in accordance with the conventions of standard steroidal structure nomenclature.*

Ethynyl estradiol is more estrogenic than its natural analogue 17-β estradiol (Gutendorf and Westendorf, 2001) and, as such, it is useful as a growth promoter in animals. Anabolic steroids act by increasing the feed to muscle ratio of the animals, as discussed previously in this dissertation, (section 1.3.3.1). Due to its potency, it is important that residues from this compound are not found in the final meat products. Some natural hormones pose risks to the end consumer, particularly in the case of prepubertal children (Opinion of the Scientific Committee on Veterinary Measures relating to Public Health, 2002).

The synthetic estrogens have also been linked to increase in breast and vaginal cancer, (estrogen dependant cancers), endometriosis, testicular and prostate cancer and reduced sperm counts in humans (Folmar *et al.*, 2000). The levels of natural, synthetic and xenoestrogens found in some surface waters and even in drinking

water supplies have been found at levels high enough to produce reproductive abnormalities.

Natural estrogens are short-lived within the body and are broken down efficiently in the liver whereas synthetic estrogens are more stable and tend to remain in the body for a longer period (Tapiero *et al.*, 2002). EE has been shown to have a 100-fold higher liver activity than estradiol, due to the fact that it is more metabolically stable in humans than the natural steroids (Diel and Michna, 1998). This can lead to a toxic build up of these substances over a period of time if contaminated foodstuffs are being consumed.

To protect the consumer the European Union (EU) has banned the use of these fattening agents in meat producing animals under EU directives 81/602/EEC and 96/22/EC. This has lead to the need for testing programmes within the European Union to ensure that the animal products being consumed are free from these residues. The links to the health problems described previously are not conclusive and the EU has a duty to prevent these substances from entering the food chain until they are proven safe.

4.1.2 Detection of EE

Many methods have been described for the detection of EE in a number of matrices using both polyclonal and monoclonal antibodies. A radioimmunoassay was developed using a monoclonal antibody to detect EE in plasma and buffer (Agasan *et al.*, 1994). This assay had low detection limits of 10ρg ml^{-1} in buffer and 43ρg ml^{-1} in plasma but the samples were extracted before use in the assay using dichloromethane. This added another step to the assay and it required time and equipment. The recovery was also not optimal as only 78% of the steroid was recovered from the spiked samples. An ether extraction method was tested but it was shown to have high backgrounds and higher variation between the assays than the dichloromethane protocol, even though it had higher recovery efficiencies of up to 96%. Throughout this assay the coefficients of variation were consistently above 10% even when buffer was used. To be of practical use an assay must be reproducible and these results show that this assay system has a high level of inherent errors. Despite this assay using a mixture of 3 anti-EE monoclonal antibodies the cross reactivities are substantial, with up to 100% cross-reactivity

against norethynodrel and high levels detected against norethisterone, EE-3-sulphate, 19-nortestosterone and levonorgestrel.

An enzyme immunoassay was described for EE (Turkes *et al.*, 1981), using polyclonal sera with a sensitivity of 2ρg per assay tube, (10ρg ml^{-1}). This polyclonal antibody showed significant cross-reaction with a number of related steroids so to avoid this problem a pre-absorption step was introduced. This allowed the extraction of the estrogens from the plasma sample but it also added another overnight incubation to the assay so that at least 2 days elapsed before a sample could be analysed.

An ELISA method has also been described for EE and compared to GC-MS analysis (Sawaya *et al.*, 1998b). This paper outlines the use of a commercial kit for the quantification of EE in both chicken muscle and sheep urine. Both matrices were subjected to lengthy extraction procedures before they were used in the assay. The average recovery of EE was 85 ± 11.8%. This study suggested an ELISA cut off point of 0.3 ng ml^{-1} to prevent false positive results using this method of analysis.

This chapter describes an assay for the detection of EE in both buffer and bile using polyclonal antibodies with low cross-reactivities with minimal pre-treatment of the sample.

4.1.3 Acceptable Limits

The maximum residue limits for anabolic steroids in general are difficult to set due to the presence of endogenous steroid in the animal itself. Some testing methods have circumvented this problem by setting action limits above which the animal is assumed to have been dosed (Draisci *et al.*, 2000). This problem is not as prevalent where the detection of synthetic steroids is concerned. By nature of the fact that these compounds are synthetic and are, therefore, not found naturally, any confirmed presence indicates that the animal was dosed. It is, therefore, imperative that any assay system for the analysis of EE be very specific and not be liable to interference from endogenous steroids. In practice, tolerance limits of between 0.5-2 ng ml^{-1} depending on the matrix have been suggested for the presence of this drug (Johansson and Hellenäs, 2001).

4.1.4 Choice of Matrix

EE has been found to be excreted rapidly into bile (Grabowski and Park, 1984). This results in very little (0.02%) EE being found in serum 18h after administration. The circulating levels in animals would therefore only be significant while the animal is being dosed. Bile is a good choice of matrix to avoid the problems associated with the rapid disappearance of EE from the plasma. It also contains large quantities of EE and, hence, it takes a significant amount of time for it to be cleared.

The metabolism of EE in the bile of rats has been investigated. EE is metabolised mainly to glucuronides but males also produced arylsulphates, all of which were found to be linked by hydroxy or methoxy groups at the 2 position (Maggs *et al.*, 1983). This indicates that bile may require an enzymatic deconjugation prior to any analysis for free EE. However, due to the fact that the derivatives are in the A-ring of the steroid nucleus an antibody with specificity for the D-ring of the steroid may supply an assay that could avoid this problem.

4.1.5 BIAcore Technology and Surface Plasmon Resonance (SPR)

The biosensor-based assay described in this chapter uses a BIAcore 3000. BIAcore covers an array of instruments produced by the company that all utilise the same sensing technology. The BIAcore 3000 uses a refractive index sensitive optical transducer based on surface plasmon resonance for mass detection (Quinn *et al.*, 1999). The mass detection occurs at a biospecific surface that is composed of a carboxymethylated dextran surface onto which biomolecules, such as antigens or antibodies, can be linked. The binding of any compounds to the ligands immobilised on this surface can be detected and quantified using a phenomenon called surface plasmon resonance.

Surface plasmon resonance (SPR) is a phenomenon dependant on the incoming light and the refractive index of the materials it is passing through and arises from the total internal reflection of light at a metal film-liquid interface (Fägerstam *et al.*, 1992). The production of an SPR angle is represented in Figure 4.2. For this explanation, SPR will be discussed in relation to its use in the BIAcore biosensor.

The sensing platform consists of a glass prism with a sensor chip docked against one side. The sensor chip has a thin gold layer, which lies next to the prism, and

has a carboxymethylated hydrogel linked to it through an inert linker layer on the opposite side (Wong *et al.,* 1997). When light is passed through an optically dense medium (the glass prism) at an angle greater than the critical angle, upon reaching an interface with the medium of a lower refractive index (e.g. buffer) the light is totally internally reflected back into the prism (Fägerstam *et al.,* 1992). Even though the light is totally reflected a small electromagnetic field component of the light called an evanescent wave penetrates into the lower refractive index medium to a distance close to one wavelength.

If the interface between the two media is coated with a thin metal layer, such as gold in the case of BIAcore, and the light is monochromatic and polarised the evanescent wave will interact with the electron cloud (plasmons) at the surface of the metal film (Quinn and O'Kennedy, 1999). Light energy is then lost to the metal film when surface plasmon resonance occurs and the intensity of the reflected light shows a decrease. This dip in intensity will occur at a sharply defined angle, SPR angle, which is dependent on the refractive index of the media on the side of the evanescent wave, once all other parameters are kept constant (Sternesjö *et al.,* 1995).

If the refractive index changes at the surface out to a distance of about 1μm, a change in the angle of the intensity dip will be seen. This is measured and it forms the basis for the response units measured in BIAcore technologies, (Figure 4.3). The measured change in refractive index can be caused by either a change in bulk refractive index or by a change in the mass concentration at a surface of the chip. The refractive index will be affected by something binding to the dextran hydrogel layer or something being removed from the surface (Wong *et al.,* 1997). All of these changes can be monitored in real-time using the SPR phenomenon. Since the SPR response is related to the mass change on the sensing surface it is usual to immobilise the smaller of the binding partners to maximise the SPR signal (Gomes and Andreu, 2002).

4.1.5.1 Biosensor-based assays

The format used in the BIAcore assay is similar to the inhibitive ELISA described in section 3.1.4. The conjugate is linked to the dextran hydrogel using EDC/NHS chemistry. The sample and the specific antibody are then mixed and allowed to

come to equilibrium outside the machine. These samples are then passed over the surface of the chip and the amount of antibody binding was recorded. The antibody was then removed from the surface with a regeneration solution ready to start a new binding cycle.

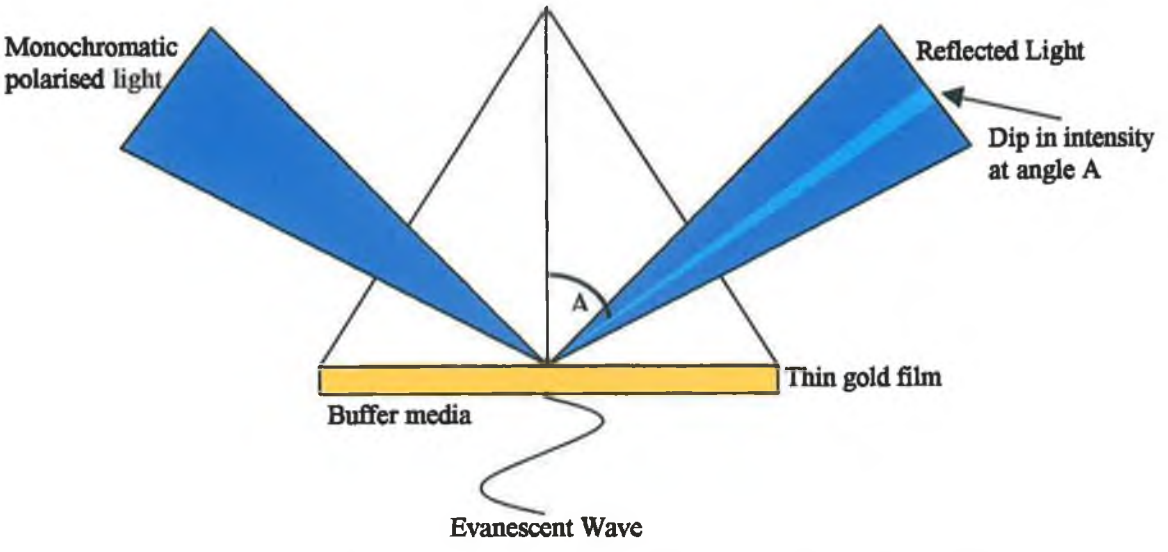


***Figure 4.2** : Diagram of the generation of the SPR signal. When the media is at a certain refractive index, the dip in light intensity due to the evanescent wave producing a surface plasmon is found at angle A. As the refractive index changes so does the SPR angle. This is monitored and converted to resonance units by BIAcore software to allow for ease of analysis.*

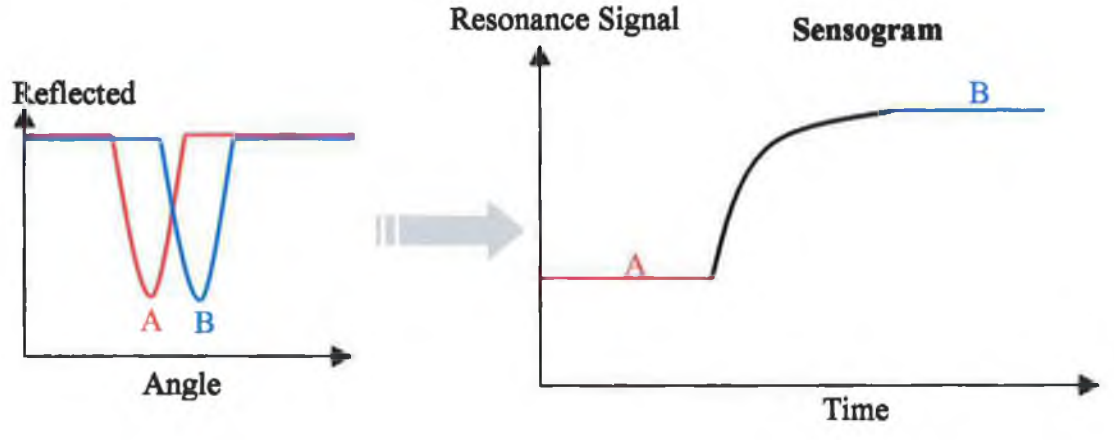


***Figure 4.3** : The intensity dips seen at different refractive indices. When the SPR angle is at angle A, a response is seen on the sensogram corresponding to the number of resonance units A. When the SPR angle shifts to B after, for example a binding cycle, a resonance signal of B is seen on the sensogram.*

4.1.6 Detection of EE using Biosensors

Johansson and Hellenäs (2001), have described a sensitive assay for the detection of EE using a BIAcore 2000, a polyclonal antibody preparation with the EE directly immobilised on the sensorchip. The drug was directly immobilised onto the surface of a BIAcore CM5 chip using an amino-modified surface and EDC/NHS chemistry. The advantage of this method of linkage is that the surface is extremely stable and can withstand many regeneration cycles. When a conjugate, consisting of a hapten and carrier, is subjected to repeated cycles of regeneration with 10-100mM NaOH or HCl, 1-20% (v/v) acetonitrile, high salt concentrations or ethanolamine it may often be less stable than the hapten linked covalently direct to the chip surface.

The anti-EE polyclonal antibody was diluted in double strength running buffer and mixed with the sample or standards. This assay showed excellent sensitivity with an IC_{50} of 0.35 ng ml^{-1} for the drug in buffer. This study proves the usefulness of both the antibody and the biosensor for the detection of free EE in buffer, but the reproducibility and accuracy of the assays were not investigated. The antibodies were also not tested in any biological matrices to show the resistance of the antibody to interference from the components of biological matrices.

This chapter describes the production, purification and characterisation of two polyclonal antibodies raised against EE-6-CMO-OVA. These antibodies were applied to the detection of EE using two ELISA formats and a biosensor-based assay. These assays were investigated for both their precision and accuracy. The test systems were then applied to the determination of EE in bovine bile.

4.2 Results

4.2.1 Preparation of Ethynyl Estradiol conjugates

The conjugates were prepared from the EE derivative EE-6-CMO (Figure 4.4) using either mixed anhydride or EDC/NHS chemistry as described in section 2.4.2. This derivative has been found to produce more specific antiserum compared to the antiserum raised when the 17 position is used to link estrogenic steroids (Lindner *et al.*, 1972; Wright *et al.*, 1973).

Conjugate	Linking Chemistry
EE-CMO-BSA	EDC/NHS
EE-CMO-OVA	Mixed anhydride
EE-CMO-HRP	Mixed anhydride
EE-CMO-THY	EDC/NHS

A minimum of 20 times excess of steroid to carrier was used in each reaction. Ethynyl estradiol has a peak at 310nm when investigated spectrophotometrically. The spectrum of each of the conjugates was recorded after dialysis and a peak at 310nm indicated the presence of the hapten coupled to the carrier. Each conjugate was then tested in an ELISA system to confirm the linkage of the hapten to the protein carrier.

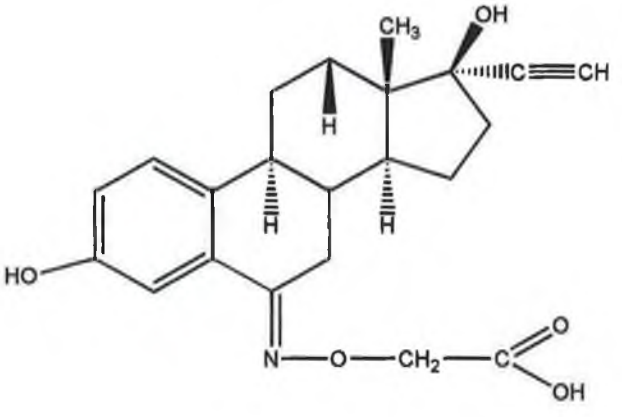


***Figure 4.4** : Structure of ethynyl estradiol-6-carboxymethyl oxime, the derivative used to link carriers to ethynyl estradiol*

4.2.2 Production and purification of rabbit anti-EE-CMO-OVA polyclonal antibodies

The protocol used for the production of the polyclonal antibodies was outlined in section 2.4.4.1. Two rabbits were immunised with 1ml of 0.5 mg ml^{-1} EE-CMO-OVA. The immunoglobulin fraction was isolated from serum after the rabbits were sacrificed. Figure 3.6. shows a typical purification profile of immunoglobulin from a Protein G affinity column. All fractions containing significant amounts of protein were pooled and dialysed. The purified samples were subjected to SDS-PAGE and each fraction was diluted to approximately 1 mg ml^{-1} before loading onto the gel, (Figure 4.5). The lane containing the final affinity-purified solution shows 2 bands corresponding to the heavy and light chains of immunoglobulin as they appear under denaturing conditions. The concentration of the purified IgG was estimated using a BCA protein assay, with an IgG standard. The polyclonal antibody from the first rabbit was designated EEAb1 and had a concentration of 4.01 mg ml^{-1}. The polyclonal antibody from the second rabbit, EEAb2, had a concentration of 3.42 mg ml^{-1}.

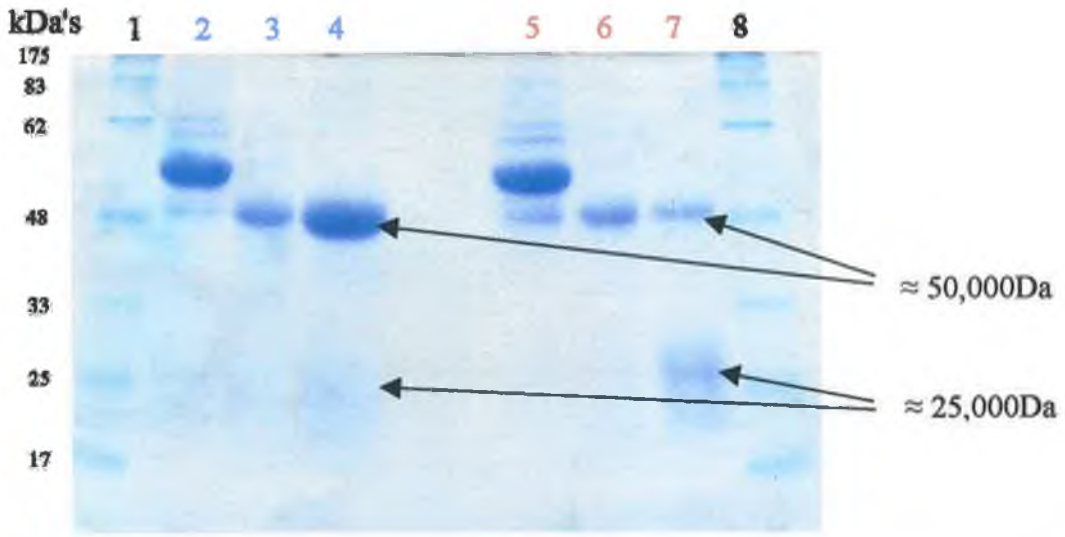


***Figure 4.5** : Coomassie stained SDS-PAGE gel with protein markers in lane 1 and 8. Lane 2: serum from rabbit 1, lane 3: dialysed SAS cut of rabbit 1 serum, lane 4: protein g affinity-purified IgG fraction (EEAb1) and lane 5: serum from rabbit 2, lane 6: dialysed SAS cut of rabbit 2 serum, lane 7: protein G affinity-purified IgG fraction (EEAb2).*

4.2.3 Optimisation of parameters for the inhibitive and competitive ELISAs for the detection of free EE

The non-competitive checkerboard ELISAs were carried out as described in sections 2.4.7 to determine the optimal coating concentration either of EE-6-CMO-BSA conjugate or anti-EE antibody, depending on the assay format, and the optimal competing solution. The lowest coating concentration that showed a steep dose response curve with strong measurable signals in the assay was chosen as the optimum. The optimal antibody dilution, (inhibitive ELISA) or HRP conjugate dilution (competitive ELISA) was also elucidated from the results of these assays. At the coating concentration chosen above, the amount of either antibody or EE-HRP conjugate that gave approximately 70% of the maximum signal was used in further assays. An example of the results of these assays is shown in Figure 4.6. The coating and competing concentrations used in the ELISAs are listed in Table 4.1.

The optimal concentrations established were used in the subsequent intra- and inter-day analysis of the two ELISA formats. A wide range of standards (0.38 - 100,000 ng ml^{-1}) was prepared and using the optimal coating and competing concentrations, the working ranges of the assays were ascertained, (Figure 4.7).

The detection ranges for the assays are listed below.

- EEAb1 Inhibitive 3.1 - 50,000 ng ml^{-1}
- EEAb1 Competitive 1.5 - 25,000 ng ml^{-1}
- EEAb2 Inhibitive 1.5 - 50,000 ng ml^{-1}
- EEAb2 Competitive 48.8 - 50,000 ng ml^{-1}

These ranges and the optimised parameters were used to conduct reproducibility studies on the ELISAs.

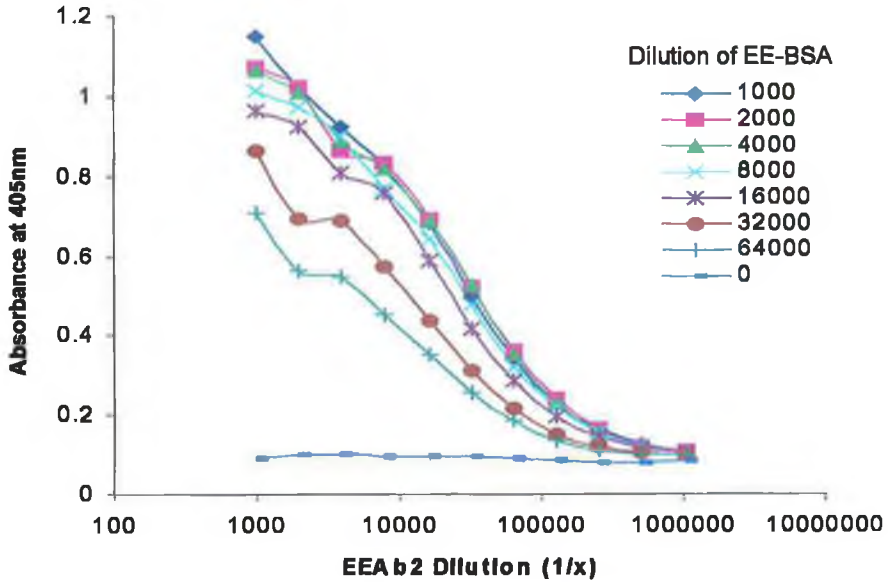


***Figure 4.6** : Checkerboard ELISA for the determination of optimum EE-6-CMO-BSA conjugate coating dilution and EEAb2 antibody dilution for an inhibitive ELISA. Dilutions of 1/16,000 EE-6-CMO-BSA (0.2 μg ml^{-1}) and 1/12,000 EEAb2 (0.57 μg ml^{-1}) were chosen as the optimum assay parameters.*

Table 4.1 : Optimal coating and competing concentrations for competitive and inhibitive ELISAs using both anti-EE polyclonal antibodies.

Assay	Coating Solution	Competing Solution
EEAb1 Inhibitive	0.16 μg ml^{-1} EE-CMO-BSA	0.50 μg ml^{-1} EEAb1
EEAb1 Competitive	2 μg ml^{-1} EEAb1	0.119 μg ml^{-1} EE-CMO-HRP
EEAb2 Inhibitive	0.2 μg ml^{-1} EE-CMO-BSA	0.57 μg ml^{-1} EEAb2
EEAb2 Competitive	1.71 μg ml^{-1} EEAb2	0.478 μg ml^{-1} EE-CMO-HRP

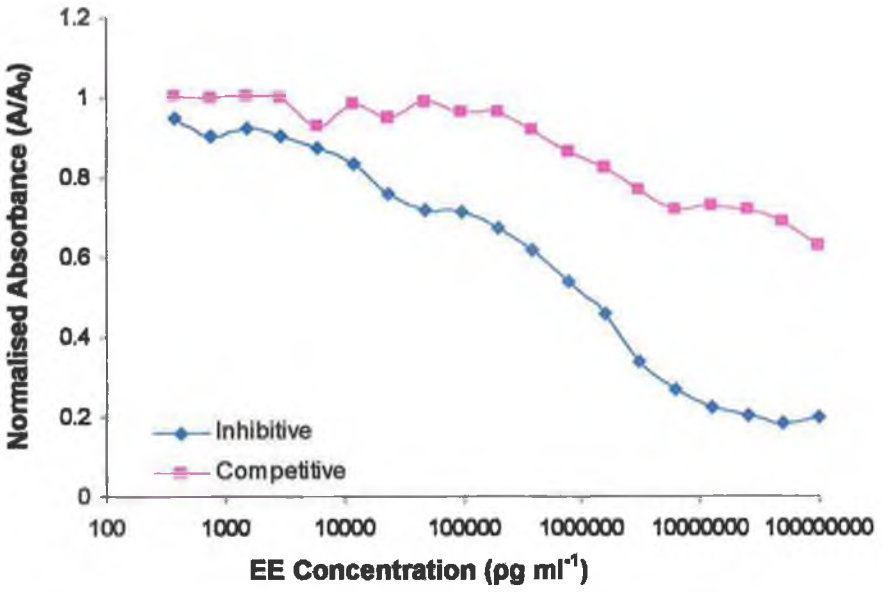


***Figure 4.7** : Determination of the range of detection for the ELISAs using EEAb1. A large number of standards were tested in the assay and the region in which they recognised EE was chosen as the detection range.*

4.2.4 Competitive and Inhibitive ELISAs using antibodies EEAb1 and EEAb2 for the detection of EE

A range of standards of EE was prepared as described in section 2.4.7.1. With the inhibitive assay these were added to an EE-CMO-BSA-coated plate at 50 µl per well together with 50 µl of anti-EE pab (EEAb1 or EEAb2) at the required dilution. After the incubation and washing steps had been completed HRP-labelled anti-rabbit pab was added to the plates. In the competitive ELISA the standards were added to an anti-EE pab-coated plate with the optimal dilution of EE-CMO-HRP. For both assays the bound HRP-conjugates were detected using the OPD substrate.

For intra-day assay variation each concentration of standard was assayed 5 times on a single day and the mean absorbance was plotted against the EE concentration of the standard. Figures 4.8, 4.9, 4.12 and 4.13 show the intra-day assays for both assay formats using both antibodies. Tables 4.2, 4.3, 4.6 and 4.7 show the means, the standard deviations, the coefficients of variation (CVs) and the percentage accuracy of the standard curve using 4-parameter curve fitting.

The inter-day assay variation was determined by carrying out the assay over 5 separate days. The A/A_0 value was compared across the 5 days. Figures 4.10, 4.11, 4.14 and 4.15 show the inter-day assays for both assay formats using both antibodies. The accompanying tables 4.4, 4.5, 4.8 and 4.9 show the means, the standard deviations, the coefficients of variation (CVs) and the percentage accuracy of the standard curve using 4-parameter curve fitting.

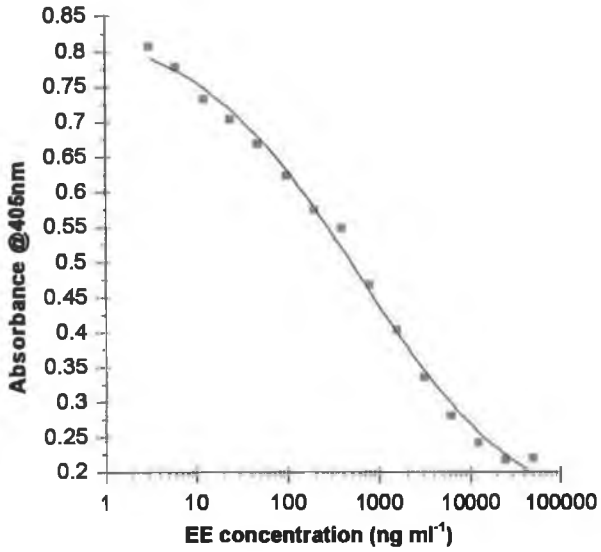


***Figure 4.8** : Intra-day studies on inhibitive ELISA using the anti-EE Pab, EEAb1 for the detection of free EE in PBS/2% (v/v) EtOH. The inhibition assay uses EE-6-CMO-BSA conjugate coated at 0.16 μg ml^{-1} and the antibody at a concentration of 0.5 μg ml^{-1}.*

Table 4.2 : Intra-day CVs and accuracies for EEAb1 inhibition ELISA in PBS. Five sets of fifteen standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (Abs)	Coefficients of variation (CVs), (%)	Accuracy, (%)
50000.0	0.222 ± 0.010	4.29	53.69
25000.0	0.219 ± 0.007	3.15	114.52
12500.0	0.243 ± 0.004	1.77	132.64
6250.0	0.282 ± 0.004	1.39	129.43
3125.0	0.337 ± 0.006	1.64	112.13
1562.5	0.404 ± 0.007	1.77	95.33
781.3	0.469 ± 0.013	2.88	89.01
390.6	0.549 ± 0.009	1.69	70.31
195.3	0.575 ± 0.016	2.72	102.80
97.7	0.624 ± 0.011	1.84	109.36
48.8	0.669 ± 0.005	0.81	113.68
24.4	0.704 ± 0.006	0.87	126.87
12.2	0.733 ± 0.012	1.63	140.77
6.1	0.779 ± 0.006	0.74	80.32
3.1	0.808 ± 0.011	1.35	42.59

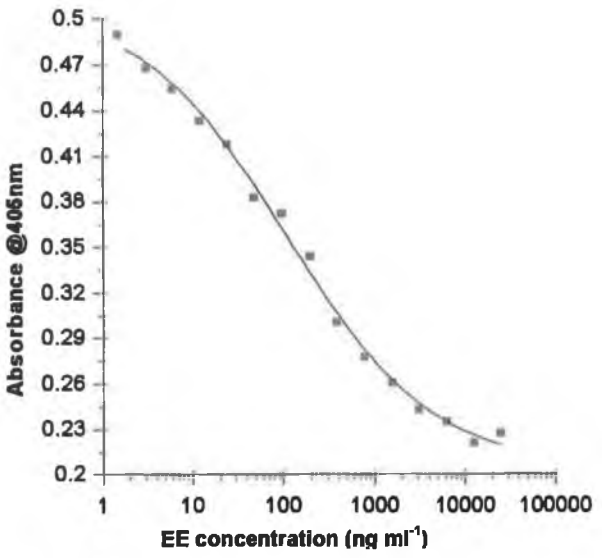


***Figure 4.9** : Intra-day studies on competitive ELISAs using the anti-EE Pab, EEAb1 for the detection of free EE in PBS/2% (v/v) EtOH. In the competition ELISA the plates are coated with 2 μg ml^{-1} solution of EEAb1 and the conjugate EE-6-CMO-HRP was used at 0.119 μg ml^{-1}.*

Table 4.3 : Intra-day CVs and accuracies for EEAb1 competition ELISA in PBS. Five sets of fifteen standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (Abs)	Coefficients of variation (CVs), (%)	Accuracy, (%)
25000.0	0.228 ± 0.009	3.87	43.10
12500.0	0.222 ± 0.010	4.55	160.07
6250.0	0.235 ± 0.011	4.66	97.01
3125.0	0.244 ± 0.010	3.95	118.71
1562.5	0.261 ± 0.002	0.83	105.54
781.3	0.278 ± 0.009	3.08	114.47
390.6	0.301 ± 0.018	5.94	116.74
195.3	0.344 ± 0.015	4.44	76.27
97.7	0.372 ± 0.016	4.30	77.72
48.8	0.383 ± 0.015	3.79	119.93
24.4	0.418 ± 0.009	2.06	95.45
12.2	0.434 ± 0.020	4.59	119.06
6.1	0.455 ± 0.015	3.32	111.80
3.1	0.469 ± 0.011	2.30	116.38
1.5	0.490 ± 0.019	3.90	49.40

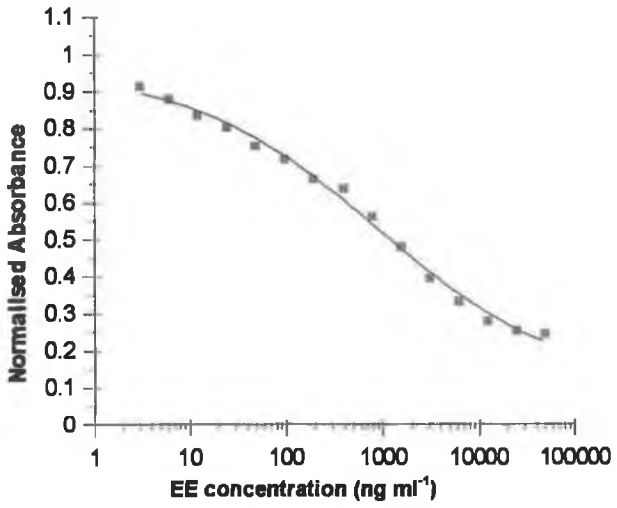


***Figure 4.10** : Inter-day studies on inhibitive ELISAs using the anti-EE Pab, EEAb1 for the detection of free EE in PBS/2% (v/v) EtOH. The inhibition assay uses EE-6-CMO-BSA conjugate coated at 0.16 μg ml^{-1} and the antibody at a concentration of 0.5 μg ml^{-1}.*

Table 4.4 : Inter-day CVs and accuracies for EEAb1 inhibition ELISA in PBS. Five sets of fifteen standards were analysed on five different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (A/A_0)	Coefficients of variation (CVs), (%)	Accuracy, (%)
50000.0	0.246 ± 0.017	6.98	62.30
25000.0	0.255 ± 0.010	4.00	105.33
12500.0	0.281 ± 0.014	4.82	137.14
6250.0	0.335 ± 0.017	5.12	126.01
3125.0	0.399 ± 0.017	4.26	115.68
1562.5	0.484 ± 0.017	3.62	92.46
781.3	0.564 ± 0.020	3.49	79.57
390.6	0.639 ± 0.005	0.84	71.76
195.3	0.667 ± 0.026	3.91	104.90
97.7	0.719 ± 0.025	3.51	111.10
48.8	0.754 ± 0.021	2.75	138.78
24.4	0.804 ± 0.010	1.28	124.64
12.2	0.837 ± 0.012	1.41	131.05
6.1	0.881 ± 0.018	2.03	84.56
3.1	0.916 ± 0.023	2.53	41.00

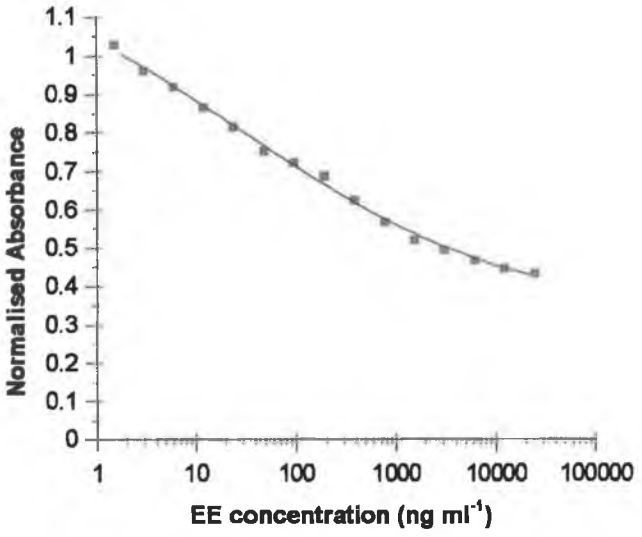


***Figure 4.11** : Inter-day studies on competitive ELISAs using the anti-EE Pab, EEAb1 for the detection of free EE in PBS/2% (v/v) EtOH. In the competition ELISA the plates are coated with 2 μg ml^{-1} solution of EEAb1 and the conjugate EE-6-CMO-HRP was used at 0.119 μg ml^{-1}.*

Table 4.5 : Inter-day CVs and accuracies for EEAb1 competition ELISA in PBS. Five sets of fifteen standards were analysed on five different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (A/A_0)	Coefficients of variation (CVs), (%)	Accuracy, (%)
25000.0	0.435 ± 0.050	11.55	73.88
12500.0	0.448 ± 0.050	11.17	97.34
6250.0	0.470 ± 0.039	8.24	108.25
3125.0	0.497 ± 0.046	9.29	113.61
1562.5	0.523 ± 0.036	6.94	131.20
781.3	0.570 ± 0.040	7.09	109.98
390.6	0.626 ± 0.049	7.84	88.30
195.3	0.688 ± 0.052	7.55	72.08
97.7	0.724 ± 0.057	7.93	87.78
48.8	0.755 ± 0.037	4.88	116.73
24.4	0.817 ± 0.047	5.73	102.51
12.2	0.868 ± 0.043	4.93	105.58
6.1	0.919 ± 0.040	4.35	104.66
3.1	0.962 ± 0.053	5.53	114.93
1.5	1.030 ± 0.080	7.80	79.75

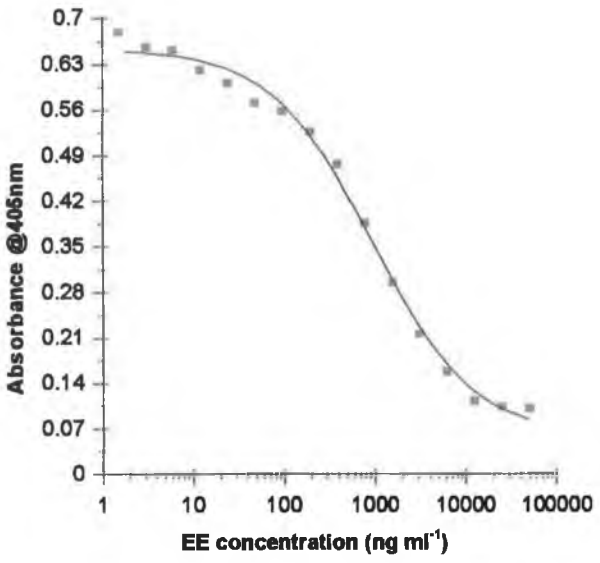


***Figure 4.12** : Intra-day studies on inhibitive ELISAs using the anti-EE Pab, EEAb2 for the detection of free EE in PBS/2% (v/v) EtOH. The inhibition assay uses EE-6-CMO-BSA conjugate coated at 0.2 μg ml^{-1} and the antibody at a dilution of 0.57 μg ml^{-1}.*

Table 4.6 : Intra-day CVs and accuracies for EEAb2 inhibition ELISA in PBS. Five sets of sixteen standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (Abs)	Coefficients of variation (CVs), (%)	Accuracy, (%)
50000.0	0.102 ± 0.003	2.77	48.47
25000.0	0.104 ± 0.004	4.14	91.04
12500.0	0.113 ± 0.004	3.27	140.46
6250.0	0.158 ± 0.007	4.59	116.20
3125.0	0.217 ± 0.006	2.94	109.55
1562.5	0.296 ± 0.007	2.29	101.37
781.3	0.387 ± 0.008	2.14	92.12
390.6	0.477 ± 0.009	1.97	79.87
195.3	0.527 ± 0.014	2.74	90.59
97.7	0.559 ± 0.020	3.50	115.34
48.8	0.572 ± 0.016	2.71	185.73
24.4	0.602 ± 0.011	1.88	192.53
12.2	0.621 ± 0.016	2.54	204.39
6.1	0.652 ± 0.014	2.15	13.84
3.1	0.657 ± 0.019	2.83	ND
1.5	0.679 ± 0.017	2.56	ND

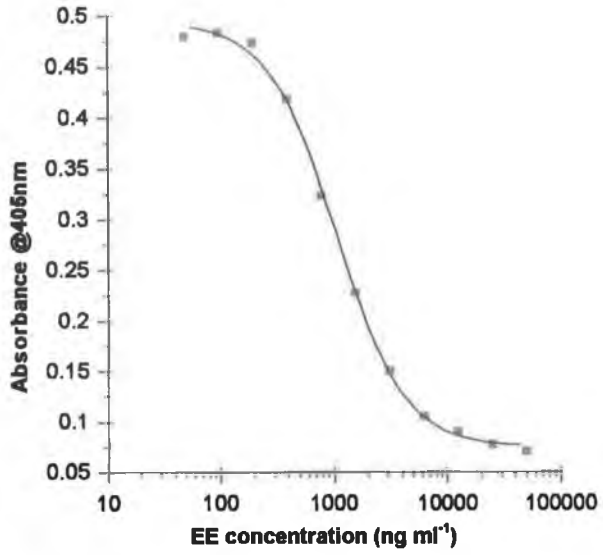


***Figure 4.13** : Intra-day studies on competitive ELISAs using the anti-EE Pab, EEAb2 for the detection of free EE in PBS/2% (v/v) EtOH. In the competitive ELISA the plates are coated with 1.71 μg ml^{-1} dilution of EEAb2 and the conjugate EE-6-CMO-HRP was used at 0.478 μg ml^{-1}.*

Table 4.7 : Intra-day CVs and accuracies for EEAb2 competition ELISA in PBS. Five sets of eleven standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (Abs)	Coefficients of variation (CVs), (%)	Accuracy, (%)
50000.0	0.071 ± 0.003	4.91	ND
25000.0	0.078 ± 0.004	5.56	135.13
12500.0	0.090 ± 0.005	5.99	80.66
6250.0	0.106 ± 0.003	3.23	97.50
3125.0	0.152 ± 0.004	2.74	95.76
1562.5	0.228 ± 0.013	5.76	100.18
781.3	0.324 ± 0.012	3.84	106.05
390.6	0.419 ± 0.030	7.24	97.40
195.3	0.474 ± 0.009	1.81	71.92
97.7	0.484 ± 0.010	2.11	92.08
48.8	0.480 ± 0.016	3.37	229.30

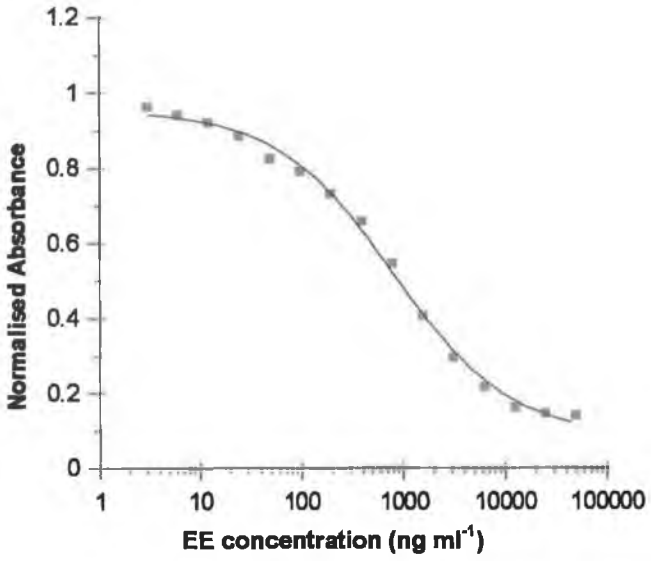


***Figure 4.14** : Inter-day studies on inhibitive ELISAs using the anti-EE Pab, EEAb2 for the detection of free EE in PBS/2% (v/v) EtOH. The inhibition assay uses EE-6-CMO-BSA conjugate coated at 0.2 μg ml^{-1} and the antibody at a dilution of 0.57 μg ml^{-1}.*

Table 4.8 : Inter-day CVs and accuracies for EEAb2 inhibition ELISA in PBS. Five sets of sixteen standards were analysed over five different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (A/A_0)	Coefficients of variation (CVs), (%)	Accuracy, (%)
50000.0	0.142 ± 0.011	7.98	49.77
25000.0	0.147 ± 0.015	10.42	89.33
12500.0	0.163 ± 0.013	8.14	133.38
6250.0	0.220 ± 0.020	8.92	123.95
3125.0	0.297 ± 0.022	7.42	117.46
1562.5	0.408 ± 0.022	5.39	102.98
781.3	0.547 ± 0.028	5.20	84.38
390.6	0.660 ± 0.033	5.07	80.75
195.3	0.733 ± 0.050	6.88	94.14
97.7	0.793 ± 0.038	4.81	111.40
48.8	0.827 ± 0.040	4.85	156.59
24.4	0.888 ± 0.028	3.18	136.08
12.2	0.923 ± 0.032	3.49	128.50
6.1	0.943 ± 0.041	4.32	134.67
3.1	0.966 ± 0.052	5.36	70.00
1.5	1.011 ± 0.053	5.29	ND

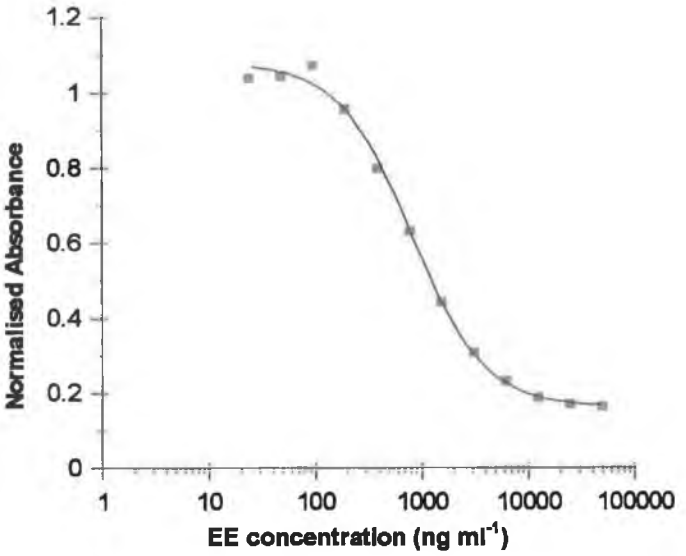


***Figure 4.15** : Inter-day studies on competitive ELISAs using the anti-EE Pab, EEAb2 for the detection of free EE in PBS/2% (v/v) EtOH. In the competitive ELISA the plates are coated with 1.71 μg ml^{-1} dilution of EEAb2 and the conjugate EE-6-CMO-HRP was used at 0.478 μg ml^{-1}.*

Table 4.9 : Inter-day CVs and accuracies for EEAb2 competition ELISA in PBS. Five sets of twelve standards were analysed over five different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (A/A_0)	Coefficients of variation (CVs), (%)	Accuracy, (%)
50000.0	0.164 ± 0.013	8.09	380.43
25000.0	0.171 ± 0.010	5.99	135.31
12500.0	0.188 ± 0.008	4.44	105.97
6250.0	0.233 ± 0.011	4.73	91.61
3125.0	0.311 ± 0.024	7.61	94.97
1562.5	0.444 ± 0.039	8.77	99.04
781.3	0.633 ± 0.066	10.40	100.50
390.6	0.801 ± 0.068	8.52	109.62
195.3	0.957 ± 0.015	1.56	97.44
97.7	1.074 ± 0.100	9.35	16.23
48.8	1.045 ± 0.100	9.53	133.89
24.4	1.040 ± 0.104	10.05	299.04

4.2.5 Detection of EE in Spiked Bile Samples

Both ELISA formats were used to detect free EE in spiked bile samples. The bile was prepared as described in section 2.4.7.2 and the EE standards were prepared using the bile as the diluent. The spiked bile was diluted with an equal volume of PBS before being applied to the assay plate. The rest of the ELISA protocols were as previously described in section 4.2.3.

Intra-day and inter-day assay variations were investigated as described for the ELISAs using PBS/2% EtOH as diluent. Figures 4.16, 4.17, 4.20 and 4.21 show the intra-assay variations along with tables 4.10, 4.11, 4.14 and 4.15. The inter-assay variations are shown in Figures 4.18, 4.19, 4.22 and 4.23 and the accompanying tables 4.12, 4.13, 4.16 and 4.17.

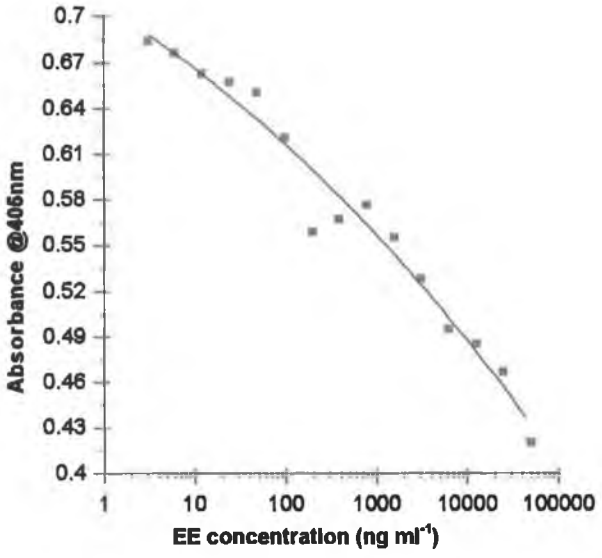


***Figure 4.16** : Intra-day studies on inhibitive ELISAs using the anti-EE Pab, EEAb1 for the detection of free EE in bile. The inhibition assay uses EE-6-CMO-BSA conjugate coated at 0.16 μg ml^{-1} and the antibody at a concentration of 0.5 μg ml^{-1}.*

Table 4.10 : Intra-day CVs and accuracies for EEAb1 inhibition ELISA in bile. Five sets of fifteen standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (Abs)	Coefficients of variation (CVs), (%)	Accuracy, (%)
50000.0	0.421 ± 0.024	5.65	137.29
25000.0	0.467 ± 0.015	3.11	72.42
12500.0	0.486 ± 0.011	2.19	82.63
6250.0	0.496 ± 0.016	3.22	122.73
3125.0	0.529 ± 0.017	3.29	83.19
1562.5	0.556 ± 0.012	2.24	66.26
781.3	0.577 ± 0.011	1.89	60.43
390.6	0.568 ± 0.021	3.78	170.29
195.3	0.559 ± 0.019	3.33	465.85
97.7	0.621 ± 0.015	2.39	83.42
48.8	0.651 ± 0.007	1.10	42.90
24.4	0.657 ± 0.012	1.84	63.76
12.2	0.663 ± 0.029	4.39	96.01
6.1	0.676 ± 0.019	2.82	98.23
3.1	0.684 ± 0.014	2.09	127.20

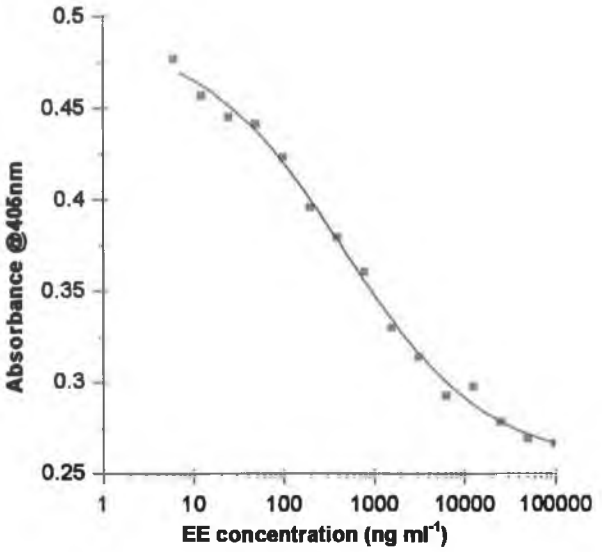


***Figure 4.17** : Intra-day studies on competitive ELISAs using the anti-EE Pab, EEAb1 for the detection of free EE in bile. The plates were coated with 2 μg ml^{-1} solution of EEAb1 and the conjugate EE-6-CMO-HRP was used at 0.119 μg ml^{-1}.*

Table 4.10 : Intra-day CVs and accuracies for EEAb1 competition ELISA in bile. Five sets of fifteen standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (Abs)	Coefficients of variation (CVs), (%)	Accuracy, (%)
100000.0	0.267 ± 0.011	4.18	112.71
50000.0	0.270 ± 0.008	2.99	136.43
25000.0	0.279 ± 0.012	4.42	104.09
12500.0	0.298 ± 0.015	5.19	56.63
6250.0	0.293 ± 0.009	3.05	148.93
3125.0	0.314 ± 0.010	3.30	107.54
1562.5	0.330 ± 0.015	4.55	117.88
781.3	0.361 ± 0.014	3.90	85.82
390.6	0.380 ± 0.015	3.90	96.17
195.3	0.396 ± 0.015	3.90	114.48
97.7	0.424 ± 0.012	2.78	90.19
48.8	0.442 ± 0.009	2.13	84.85
24.4	0.446 ± 0.018	4.11	140.12
12.2	0.457 ± 0.013	2.85	149.27
6.1	0.477 ± 0.012	2.44	51.34

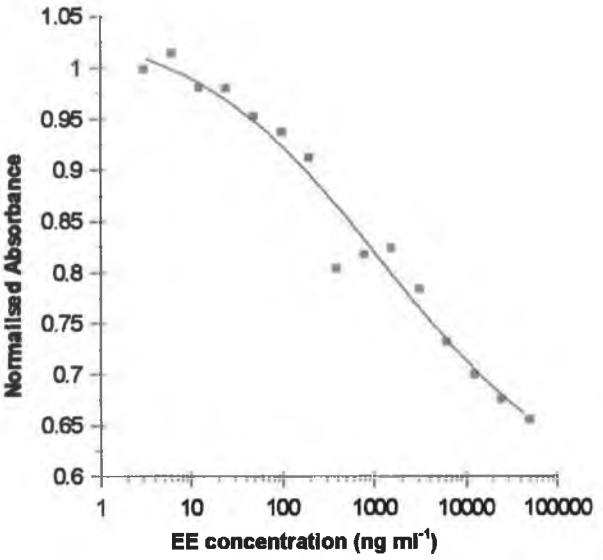


***Figure 4.18** : Inter-day studies on inhibitive ELISAs using the anti-EE Pab, EEAb1 for the detection of free EE in bile. The inhibition assay uses EE-6-CMO-BSA conjugate coated at 0.16 μg ml^{-1} and the antibody at a concentration of 0.5 μg ml^{-1}.*

Table 4.12 : Inter-day CVs and accuracies for EEAb1 inhibition ELISA in bile. Five sets of fifteen standards were analysed on five different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (A/A_0)	Coefficients of variation (CVs), (%)	Accuracy, (%)
50000.0	0.656 ± 0.068	10.30	107.55
25000.0	0.677 ± 0.072	10.70	109.73
12500.0	0.701 ± 0.067	9.52	114.41
6250.0	0.733 ± 0.070	9.59	104.28
3125.0	0.785 ± 0.069	8.80	68.62
1562.5	0.824 ± 0.067	8.19	61.55
781.3	0.818 ± 0.066	8.01	138.61
390.6	0.805 ± 0.038	4.72	362.91
195.3	0.913 ± 0.016	1.79	70.81
97.7	0.938 ± 0.027	2.87	74.04
48.8	0.952 ± 0.036	3.82	96.93
24.4	0.980 ± 0.027	2.71	72.83
12.2	0.981 ± 0.020	2.09	140.13
6.1	1.014 ± 0.031	3.01	47.21
3.1	0.999 ± 0.033	3.27	246.08

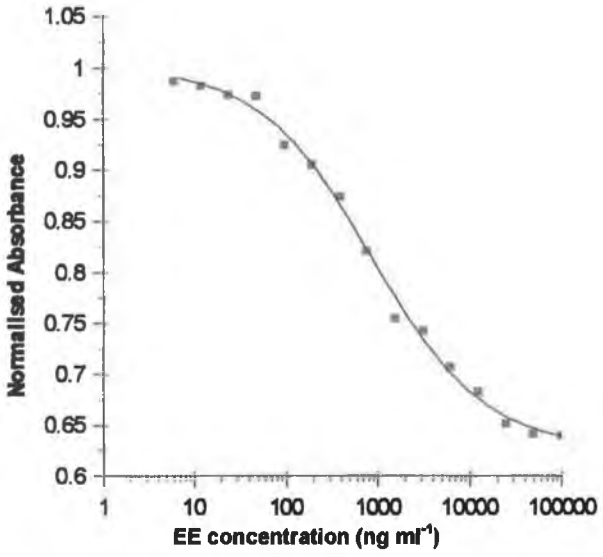


Figure 4.19 *: Inter-day studies on competitive ELISAs using the anti-EE Pab, EEAb1 for the detection of free EE in bile. The plates are coated with 2 μg ml^{-1} solution of EEAb1 and the conjugate EE-6-CMO-HRP was used at 0.119 μg ml^{-1}.*

Table 4.13 : Inter-day CVs and accuracies for EEAb1 competition ELISA in bile. Five sets of fifteen standards were analysed on five different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (A/A_0)	Coefficients of variation (CV_B), (%)	Accuracy, (%)
100000.0	0.640 ± 0.060	9.31	89.79
50000.0	0.642 ± 0.069	10.80	146.18
25000.0	0.652 ± 0.056	8.63	143.10
12500.0	0.684 ± 0.042	6.21	77.89
6250.0	0.707 ± 0.056	7.97	85.45
3125.0	0.744 ± 0.050	6.70	84.06
1562.5	0.755 ± 0.029	3.87	138.20
781.3	0.821 ± 0.034	4.09	99.38
390.6	0.875 ± 0.071	8.16	85.77
195.3	0.906 ± 0.036	3.97	97.56
97.7	0.926 ± 0.027	2.92	126.35
48.8	0.973 ± 0.022	2.25	50.26
24.4	0.974 ± 0.061	6.28	94.81
12.2	0.982 ± 0.048	4.89	105.19
6.1	0.987 ± 0.060	6.11	139.22

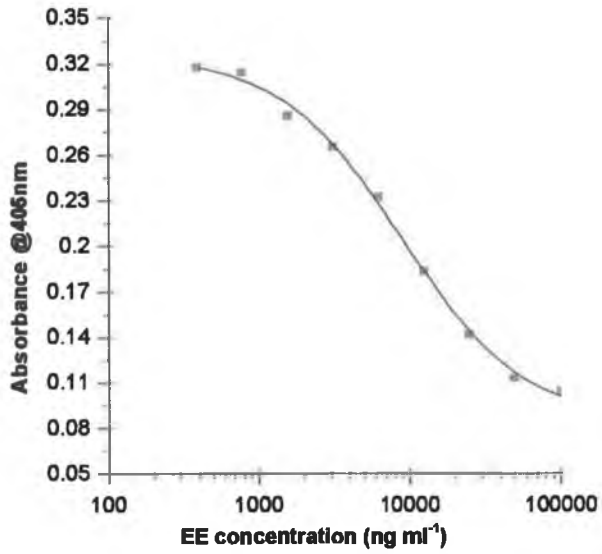


***Figure 4.20** : Intra-day studies on inhibitive ELISAs using the anti-EE Pab, EEAb2 for the detection of free EE in bile. The inhibition assay uses EE-6-CMO-BSA conjugate coated at 0.2 μg ml^{-1} and the antibody at a dilution of 0.57 μg ml^{-1}.*

Table 4.14 : Intra-day CVs and accuracies for EEAb2 inhibition ELISA in bile. Five sets of ten standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (Abs)	Coefficients of variation (CVs), (%)	Accuracy, (%)
100000.0	0.104 ± 0.002	1.99	84.38
50000.0	0.114 ± 0.004	3.21	113.45
25000.0	0.142 ± 0.004	2.87	104.63
12500.0	0.184 ± 0.001	0.62	99.86
6250.0	0.233 ± 0.003	1.19	92.75
3125.0	0.266 ± 0.003	1.19	102.29
1562.5	0.286 ± 0.013	4.37	126.84
781.3	0.315 ± 0.011	3.56	70.55
390.6	0.318 ± 0.003	0.99	100.27
195.3	0.295 ± 0.007	2.43	784.49

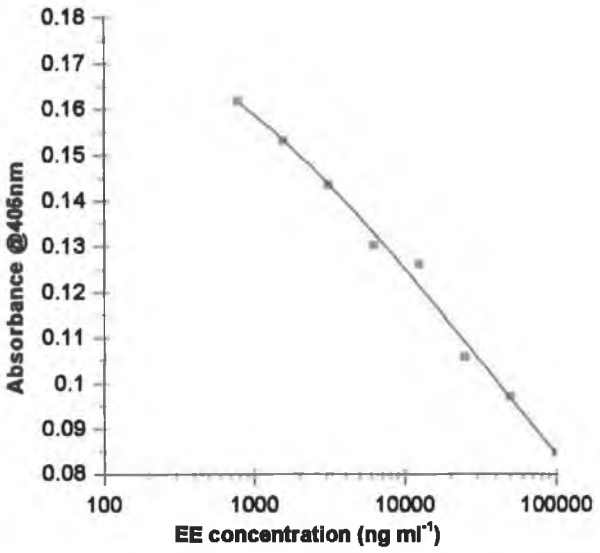


***Figure 4.21** : Intra-day studies on competitive ELISAs using the anti-EE Pab, EEAb2 for the detection of free EE in bile. In the competitive ELISA the plates are coated with 1.71 μg ml^{-1} dilution of EEAb2 and the conjugate EE-6-CMO-HRP was used at 0.478 μg ml^{-1}.*

Table 4.15 : Intra-day CVs and accuracies for EEAb2 competition ELISA in bile. Five sets of eight standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (Abs)	Coefficients of variation (CVs), (%)	Accuracy, (%)
100000.0	0.085 ± 0.002	2.11	99.96
50000.0	0.097 ± 0.003	3.29	98.49
25000.0	0.106 ± 0.002	2.35	121.69
12500.0	0.126 ± 0.002	1.97	76.01
6250.0	0.130 ± 0.004	2.85	118.25
3125.0	0.144 ± 0.004	3.02	101.97
1562.5	0.153 ± 0.006	3.98	102.51
781.3	0.162 ± 0.007	4.36	101.25

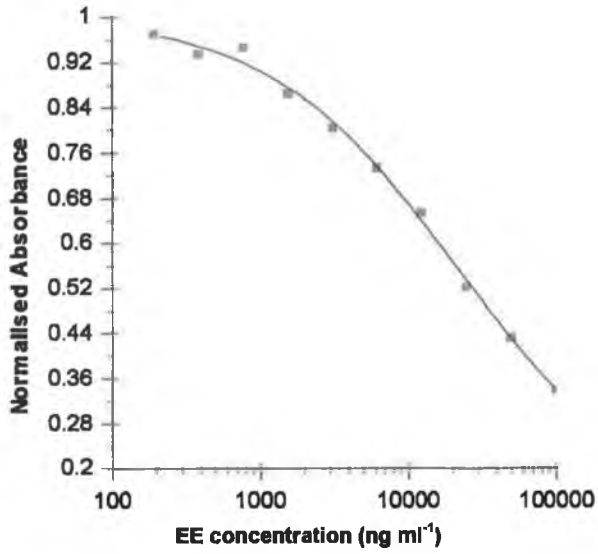


***Figure 4.22** : Inter-day studies on inhibitive ELISAs using the anti-EE Pab, EEAb2 for the detection of free EE in bile. The inhibition assay uses EE-6-CMO-BSA conjugate coated at 0.2 μg ml^{-1} and the antibody at a dilution of 0.57 μg ml^{-1}.*

Table 4.16 : Inter-day CVs and accuracies for EEAb2 inhibition ELISA in bile. Five sets of ten standards were analysed on five different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (μg ml^{-1})	Calculated mean ± SD, (A/A_0)	Coefficients of variation (CVs), (%)	Accuracy, (%)
100000.0	0.340 ± 0.021	6.16	98.22
50000.0	0.432 ± 0.029	6.69	97.77
25000.0	0.523 ± 0.028	5.40	106.12
12500.0	0.656 ± 0.041	6.33	87.82
6250.0	0.735 ± 0.028	3.78	98.36
3125.0	0.807 ± 0.038	4.66	105.23
1562.5	0.866 ± 0.033	3.79	109.47
781.3	0.947 ± 0.041	4.37	48.10
390.6	0.935 ± 0.024	2.61	130.94
195.3	0.971 ± 0.026	2.73	76.55

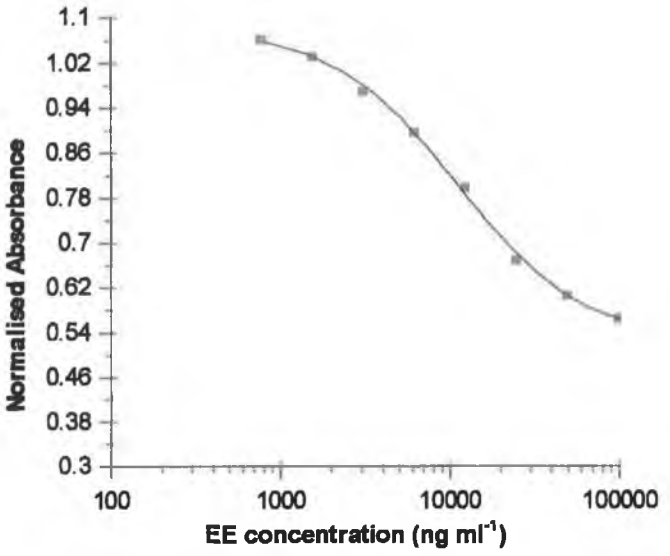


***Figure 4.23** : Inter-day studies on competitive ELISAs using the anti-EE Pab, EEAb2 for the detection of free EE in bile. In the competitive ELISA the plates are coated with 1.71 μg ml^{-1} dilution of EEAb2 and the conjugate EE-6-CMO-HRP was used at 0.478 μg ml^{-1}.*

Table 4.17 : Inter-day CVs and accuracies for EEAb2 competition ELISA in bile. Five sets of eight standards were analysed on five different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (A/A_0)	Coefficients of variation (CV_5), (%)	Accuracy, (%)
100000.0	0.566 ± 0.046	8.17	93.46
50000.0	0.607 ± 0.044	7.24	98.91
25000.0	0.669 ± 0.039	5.80	109.79
12500.0	0.799 ± 0.046	5.79	92.25
6250.0	0.898 ± 0.040	4.50	98.30
3125.0	0.970 ± 0.045	4.68	109.50
1562.5	1.032 ± 0.043	4.17	96.44
781.3	1.062 ± 0.050	4.73	78.64

4.2.6 Cross Reactivity Studies on EEAb1 and EEAb2

Cross reactivity studies were carried out as described in section 2.4.7.8. Both antibodies were tested against a number of structurally related compounds to identify the extent of their cross reactivity. Sets of standards were prepared using EE and each of the steroids to be tested. These were all run in duplicate and in parallel in the optimised inhibitive ELISA. For each compound the concentration that suppressed 50% of the zero signal was calculated. This value for each drug was compared to that obtained for EE. The percentage cross reactivity was calculated according to the following formula;

$$\%\,\text{Cross reactivity} = \frac{\text{IC50 of EE}}{\text{IC50 of compound}} \times 100$$

The drugs tested and the calculated cross reactivities are shown in Table 4.18.

Table 4.18 : Cross reactivity of the polyclonal antisera to EE and other structurally related steroid hormones and cross-reacting compounds in inhibition ELISAs. [a]Percentage cross reactivity was calculated as (IC_{50}[EE]/IC_{50}[test compound])*100.

Compounds Tested	EEAb1	EEAb2
	% Cross Reactivity[a]	
EE	100.00	100.00
Trenbolone	1.24	<0.10
Diethylstilbestrol	<0.03	<0.10
α-Estradiol	<0.03	<0.10
β-Estradiol	1.78	1.51
Estrone	<0.03	<0.10
Norgestrol	<0.03	14.35
19-Nortestosterone	<0.47	6.20
Methyl Testosterone	1.42	12.19
Zeranol	0.03	<0.10
Norethisterone	0.50	73.46

4.2.7 Optimisation of BIAcore 3000 assay for the detection of EE

4.2.7.1 Optimisation of pH for the chip coupling reaction

This was carried out as described in section 2.4.9.1. The solutions containing EE-CMO-BSA at various pHs were passed over a fresh chip surface and the amount of conjugate that associated with the surface was noted. The pH at which the highest response was seen was taken to be the optimum pH for the EDC/NHS coupling reaction. Figure 4.24 shows this response curve and it can be noted that pH 4.2 gave the optimal response. This was used in all subsequent chip coatings.

4.2.7.2 Immobilisation of EE-CMO-BSA

A 50 μg ml^{-1} solution of EE-CMO-BSA was prepared in 10mM sodium acetate buffer, pH 4.2. This was immobilised to the surface of a sensor chip as described in section 2.4.9.2. Figure 4.25 shows a typical profile for the immobilisation of this drug onto the dextran surface.

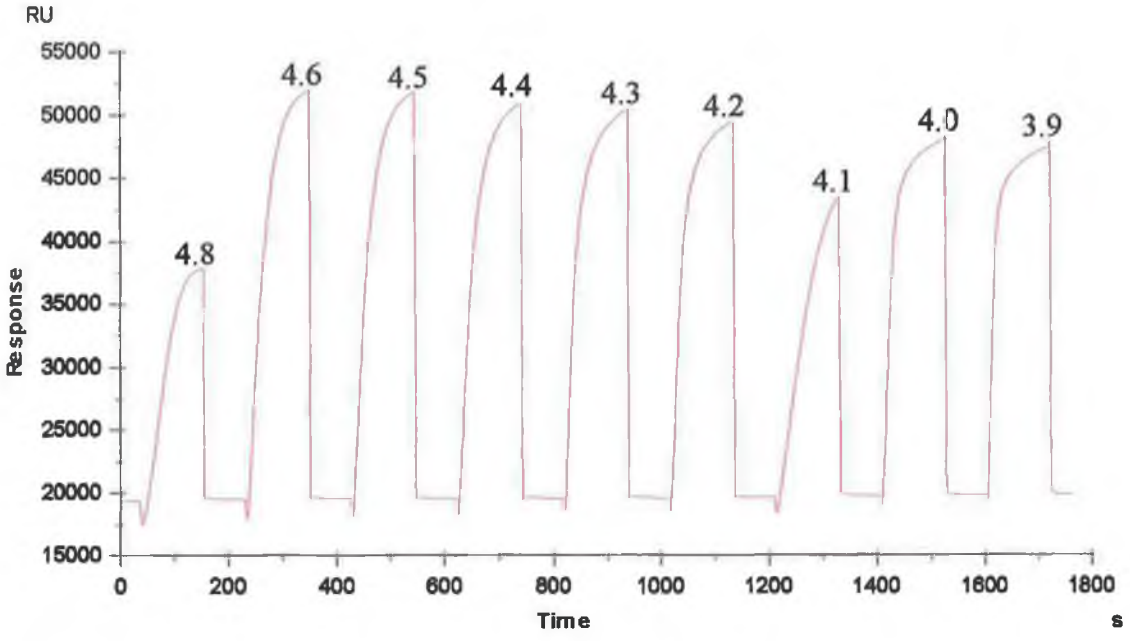


***Figure 4.24** : Preconcentration curve for the determination of the optimum buffer pH to carry out the immobilisation of EE-CMO-BSA to BIAcore CM5 sensor chip.*

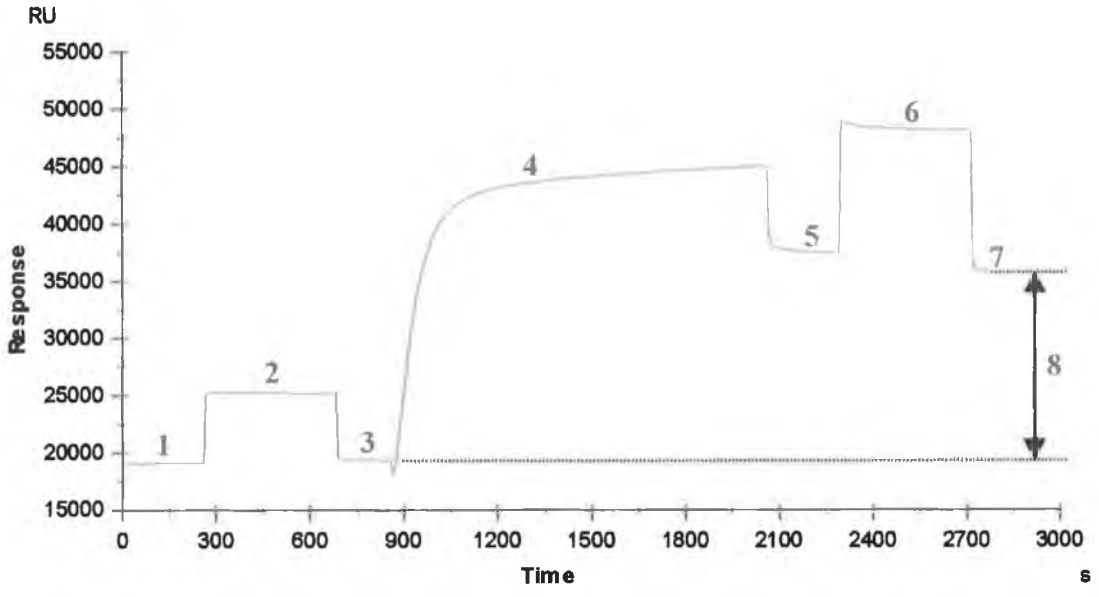


***Figure 4.25** : Typical BIAcore sensogram for the immobilisation of EE-CMO-BSA to the surface of the CM5 sensor chips. The numbered areas refer to the different stages of the immobilisation procedure. Section 1 is the machine baseline, with HBS buffer flowing over the surface of the chip. 2 refers to the refractive index change in the liquid when the EDC/NHS mixture is passed across the chip and 3 is the return to HBS buffer. A minimal increase in response units is seen at 3. Section 4 shows the binding of the conjugate to the activated dextran matrix and 5 is the return to HBS buffer flow. The surface is then capped with ethanolamine to remove any loosely bound conjugate and to deactivate any remaining active sites on the dextran surface (6 - 7). The total amount of conjugate bound is the difference between 3 and 7, labelled 8, (approximately 17,000RU in this example).*

4.2.7.3 Background binding of both EE polyclonal antibody preparations in the BIAcore-based assay

It is vital to show that the antibody is not binding to any other component present on the surface of the sensor chip other than the immobilised drug, EE. Solutions of the polyclonal antibodies were prepared in HBS buffer and were passed over three chip surfaces, a dextran chip surface, a BSA-immobilised surface and an EE-CMO-BSA-immobilised surface. A specific antibody would be expected to only bind to the conjugate-immobilised surface. The amount of binding to the other surfaces is an indication of the background binding, if any, that can be expected in the biosensor assay. Figure 4.26 shows these binding curves and it can be seen that only the conjugate surface had antibody binding in appreciable amounts. The results obtained for EEAb2 in these controls are not shown, as they were similar to those displayed for EEAb1.

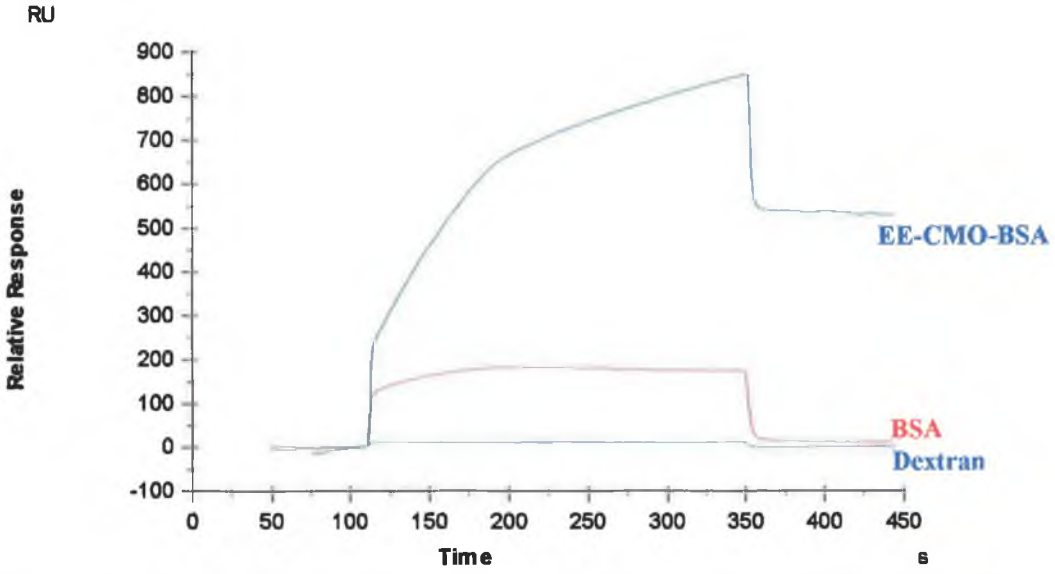


***Figure 4.26** : Binding curves of EEAb1 to a dextran surface, a BSA-immobilised surface and a BSA-conjugate-coated surface. The amount of antibody binding to the dextran and the BSA surfaces is very low. Both the BSA and dextran surfaces show less than 5 RU of antibody binding compared to more than 500 RU for the EE conjugate-coated surface.*

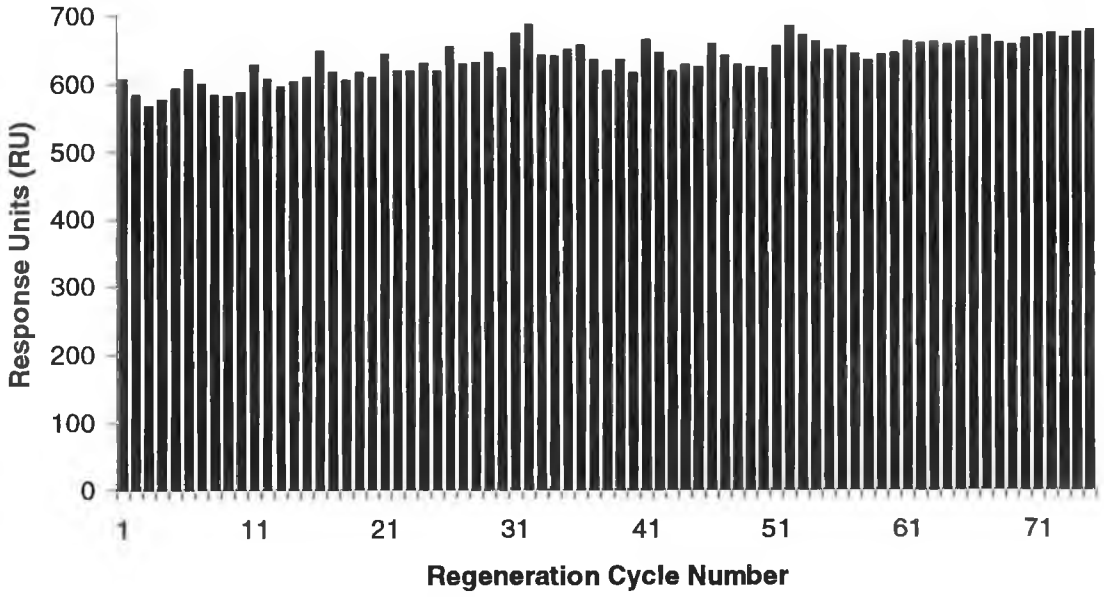


***Figure 4.27** : Regeneration study for EEAb1 using 1M ethanolamine, pH 13.6. 20 cycles of the regeneration protocol were performed to equilibrate the surface before the regeneration study or any assays were carried out. Over the course of the 75 regeneration cycles this surface actually begins to bind more antibody. An increase of 60 RU in binding occurs over the 75 regeneration cycles.*

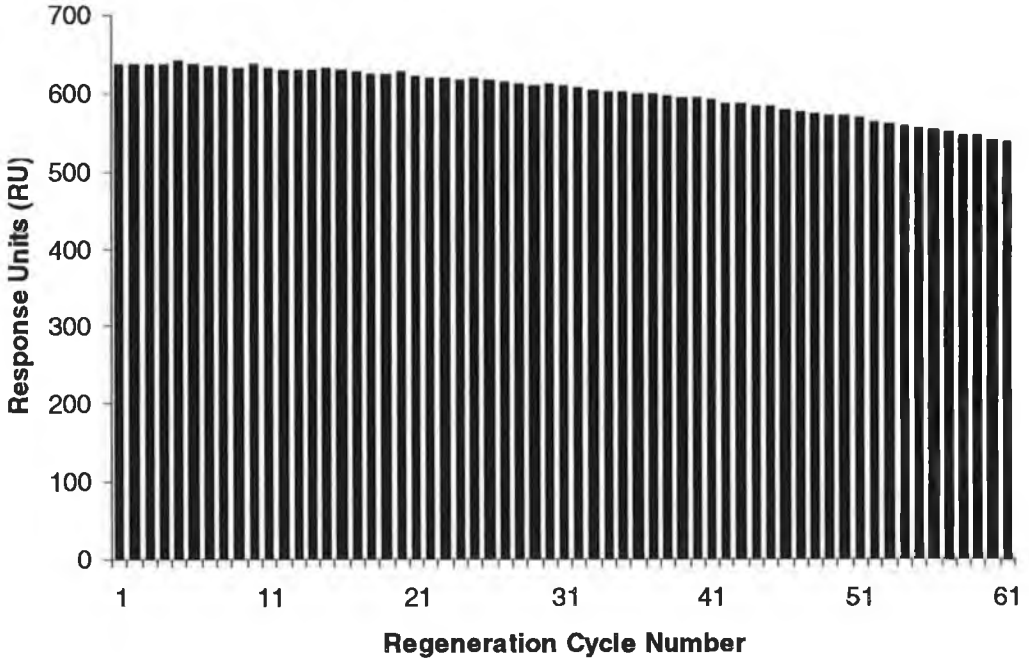


***Figure 4.28** : Regeneration curve for EEAb2 using 30mM HCl followed by 30mM NaOH/10% acetonitrile. 12 cycles of the regeneration protocol were performed to equilibrate the surface before the regeneration study or any assays were performed. The drop in response over the 60 regeneration cycles is 102 RU.*

4.2.8 Biosensor-based assays for the detection of EE using a BIAcore 3000 sensor

The BIAcore was used as the system to produce the biosensor assay. The carboxymethylated dextran surfaces of the sensor chips were activated and the EE-CMO-BSA conjugate was covalently attached to the surface. The samples were prepared in either HBS/2% (v/v) EtOH or bile depending on the matrix being investigated. The bile was diluted with an equal volume of HBS before use in the assay. The samples were mixed 1:1 with the appropriate antibody dilution and incubated for 1 hour at 37°C. They were then applied to the biosensor where each sample was assayed in triplicate. Figure 4.29 shows the typical binding curves obtained for a number of different EE concentrations. The differences in signal are clearly visible.

Figures 4.30 to 4.33 show the intra and inter-day assay standard curves for the BIAcore-based assay using EEAb1 and EEAb2 to detect free EE in HBS/2% (v/v) EtOH. Figures 4.34 to 4.37 show the intra and inter-day assay standard curves for the BIAcore-based assay using EEAb1 and EEAb2 to detect free EE in bile/HBS.

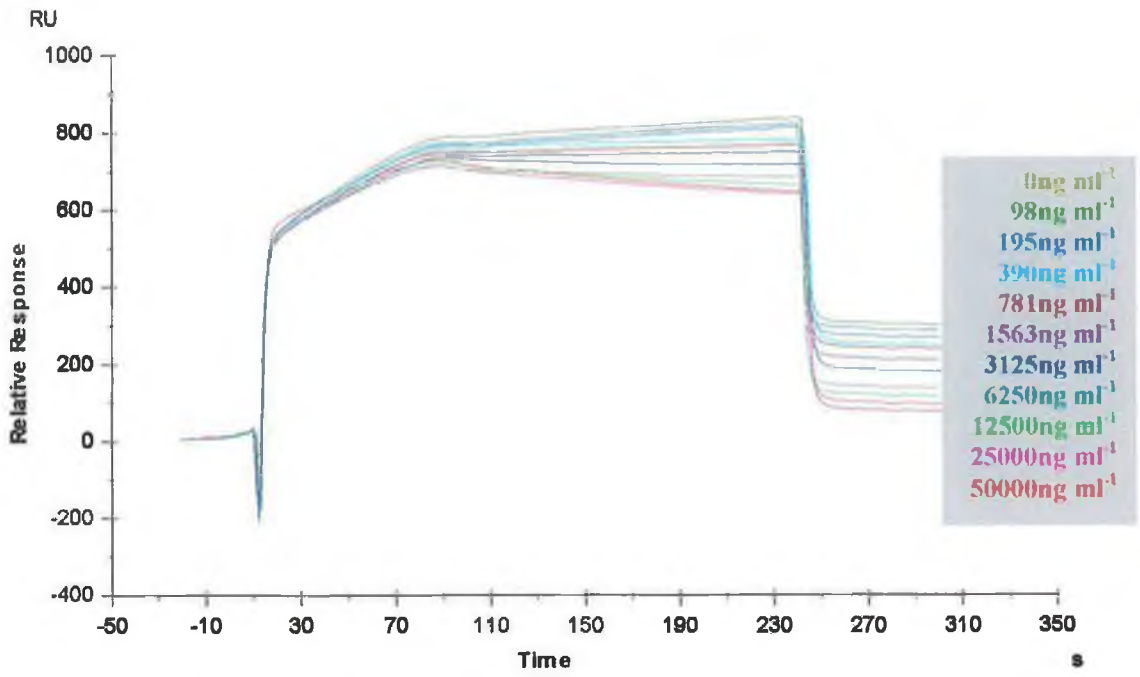


***Figure 4.29** : Binding curves for inhibitive BIAcore assay at different concentrations of free EE. As in all competitive immunoassays the highest signal is obtained at the lowest concentration of free drug.*

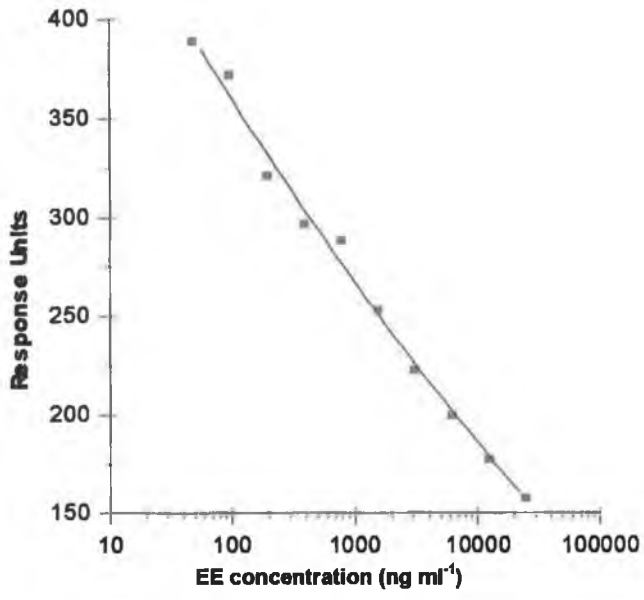


***Figure 4.30** : Intra-day BIAcore assays for EE in HBS/2% (v/v) EtOH using EEAb1 polyclonal antibody. The BSA conjugate was coated at 50 μg ml^{-1} onto the surface of the chip for the assays. The antibody was used at a concentration of 16 μg ml^{-1}.*

Table 4.19 : Intra-day CVs and accuracies for EEAb1 inhibition BIAcore assay in HBS. Three sets of thirteen standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (RU)	Coefficients of variation (CVs), (%)	Accuracy, (%)
25000.0	158.0 ± 1.1	0.71	95.20
12500.0	177.6 ± 1.5	0.82	102.15
6250.0	200.0 ± 1.0	0.51	103.69
3125.0	223.0 ± 1.5	0.65	106.55
1562.5	253.2 ± 1.0	0.40	92.43
781.3	288.4 ± 3.0	1.02	73.30
390.6	296.9 ± 3.7	1.25	118.10
195.3	321.2 ± 9.4	2.94	129.00
97.7	372.4 ± 1.9	0.52	76.27
48.8	389.3 ± 4.9	1.25	103.56
24.4	364.1 ± 3.7	1.00	370.32

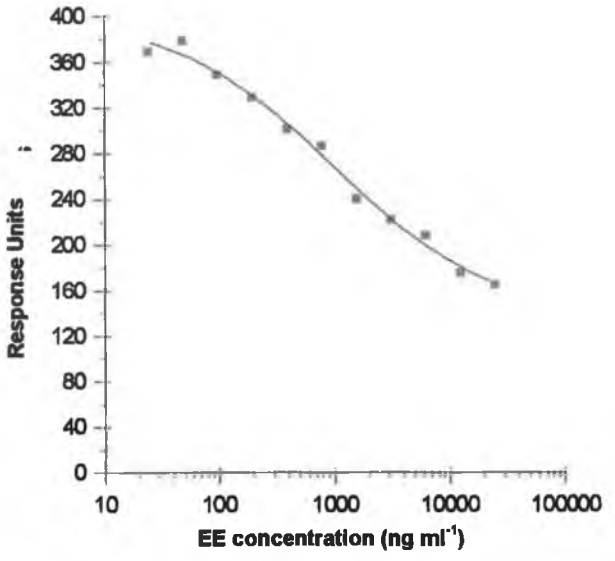


***Figure** 4.31 : Inter-day BIAcore assays for EE in HBS/2% (v/v) EtOH using EEAb1 polyclonal antibody. The BSA conjugate was coated at 50 μg ml^{-1} onto the surface of the chip for the assays. The antibody was used at a concentration of 16 μg ml^{-1}.*

Table 4.20 : Inter-day CVs and accuracies for EEAb1 inhibition BIAcore assay in HBS. Three sets of eleven standards were analysed on three different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (RU)	Coefficients of variation (CVs), (%)	Accuracy, (%)
25000.0	165.5 ± 7.8	4.70	103.52
12500.0	175.8 ± 14.2	8.08	121.61
6250.0	209.0 ± 8.5	4.05	73.56
3125.0	222.4 ± 14.6	6.57	100.30
1562.5	240.9 ± 16.6	6.89	123.98
781.3	286.7 ± 3.0	1.05	81.30
390.6	301.6 ± 4.2	1.40	111.93
195.3	329.7 ± 11.8	3.59	102.75
97.7	349.3 ± 25.4	7.26	105.88
48.8	379.3 ± 15.7	4.13	43.92
24.4	369.7 ± 15.7	4.26	166.34

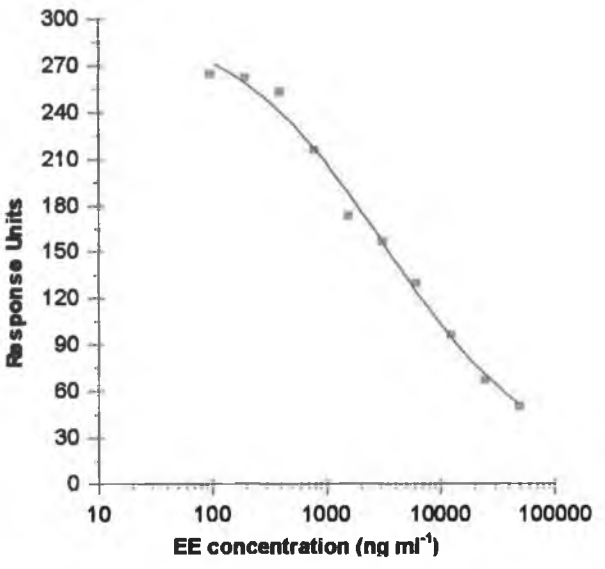


***Figure 4.32** : Intra-day BIAcore assays for EE in HBS/2% (v/v) EtOH using EEAb2 polyclonal antibody. The BSA conjugate was coated at 50 μg ml^{-1} onto the surface of the chip for the assays. The antibody was used at a concentration of 15.2 μg ml^{-1}.*

Table 4.21 : Intra-day CVs and accuracies for EEAb2 inhibition BIAcore assay in HBS. Three sets of ten standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (RU)	Coefficients of variation (CVs), (%)	Accuracy, (%)
50000.0	51.0 ± 5.2	10.13	102.09
25000.0	67.7 ± 5.0	7.35	108.56
12500.0	96.9 ± 6.3	6.48	94.29
6250.0	130.6 ± 6.6	5.02	86.19
3125.0	157.2 ± 4.8	3.08	97.50
1562.5	173.9 ± 9.2	5.27	136.31
781.3	216.0 ± 1.7	0.80	102.11
390.6	253.6 ± 6.3	2.50	62.47
195.3	262.8 ± 3.4	1.30	83.59
97.7	265.2 ± 0.6	0.21	148.32

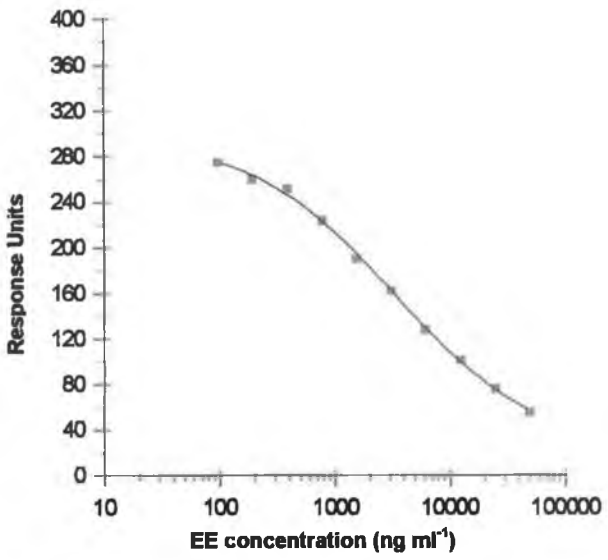


***Figure** 4.33 : Inter-day BIAcore assays for EE in HBS/2% (v/v) EtOH using EEAb2 polyclonal antibody. The BSA conjugate was coated at 50 μg ml^{-1} onto the surface of the chip for the assays. The antibody was used at a concentration of 15.2 μg ml^{-1}.*

Table 4.22 : Inter-day CVs and accuracies for EEAb2 inhibition BIAcore assay in HBS. Three sets of ten standards were analysed on three different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (RU)	Coefficients of variation (CVs), (%)	Accuracy, (%)
50000.0	55.3 ± 4.5	8.05	108.40
25000.0	76.8 ± 15.9	20.74	95.92
12500.0	101.8 ± 10.0	9.77	95.15
6250.0	128.3 ± 8.3	6.50	102.81
3125.0	162.5 ± 13.8	8.52	99.65
1562.5	190.2 ± 17.7	9.30	110.62
781.3	224.5 ± 10.5	4.69	97.70
390.6	252.0 ± 4.6	1.81	83.01
195.3	260.5 ± 5.5	2.12	117.91
97.7	275.5 ± 9.9	3.58	105.35

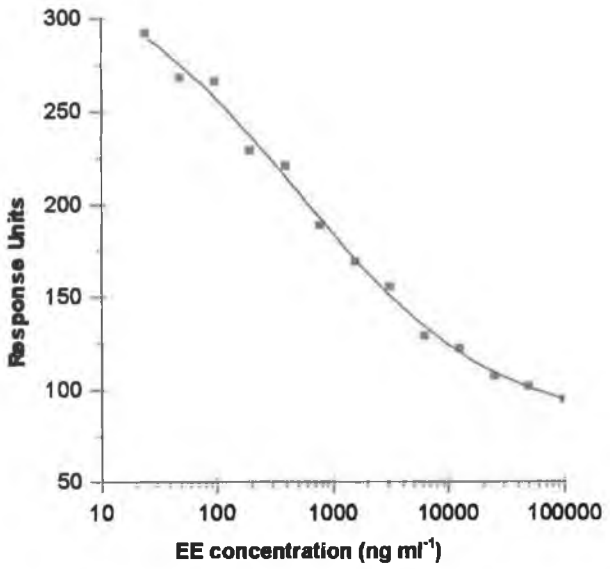


***Figure 4.34** : Intra-day BIAcore assays for EE in bile using EEAb1 polyclonal antibody. The BSA conjugate was coated at 50 μg ml^{-1} onto the surface of the chip for the assays and the antibody was used at a concentration of 40 μg ml^{-1}.*

Table 4.23 : Intra-day CVs and accuracies for EEAb1 inhibition BIAcore assay in bile. Three sets of fourteen standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (RU)	Coefficients of variation (CVs), (%)	Accuracy, (%)
100000.0	95.3 ± 3.3	3.41	98.24
50000.0	102.5 ± 11.3	11.01	89.46
25000.0	107.5 ± 2.8	2.59	116.61
12500.0	122.6 ± 2.8	2.29	88.17
6250.0	129.6 ± 0.5	0.36	123.47
3125.0	156.1 ± 0.9	0.57	82.40
1562.5	169.5 ± 2.8	1.68	103.63
781.3	189.2 ± 0.5	0.28	110.20
390.6	221.2 ± 1.3	0.60	82.63
195.3	229.3 ± 9.9	4.30	128.60
97.7	266.6 ± 3.8	1.43	71.56
48.8	268.8 ± 7.1	2.64	131.11
24.4	292.5 ± 3.3	1.11	87.97

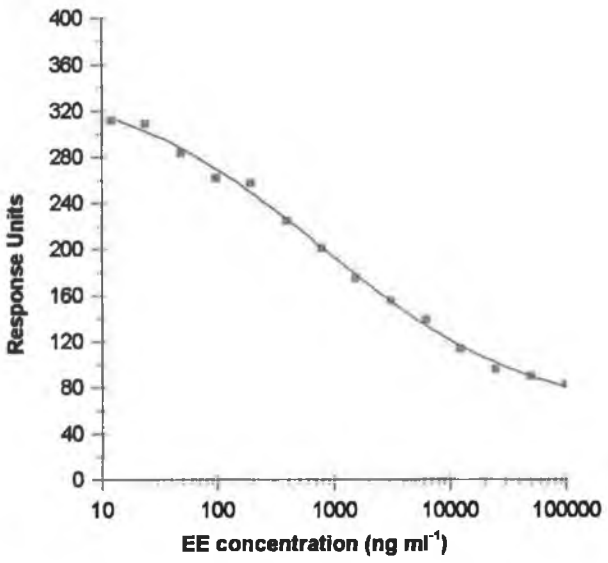


***Figure 4.35** : Inter-day BIAcore assays for EE in bile using EEAb1 polyclonal antibody. The BSA conjugate was coated at 50 μg ml^{-1} onto the surface of the chip for the assays. The antibody was used at a concentration of 40 μg ml^{-1}.*

Table 4.24 : Inter-day CVs and accuracies for EEAb1 inhibition BIAcore assay in bile. Three sets of fourteen standards were analysed on three different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (RU)	Coefficients of variation (CVs), (%)	Accuracy, (%)
100000.0	83.3 ± 12.3	14.82	81.92
50000.0	90.7 ± 10.5	11.63	95.69
25000.0	96.4 ± 13.0	13.47	134.40
12500.0	113.9 ± 9.5	8.30	111.14
6250.0	139.4 ± 12.0	8.61	82.76
3125.0	156.2 ± 0.9	0.58	95.68
1562.5	175.2 ± 5.0	2.87	107.98
781.3	201.3 ± 10.6	5.26	102.40
390.6	225.2 ± 5.3	2.34	104.06
195.3	257.7 ± 25.7	9.98	77.20
97.7	262.5 ± 30.7	11.71	131.59
48.8	284.3 ± 40.7	14.32	118.06
24.4	309.8 ± 14.9	4.82	68.34
12.2	312.3 ± 61.5	19.70	117.29

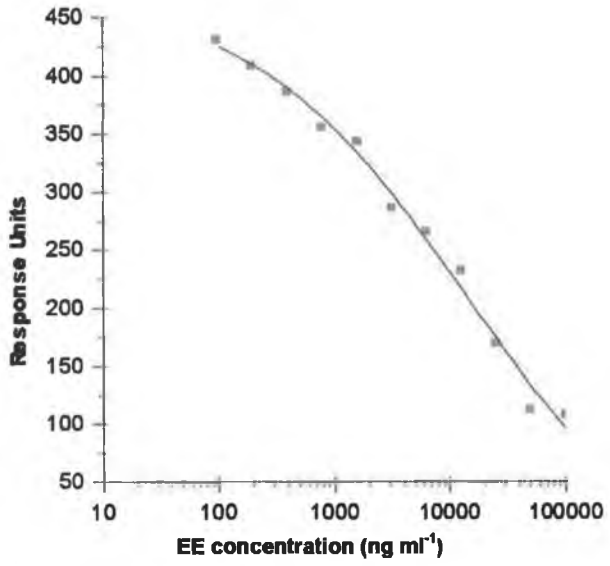


***Figure 4.36** : Intra-day BIAcore assays for EE in bile using EEAb2 polyclonal antibody. The BSA conjugate was coated at 50 μg ml^{-1} onto the surface of the chip for the assays and the antibody was used at a concentration of 34.2 μg ml^{-1} for EEAb2.*

Table 4.25 : Intra-day CVs and accuracies for EEAb2 inhibition BIAcore assay in bile. Three sets of eleven standards were analysed on the same day and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (RU)	Coefficients of variation (CVs), (%)	Accuracy, (%)
100000.0	108.6 ± 4.6	4.24	78.82
50000.0	112.8 ± 3.2	2.85	145.76
25000.0	169.8 ± 4.8	2.80	108.41
12500.0	232.8 ± 6.1	2.62	77.73
6250.0	266.5 ± 3.8	1.43	88.77
3125.0	287.0 ± 15.4	5.37	124.50
1562.5	344.4 ± 21.3	6.17	81.02
781.3	356.5 ± 1.5	0.42	123.17
390.6	387.0 ± 29.1	7.53	110.63
195.3	408.9 ± 7.4	1.80	106.80
97.7	431.4 ± 14.2	3.28	77.24

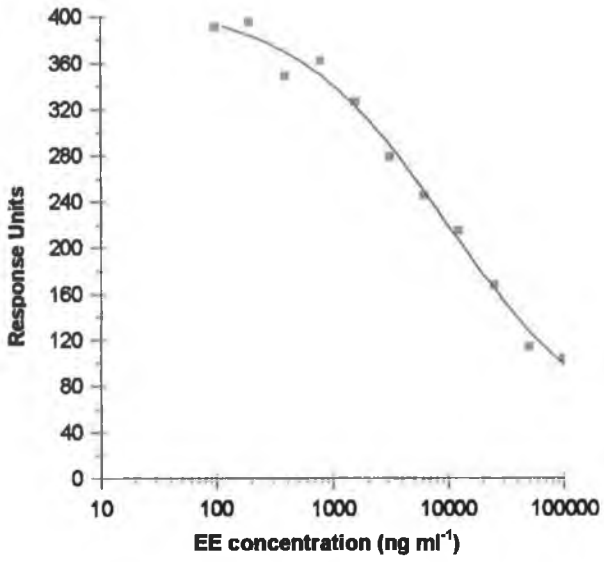


***Figure 4.37** : Inter-day BIAcore assays for EE in bile using EEAb2 polyclonal antibody. The BSA conjugate was coated at 50 μg ml^{-1} onto the surface of the chip for the assays. The antibody was used at a concentration of 34.2 μg ml^{-1}.*

Table 4.26 : Inter-day CVs and accuracies for EEAb2 inhibition BIAcore assay in bile. Three sets of eleven standards were analysed on three different days and the CVs were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

Ethynyl Estradiol Concentration, (ng ml^{-1})	Calculated mean ± SD, (RU)	Coefficients of variation (CVs), (%)	Accuracy, (%)
100000.0	104.8 ± 3.6	3.46	84.70
50000.0	114.5 ± 7.5	6.57	134.46
25000.0	168.4 ± 12.7	7.51	93.44
12500.0	215.3 ± 17.4	8.10	86.15
6250.0	246.1 ± 19.4	7.88	104.75
3125.0	279.5 ± 6.6	2.35	118.86
1562.5	327.0 ± 43.9	13.41	91.85
781.3	362.6 ± 24.3	6.70	66.90
390.6	349.2 ± 39.7	11.38	205.95
195.3	396.0 ± 39.4	9.94	40.43
97.7	391.0 ± 35.6	9.10	126.14

4.3 Discussion

The structure of EE has no side groups which could be easily conjugated that are not involved in either the function of the molecule or its uniqueness compared to other steroids. To allow the conjugation of this small molecule to a carrier a common derivative, EE-6-CMO, is used. This molecule provides a carboxy group at the 6 position through which attachment can be carried out without affecting the recognition sites on the molecule. Two chemistries were used for conjugation of EE-6-CMO, i.e. a mixed anhydride and a NHS-ester method. Both of these chemistries form an amide bond between the -COOH group of EE-6-CMO and -NH_2 groups located on the carrier molecule.

All conjugates were dialysed extensively in dialysis tubing with a molecular weight cut-off of 10,000. This allows any unconjugated steroids to pass into the dialysis vessel, as EE-6-CMO has a M.W. of 383.4, whereas all the protein and any EE bound to it will remain inside the tubing. This ensures that any further analysis of the conjugation will not show any interference from any free EE-6-CMO. The spectrums of the conjugates were analysed to confirm the success of the conjugation. EE-6-CMO shows a distinct peak at 310nm that is not a characteristic of any of the protein carriers. The presence of this peak in the spectrogram of the dialysed conjugate indicated the presence of the bound hapten. The conjugates were also tested against commercial antibodies (data not shown) to further confirm the presence of the bound hapten.

Polyclonal antibodies were produced using New Zealand White Rabbits and the EE-6-CMO conjugates. The rabbits were boosted until the titre reached at least 1/256,000 and were then sacrificed. Rabbit two was sacrificed after two booster injections and rabbit one was left for a further three boosts. The antibodies were purified by saturated ammonium sulphate precipitation followed by protein G affinity chromatography, using low pH elution conditions. The purified proteins were characterised by SDS-PAGE, (Figure 4.5) and the two bands corresponding to the heavy and light chains of the antibody can be seen. The heavy and light chains are dissociated and separated clearly as SDS-PAGE separates proteins by size under denaturing conditions. No other bands are seen in the purified sample.

The polyclonal antibodies were produced for use in testing systems for the detection of free EE in solution. The ELISA test systems were developed first with

competitive and inhibitive formats (Figure 3.1.4). With the competitive assay free EE and EE-6-CMO-HRP compete for binding to the polyclonal antibody that is absorbed on the surface of a 96 well immunoplate. The amount of bound HRP is then detected. To optimise the antibody concentration required for the coating the antibody must be the limiting reagent in the assay. If too much antibody is available to bind the free and conjugated EE, the assay will not show a significant change in signal with small increases in free EE concentration. The optimal working dilutions for the EE-6-CMO-HRP conjugate were taken from the linear region of the conjugate dilution curve, at approximately 50-70% of maximum binding. At this stage of the response curve small changes in conjugate concentration give the maximum change in signal. Non-competitive ELISAs were carried out to determine the optimum dilutions for the assays and the results were a coating of 2 $\mu g\ ml^{-1}$ of EEAb1 with an EE-6-CMO-HRP concentration of 0.119 $\mu g\ ml^{-1}$, and 1.71 $\mu g\ ml^{-1}$ of EEAb2 and 0.478 $\mu g\ ml^{-1}$ of EE-6-CMO-HRP.

In the inhibition format the EE-6-CMO-BSA conjugate is absorbed onto the plate and the antibody and free EE is added. The free EE inhibits the amount of antibody that can bind to the conjugate on the surface of the plate. A HRP-labelled secondary antibody then detects the amount of antibody bound. In the inhibition assay it is also necessary to have the concentrations of the coating and the added components optimised. The antibody is still the limiting reagent even though it is no longer bound to the surface of the plate but it is also necessary to ensure that the amount of conjugate absorbed to the surface of the plate is not too high. This would result in the antibody no longer being the limiting factor as it could all be bound to the immobilised conjugate. Non-competitive ELISAs were carried out to determine the optimum concentrations of both the antibody and the coating conjugate and they resulted in an EE-6-CMO-BSA concentration of 0.16 $\mu g\ ml^{-1}$ being used with an EEAb1 concentration of 0.5 $\mu g\ ml^{-1}$ and 0.57 $\mu g\ ml^{-1}$ of EEAb2 with a coating of 0.2 $\mu g\ ml^{-1}$ BSA conjugate.

Using the optimised conditions for the assays the detection ranges of the assays were determined. A wide range of free EE concentrations from 0.38 - 100,000 ng ml^{-1} were prepared in PBS/2% (v/v) EtOH and assayed within the ELISA test systems. The antibodies showed competition in these ranges for both assay formats. The EEAb1 competitive assay had a range of detection of 1.5 - 25,000 ng ml^{-1}. The

inhibitive assay had a range of 3 - 50,000 ng ml^{-1}. The EEAb2 antibody had a range of 48.8 - 50,000 ng ml^{-1} in the competitive assay and 1.5 - 50,000 ng ml^{-1} in the inhibitive format.

The results of these assays were statistically analysed to show their reproducibility. Intra-assay (5 replicates within an assay) and inter-assay (5 assays over 5 days) variations were analysed. The coefficients of variations (standard deviation of the data set as a function of the mean) were calculated and Table 4.27 shows a summary of the ranges of CVs found in each assay.

Table 4.27 : CV ranges of the inhibitive and competitive ELISAs for the detection of free EE in PBS/2% (v/v) EtOH.

	Competitive		Inhibitive	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
EEAb1	0.83 - 5.94%	4.35 - 11.55%	0.74 - 4.29%	0.84 - 6.98%
EEAb2	1.81 - 5.99%	1.56 - 10.40%	1.88 - 4.59%	3.48 - 10.42%

The CVs for all these assays are well within the suggested acceptable limits for the precision of an immunoassay of below 20% (Findlay *et al.*, 2000).

The data sets showed good agreement with a 4-parameter model fit. This model was used to fit trendlines to the data using BIAevaluation 3.1 software.

While the CVs are acceptable for the majority of these assays, at the extreme points of the range the accuracies become unworkable. At the present time an accuracy range of 80-120% is recommended, although some workers have suggested a less stringent criteria of 75-125% for immuno-based analytical techniques (Findlay *et al.*, 2000).

The accuracies of these assays can be improved by reducing the working range of the assays to one that does not include the inaccurate outlying points. These points are tending towards the asymptotes of the dose response curve and in these areas a large change in concentration may only affect a minimal difference in measurable signal, leading to large errors. The working range of the assays should not contain these areas of the dose response curves, as the assay cannot assess the concentration of a sample accurately at these extremes. The following list gives the recommended ranges for the detection of free EE in PBS/2% (v/v) EtOH in each of the ELISAs.

- EEAb1 Inhibitive 6.1 - 25,000 ng ml^{-1}
- EEAb1 Competitive 3.1 - 6,250 ng ml^{-1}
- EEAb2 Inhibitive 97 - 25,000 ng ml^{-1}
- EEAb2 Competitive 195 - 12,500 ng ml^{-1}

Once the principle of the ELISA assays had been proven using these antibodies and conjugates, the ability of the reagents to detect EE within a biological matrix was assessed. There are many different matrices that could have proven useful for the detection of illegally administered steroid hormones including urine, hair and serum, but it was decided to use bile for these assays. Bile is the natural storage area for steroid hormones in the body and it has been shown that some residues from administered steroids persist there longer than in other biological fluids (M^{c}Evoy *et al.*, 1998).

The bile reduced the measurable signal when it was used neat in the assays so to minimise this effect it was diluted 50:50 with PBS before use. By carrying out this slight pre-treatment of the matrix many of the interference effects were reduced. However, it would also raise the overall lower limit of detection of the assay. The assay graphs (Figures 4.16 - 4.23) and accompanying statistical tables (Tables 4.10 - 4.17) are shown for the assays when bile was used as the sample diluent.

The CVs for the assays are comparable with those found in the assays using PBS as a sample diluent. A comparison of the CV ranges (Table 4.27 and 4.28) shows that the variation within the assay when bile is used as a sample matrix is almost as low as that found in the model assay.

Table 4.28 : CV ranges of the inhibitive and competitive ELISAs for the detection of free EE in bovine bile.

	Competitive		Inhibitive	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
EEAb1	2.13 - 5.19%	2.25 -10.80%	1.10 - 5.65%	1.79 -10.70%
EEAb2	1.97 - 4.36%	4.17 - 8.17%	0.62 - 4.37%	2.61 - 6.69%

The 4-parameter model was used to fit standard curves to the ELISA data and used to calculate the accuracies of the assays. The inaccuracy of the bile assays is greater than those found for the assays using PBS/2% (v/v) EtOH. As seen for the model

assays the elimination of inaccurate points at the extreme ends of the limits of detection can bring about some improvement for some of the assays but not all.

- EEAb1 Inhibitive 48 - 50,000 ng ml^{-1}
- EEAb1 Competitive 12 - 12,500 ng ml^{-1}
- EEAb2 Inhibitive 390 - 100,000 ng ml^{-1}
- EEAb2 Competitive 781 - 100,000 ng ml^{-1}

The assays in general fit well to the 4-parameter model but the inhibitive assay using EEAb1 antibody for the detection of EE in bile shows a very inaccurate mid-section. This trend was seen throughout both the intra- and inter-assay tests. This dip was not seen in the same assay when PBS was used as a diluent and it was not seen in the competitive assay using the same antibody and diluent. If it were a problem inherent with the antibody it would be expected that the error would be present in other assays using it and this is not the case. Is the bile matrix affecting the assay? This is possible but again the trend is not seen in the other assays using bile. One theory to explain the anomaly may be the interaction of the polyclonal antibody population with the bile but again the use of the same antibody but with a different assay format did not show this anomaly. It is possible that in the competitive assay format the fact that the antibody was immobilised on the surface may confer some immunity to this error. These standards were also run on the edge of a plate, one across the bottom and one across the top. This seems like a possible explanation but these wells were used in other assays and the inaccuracy was not seen. The assay was also run changing the positioning of the standards but this did not alter the outcome. It is recommended, in the light of these points, not to use this assay for the detection of EE in the range where the anomaly is present.

The use of bile as the sample matrix alters the limit of detection of the assays. It affects all of the assays in a similar way. In all cases the assays lose sensitivity, being 4-fold for both competitive assays and the EEAb2 inhibitive assay and 8-fold in the case of EEAb1 inhibitive assay. The upper limit of detection is also raised but not necessarily to a similar extent as the lower limit. This means that the dose response curve has been shifted to the right, (comparing Figures 4.8 - 4.15 to 4.16 - 4.23). This may be due to some of the proteins contained within the bile that would ordinarily associate with natural estradiols. These may bind to free, conjugated or

immobilised EE and not allow the binding reactions with the antibodies to occur as they do in the assays using PBS/2% (v/v) EtOH as the diluent. The constituents of the bile fluid may also be inhibiting the antibody binding reaction by occupying the antigen binding sites of the antibodies or binding the steroids themselves. Despite this, the assays described are reproducible and maintain a good level of accuracy with the spiked bile samples.

To assess the specificity of the anti-EE polyclonal antibodies that were produced for this project they were both tested against a number of related compounds (as listed in Table 4.18). EEAb1 contains a more specific antibody population as it has much lower cross reactivities to related molecules with all the steroids tested having a cross-reaction value of less than 2%. EEAb2 shows much greater levels of cross-reaction with the same panel of drugs, e.g. 73.46% with norethisterone. This could have major implications for the use of this antibody for the detection of EE in different contexts. EE is often used in concert with norethisterone in the contraceptive pill (Dyas *et al.,* 1981). Each of the compounds that cross-react with the polyclonal antibodies to any extent are similar to EE about the D ring. They each have a similar group at the 17 position and in addition they may have the same groups with the same orientation at positions 13 and 14. This indicates that the antibodies are directed against this end of the molecule. The point of attachment for the immunogen was position 6, which allows more of the D-ring to be exposed to the immune system than any other part of the molecule.
The data also shows much higher cross reactivities with EEAb2, the antibody that was harvested after only 2 booster injections. This kind of high cross reaction could be due to the shorter immunisation schedule. For example, norethisterone cross-reacts with EEAb2 at a level of 73% and this falls to 0.5% after a further 3 booster injections. This difference could be a function of the different immune systems of the two rabbits but it may also be affected by longer exposure of the antigen to the immune system, allowing for a refinement of the antibody population.

The most sensitive assay developed in this study is a competitive assay using the EEAb1 anti-EE polyclonal antibody. It can detect as little as 12 ng ml^{-1} of EE in a sample of bile with minimal pre-treatment.

The radioimmunoassay described by Agasan *et al.* in 1994 is able to detect much lower amounts of EE in both buffer and plasma. However, the monoclonal antibodies used showed high cross-reactivity with norethynodrel (25.7-100%), norethisterone (5.5-26.1%) and ethynylestradiol-3-sulphate (11.4-52.3%) (Agasan *et al.,* 1986). The assays developed here may not be as sensitive but they do not show the same high levels of cross-reactivity and the are also free from radio-active labels and so the assays are easier to carry out and safer for the workers involved.

Turkes *et al.* (1981) described an enzyme immunoassay for the detection of EE in plasma samples. The plasma was extracted and subjected to the EIA procedure. The sensitivity again was much lower than the ELISAs developed in this chapter but this assay uses 2 overnight incubations, one in the extraction procedure and one in the assay itself. This is a substantial time lag if results are required to prevent food entering the food chain. Also this assay is run in immunotubes and so requires more reagents and space to run assays. Microtitre plates are much easier to handle and can be used to screen many samples simultaneously.

The commercial ELISA kit used by Sawaya *et al.* (1998b) to measure the amounts of EE in sheep urine and chicken muscle was produced by R-Biopharm. This assay was sensitive to a low $\rho g\ ml^{-1}$ level. After investigation of the two matrices a cut-off concentration of 0.3ppb was chosen. Below this amount the false positive rate was substantial and the introduction of the cut-off point reduced the number of negative results being treated as positive. The ELISA assays in this chapter are not as sensitive as this kit assay but they again have the advantage of simple sample pre-treatment.

The assays developed here must be improved further to obtain an assay that can compete with those described above. The assay must be able to detect low nanogram quantities of EE in samples. The EU has a zero tolerance for this compound in animals but in practice this translates to action values of 2ppb in urine and 0.5ppb in muscle. Any screening assay must be able to detect at these levels to be useful in the elimination of residues of this steroid from the food chain. The use of chemiluminescent or fluorescent-based assays may improve the sensitivity of the assays described. Delayed fluorescent immunoassays (DELFIA) may also prove to enhance the detection capabilities of these reagents.

The BIAcore chip (CM5) used in this project has a carboxymethylated dextran surface to which the conjugate was immobilised. To establish the optimum pH for the immobilisation of the EE-CMO-BSA conjugate to the surface of the chip a preconcentration study was performed. Figure 4.24 shows the results of this experiment. A pH of 4.2 was chosen to perform the immobilisation. This was not the highest peak but as no peak between 4.2 and 4.6 was significantly better than another, a pH that suited the linking reagents was selected. Using this pH the conjugate was linked to the surface of the chip using a NHS-ester method. An example of the binding curve obtained with the immobilisation protocol is shown in Figure 4.25. The total amount of conjugate bound to the surface, in RU, is the change in values between the baseline before conjugate addition and the baseline after ethanolamine capping.

The ability of the antibodies to bind to this surface was evaluated. A number of solutions containing different amounts of antibody were passed across the surface until a response of approximately 500 units was seen. This concentration of antibody was used for all subsequent experiments using HBS as sample diluent, being 16 $\mu g\ ml^{-1}$ for EEAb1 and 15.2 $\mu g\ ml^{-1}$ for EEAb2

The non-specific binding of the antibodies in this system were investigated. They were passed over an unmodified dextran surface, a BSA-immobilised surface and surface with the EE conjugate immobilised. The results (Figure 4.26) show that the antibody has negligible binding to either of the non-specific surfaces, but shows substantial binding to the conjugate-coated surface.

Each time the antibody binds to the surface of the chip it must be removed to allow another cycle to begin. The solutions required to remove the antibody and yet not denature the binding surface of the chip must be determined. A number of solutions were investigated including HCl and NaOH (10-100mM), alone and in combination, NaOH supplemented with 5-20% (v/v) acetonitrile and 1M ethanolamine, pH 13.6. Regeneration studies were carried out by determining the reproducibility of antibody binding to the surface over a course of antibody binding/regeneration cycles. If the amount of antibody binding remained relatively stable over a substantial number of regeneration cycles the regeneration protocol was taken to be sufficient. Figures 4.27 and 4.28 show the regeneration profile of the two antibodies using 1M ethanolamine pH 13.6 for EEAb1 and 30mM HCl

followed by 30mM NaOH/10% (v/v) acetonitrile for EEAb2. Both regeneration protocols require the surface to be equilibrated before any assay is performed and this is achieved by passing a number of regeneration cycles across the surface. This ensures that any loosely immobilised conjugate is removed and the regeneration is only disrupting the antibody to surface binding.

The regeneration curve in Figure 4.28 shows the typical regeneration profile of most conjugate-bound surfaces. A slight drop is seen across the extent of the study. It has been proposed that once the surface loses no more than 20% of its original binding over the course of an assay then it can be deemed within tolerances (Wong *et al.*, 1997). The profile seen in Figure 4.27 for the regeneration of EEAb1 shows a different trend. This seems to have more binding after repeated regeneration cycles. This is not actually the case and it is purely an effect of the ethanolamine on the surface matrix. The same trend is seen if ethanolamine is passed over an immobilised surface without any antibody binding at all, (data not shown). The regeneration solution may cause a stretching or deformation of the dextran matrix that results in this slight increase in the amount of binding measured over time. Even though this is an unusual effect it would still lie within the 20% variation that has been proposed as agreeable.

Assays were carried out using the antibodies and EE spiked into HBS/2% (v/v) EtOH. The antibody solution in HBS was mixed in equal volumes with the EE samples and incubated for 1h at 37°C. The samples were then passed over an immobilised surface for the required time in triplicate and in random order. The random order is to facilitate the removal of any machine or assay bias that may occur if the samples are run consecutively.

For these assays, like the previous ELISAs, the reproducibility and accuracy of the assay was investigated. Each assay was run in triplicate and repeated three times. This lead to intra- and inter-day precisions being calculated along with the standard curves for the assays, Figures 4.30 - 4.33 and Tables 4.19 - 4.22. Table 4.29 below gives the ranges of the percentage coefficients of variation for the assays. The antibody response curves for the BIAcore assays also followed the 4-parameter model and this was used to fit the standard curve and calculate the percentage accuracy of it.

Table 4.29 : CV ranges of the inhibitive BIAcore assays for the detection of free EE in HBS/ 2% (v/v) EtOH and in bovine bile.

	HBS/ 2% (v/v) EtOH		Bovine Bile	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
EEAb1	0.51 - 2.94%	1.05 - 8.08%	0.28 - 11.01%	0.58 - 19.70%
EEAb2	0.21 - 10.13%	1.81 - 20.74%	0.42 - 6.17%	2.35 - 13.41%

The majority of the % CV ranges for the BIAcore assays are higher than those of the corresponding ELISAs but that fact is deceptive as most of the figures are at the lower end of these ranges allowing them better overall precision. The accuracies are also closer to the value of a 20% deviation of normal. Again there are some inaccuracies at the extremes of the assay where it is approaching the asymptotes of the fit. These outliers may be removed to provide a more accurate assay system. The suggested ranges for the detection of free EE in HBS/ 2% (v/v) EtOH are

- EEAb1 BIAcore Inhibitive 97 - 25,000 ng ml^{-1}
- EEAb2 BIAcore Inhibitive 97 - 50,000 ng ml^{-1}

After developing the working BIAcore assay the chosen matrix, bile, was tested in the system. The assays again look like they have higher % CV values than the corresponding ELISAs, (Table 4.29) but again the majority of the CVs are at the lower end of the range. Each of the BIAcore assays in the bile matrix show greater inaccuracies at the lower range of the assay. This leads to a loss in sensitivity and reliability of the assay in this region. As before the assay ranges given below show the most accurate range for the BIAcore assays.

- EEAb1 BIAcore Inhibitive 48 - 100,000 ng ml^{-1}
- EEAb2 BIAcore Inhibitive 1,563 - 100,000 ng ml^{-1}

These detection ranges for EE represent assays that have accuracies close to the less stringent 75 - 125% limits that have been proposed for immunoassays. Some refinement of the pre-incubation procedure may increase the accuracy although assays that did not use the pre-incubation and instead used a 10min incubation

within the machine for each sample did not show any great improvement in accuracy.

Previously, a BIAcore assay for the detection of EE has been described (Johansson and Hellenäs, 2001). This assay showed a standard curve between 0.1 - 3 ng ml^{-1} allowing the detection of EE below the action limits of 0.5 - 2 ng ml^{-1} depending on the matrix. The major advantage of this assay was the direct immobilisation of the steroid to the sensorchip surface. This gave the chip greater regeneration stability and the ability to be used for hundreds or even thousands of assays. This will make the assay very cost effective. The assays described in this study do not have unlimited regeneration capability because the proteins through which the steroids are linked to the surface are prone to degradation during regeneration protocols. Further work must be carried to investigate whether the immunoreagents developed in this study could be used directly immobilised to the chip and in this way produce a more sensitive and longer lasting assay (Gillis *et al.*, 2002).

Using EEAb1 the competitive ELISA format showed a slightly lower assay range in both the model assay and the assay when bile was used as a sample matrix than that seen for the inhibitive format. The ranges in both EEAb1 ELISAs are substantially better than those seen with the second antibody, EEAb2. Unlike the first antibody, EEAb2 gives a more sensitive assay using the inhibitive ELISA format, but it is still at least 8-fold less sensitive than the corresponding assay using EEAb1. This trend was also found in the BIAcore assay results. The two assays show similar detection ranges for the model biosensor assay but EEAb1 shows substantially better detection for free EE than EEAb2 when the bile sample matrix is used.

The ELISA assays can be carried out quickly and simply with minimal amounts of equipment. They can screen many samples simultaneously and they would be ideally used where large numbers of animals were to be tested. The use of bile as the sample matrix lends the assay to the detection of bile in slaughter houses where this matrix is readily accessible. It will provide results within a couple of hours and at that stage carcasses can be held for further confirmatory testing.

The BIAcore assay could also be used in this manner and it would provide results as each sample is analysed and not in a batch fashion as the ELISA system. This

would provide an even faster response to residue containing meat. The BIAcore assay can take anything between 5-10 mins to complete the analysis of each sample and so depending on the numbers of samples to be tested the ELISA may actually provide analysis of more samples in a day than the BIAcore assay.

In summary, EE-6-CMO was used to produce protein conjugates as both immunogens and assay reagents. These conjugates were used to produce anti-EE polyclonal antibodies. These antibodies were isolated from the rabbit serum and used to develop competitive and inhibitive ELISAs along with a BIAcore-based immunoassay to detect nanogram amounts of free EE in samples. Analysis of these assays shows both to be reproducible and accurate. These assay systems were then tested using bovine bile, a complex sample matrix. The results show that the antibodies and the systems developed can be used to detect free EE in bile with close to the same sensitivity as the model assay. The assays are not sensitive enough to provide screening at the recommended levels of 2ppb but further work such as the use of chemiluminescent substrates may increase the sensitivity of the assays to this required level (Kohen *et al.,* 1983; Adamczyk *et al.,* 2000). The studies on the cross-reactivities of these antibodies show that EEAb1 has a high specificity for EE, but EEAb2 exhibits much higher interference from related compounds.

CHAPTER 5

DEVELOPMENT OF A RAPID ELISA METHOD USING A PORTABLE AUTOMATED DEVICE

5.1 Introduction

5.1.1 Rapid Assays

Until recently the predominant usage of immunoassay methods had been confined to laboratories with the correct equipment and trained personnel (Paek *et al.,* 2000). However, immuno-analysis has evolved from this starting position to one where it can be used in a variety of surroundings by relatively untrained operators. The current trends in the development of such on-site testing systems are moving towards the use of two technologies, the immunochromatographic test strip or lateral flow immunoassay and the immunosensor (Choi *et al.,* 1999). The theory behind these assay formats is described in section 1.4. The automation of existing immunoassays is also being investigated for use in areas outside the lab environment.

Lateral flow immunoassays can be used to detect a wide range of analytes, e.g. digoxin (Choi *et al.,* 1999), cannabinoids (Wennig *et al.,* 1998) and trinitrotoluene (TNT, Heiss *et al.,* 1999), using the formats described in section 1.4.5. A dipstick dye immunoassay for schistosomiasis diagnosis has also been developed which would be of great benefit in the areas where this disease is prevalent (Zhu *et al.,* 2002). The lateral flow immunoassay can also be improved by the addition of extra steps within the test strip. A lateral flow immunoassay has recently been described that separates isoforms of proteins before analysis, all on a single assay strip (Lönnberg and Carlsson, 2000). In this form, the sample is first subjected to an anion exchange membrane, which will retard the flow of the unwanted protein isoforms and let the active isoform to proceed along the strip. The fraction containing the target isoform will continue along the strip and bind at the immobilised capture antibody. A second labelled antibody is then added to the base of the strip (post-anion exchange section) and will bind to any immobilised protein caught by the capture antibody. The label can then be visualised and the amount of protein determined. This system could be used in many different applications where samples may need to be separated before analysis. By altering the primary exchange system from an anion to a cation exchange, size exclusion or affinity system the strip could be used to separate many different types of analytes to provide more accurate and specific test strips.

Portable immunosensors may also be developed in the near future in formats that can be readily transported and used at different sites. Examples of this type of technology would be the BIAprobe (Figure 5.1) and the Texas Instruments Spreeta device (Figure 5.2). Both of these systems contain a sensing surface to which one half of a binding couple is immobilised. The BIAprobe can be immersed in the sample and any binding occurring will be detected through the same SPR phenomenon used for the BIAcore systems (section 4.1.5). The bound molecules can then be stripped and the probe used again. It may also be possible to mass-produce the sensing tips of the probe and so provide a disposable sensing platform that can be changed after each determination. It would also be possible to provide probes with different specificities to make this a universally useful assay system.

The Spreeta device works more like a miniature version of the BIAcore system. It utilises a flow system to bring the sample into contact with the sensing surface to allow binding and also to remove the bound molecules for the re-use of the surface. Both of these systems can be connected to a laptop computer for data acquisition and analysis outside the laboratory. With the correct immunoreagents these systems can be used in many areas to provide fast and accurate determinations of the presence or absence of numerous analytes that require monitoring.

A reduction in the time needed to perform an assay is also of interest. If these assays can be carried out in shorter times they become candidates for use in the field. Some workers have looked at the application of enzyme immunoassays to detect analytes in less than 30min (Yolken and Leister, 1981; Claycomb *et al.*, 1998). There are numerous automated immunoassay systems on the market but most of them are designed for use in a laboratory environment (Wild, 1994). Many of these systems will become very useful as the trend towards home and bedside testing continues in the health sector (Lehmann, 2002).

The enzyme immunoassay device used in this work was designed to be easy to use and portable. It is small and can be mounted in the boot of a car or even on the dashboard (Figure 5.1). This device combines the shortening of the immunoassay procedure with a portable automatic system to provide a useful analytical device for use in the field. This device is explained in detail in the section 5.1.3.

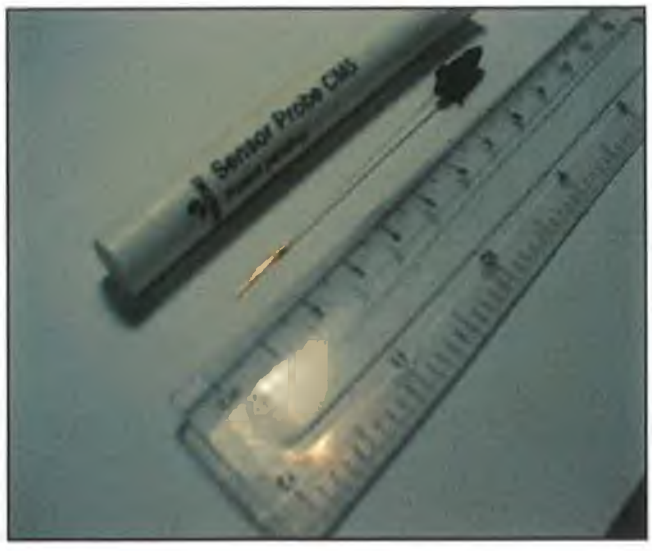


***Figure 5.2** : The BIAprobe. A small thin fibre optic that has had one end coated with a gold layer. This is the sensing end of the probe. It is immersed and the signal is passed from the probe to a data acquisition device. Binding is detected using a combination of the light passed through the fibre optic and SPR.*

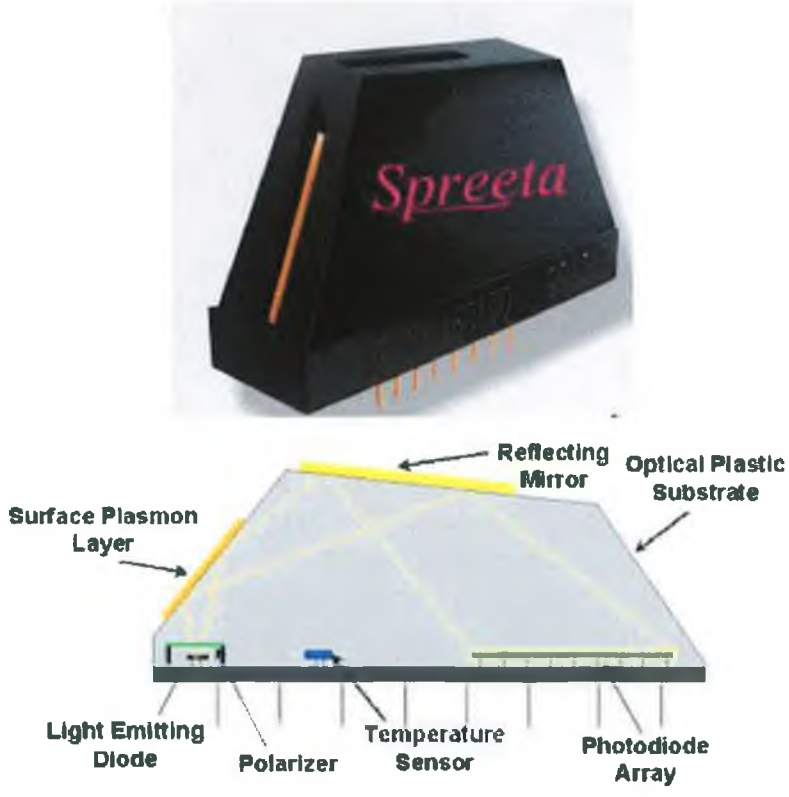


***Figure 5.1** : Picture of the Spreeta biosensor from Texas Instruments. This device uses SPR to detect the required change in the sensing environment. The entire assembly is small and fits easily into the palm of a hand. The components of the device are shown in the schematic above.*

5.1.2 Use of Rapid Assay Systems for the Detection of Steroids

Rapid assays, developed for the detection of steroids in less than 30min, are useful because of the conditions under which many of these analyses would be carried out, i.e. in slaughterhouses and on farms. Test strips, rapid enzyme immunoassays and an antibody-based electrodes have all been used as approaches to measure steroid concentration.

Ploum *et al.* (1991) described the development of prototype test strip immunoassays for the detection of nortestosterone and clenbuterol. For this format the drug-specific antibody was dried onto a nitrocellulose strip and the strip was then blocked with BSA and dried. The strip was held in a glass test tube and 1 ml of sample was added to the tube along with 15 µl of the drug-HRP conjugate. The strip was washed in water after a 45-60 min incubation period. The strip was transferred to a tube holding 0.8 ml of substrate and the colour reaction proceeded for 1min. The strip was washed and the presence of colour indicated a negative result and a clear strip indicated a positive result. This assay could take almost an hour to complete and it requires the use of multiple tubes containing different solutions and the strip requires washing between steps. The strip tests were sensitive enough to detect 1-2 ng ml^{-1} of nortestosterone and 10 ng $ml^{-1}$of clenbuterol in urine.

A rapid plate-based enzyme immunoassay for the detection of progesterone has been described (Claycomb *et al.*, 1998). This assay is similar to the competitive ELISA described in Chapters 3 and 4 except for the time it takes to run the assay. This assay immobilises a monoclonal antibody on hydrazine-activated immunoplates. After the wells were washed and blocked, 50 µl of sample is added to wells in combination with 50 µl of a progesterone-HRP conjugate. The plate is incubated for 5min at room temperature with agitation. The plate is washed and the substrate is added and read every 10s for 3min. This assay had a dynamic range of 0.2-20 ng ml^{-1} for this steroid in milk.

The development of an antibody-based electrode that could be used to measure corticosteroids in animals was reported in Nature Bioterchnology (Cook, 1997). Antibodies specific for this group of steroids are immobilised on a platinum electrode. This is small probe encased in a membrane that separates the electrode environment from the sampling one. The electrode measures the peroxidase

activity of the corticosteroid-HRP conjugate. When the electrode is immersed in a solution containing the steroid, it competes for binding to the electrode. The more endogenous steroid present the lower the signal measured by the sensor. The whole assembly is small (350μm) and can be implanted to allow for 'real-time' measurements within the circulating system of an animal. This particular electrode is viable for 200-400 sequential measurements and can detect between 0.2-0.6 μg of cortisol or corticosterone per 100 ml. It is not very sensitive but it does show the possibility of creating small implantable sensors to monitor many compounds of biological significance.

5.1.3 The Prototype Rapid ELISA Device

This device has the ability to automate the steps involved in a competitive ELISA. The machine used a standard competitive ELISA protocol, where sample and conjugate are in competition for the binding positions of the immobilised antibody. The reagents are all prepared in advance, the coated wells, the blank wells, the conjugate solution and substrate. The operator of the machine needs only to put the wells and eppendorfs with their solutions in the correct position and press start. The machine aliquots the sample, mixes it with the conjugate and then transfers it to the reaction wells. It shakes the wells during all the incubation steps to ensure even binding and washes the wells after the reaction has occurred. It then adds the substrate to the wells and the operator reads the result. The device requires no further input from the operator after the initial set-up.

The protocol used in this device is described in detail in section 2.4.8.3. Briefly, it has antibody-coated wells into which it adds a mixture of the sample and a drug-HRP conjugate. After a short incubation period, (4min at 35°C with rapid shaking) the machine washes the wells and tetramethylbenzidine (TMB) substrate is added. The device reads the transmission of the substrate just after it is added to the wells and again after a 5min incubation. These two values are compared to give the percentage transmission result for each well. Figure 5.2 gives the layout of the wells and the eppendorf tubes on the reaction wheel of the machine.

The machine takes 20min to complete each analysis, including the arrangement of all the wells and solutions. Initially all the coated wells contained antibody of

the same specificity. This resulted in an assay that performed each analysis in quadruplicate and a single blank control well. The blank well can be used to relate different runs to each other. The 4 values for the sample are averaged and are then divided by the ratio for the blank well. This gives the analytical result for that particular sample. Each run was analysed in the same manner. To give these results meaning a blank sample (buffer) is analysed. Any sample that gives a value above that calculated for the zero concentration sample is considered positive.

If reaction wells coated with a number of specific antibodies are used the usefulness of this machine can be extended. The relevant conjugate solution can be used and the same sample can be analysed for up to five different substances. This format does not allow for a blank well and so the assay must be optimised and validated precisely. To show the principle of multianalyte testing using this machine an assay for the co-analysis of two steroids within a single sample was developed.

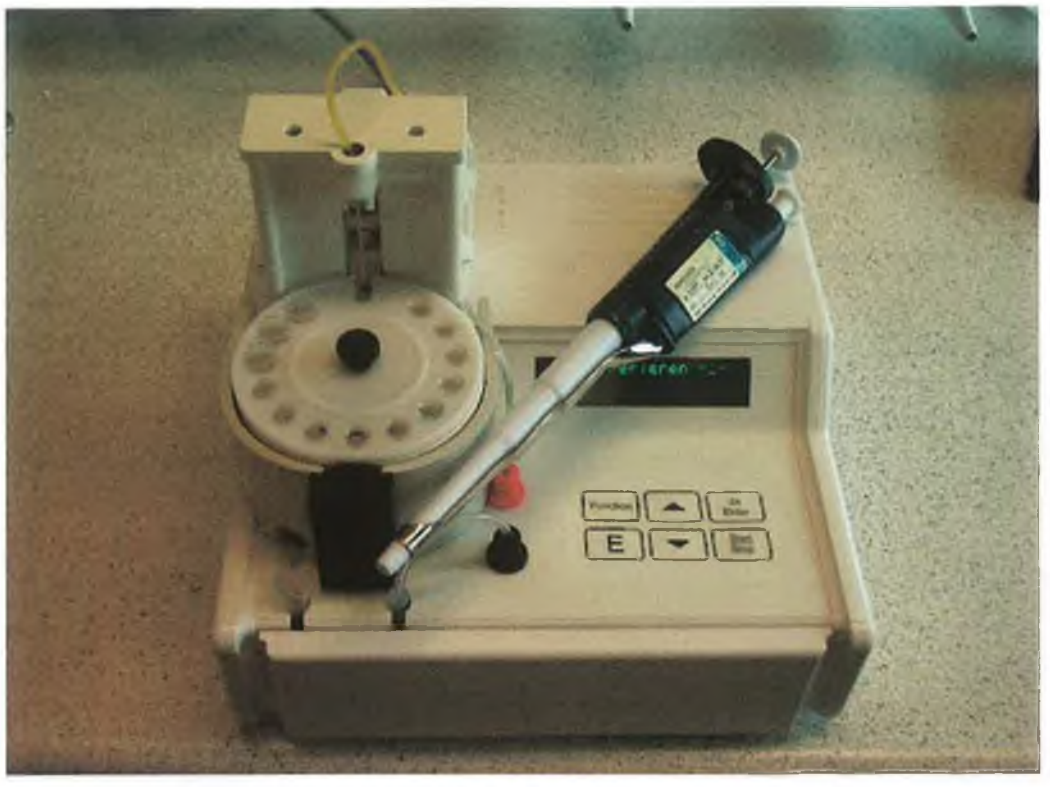


***Figure 5.1** : The prototype rapid ELISA device. The buffer and waste reservoirs are located at the front of the device under the reagent wheel. The placement of the wells on the reagent wheel is described in Figure 5.2.*

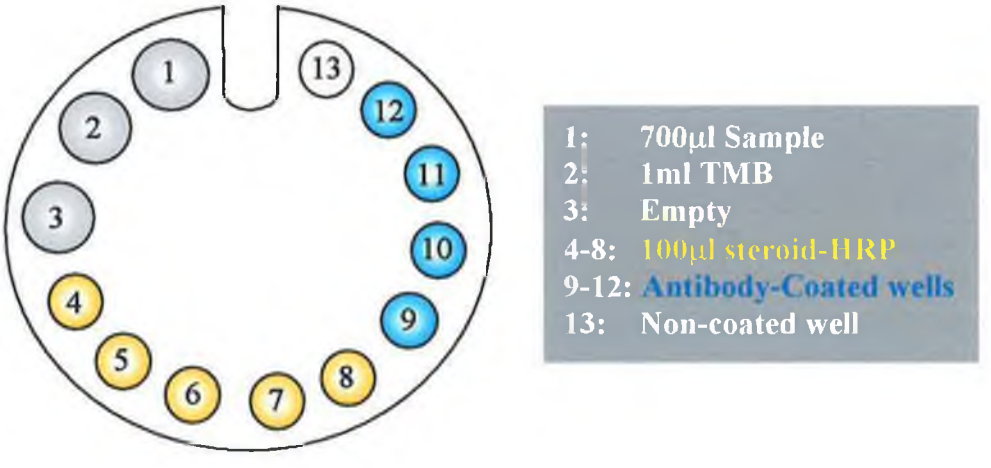


***Figure 5.2** : Layout of the wells as they are positioned on the wheel of the rapid ELISA device.*

5.1.4 The Co-analysis of Two Steroids using the Rapid Assay Device

The use of the rapid assay device is quite limited if it only detects the presence of a single residue. Multi-analyte testing would allow users to reduce the amount of time spent in testing and increase the amount of information produced from each assay run.

The assay format developed in this chapter aims to detect both trenbolone and diethylstilbestrol residues in a sample in a single assay. To achieve this wells were coated with anti-DES and others with anti-TR antibodies. The blank well was retained to allow for comparison between runs.

The conjugate solution was also altered. In the single analyte assay the same conjugate solution is added to all five mixing wells (4-8). This ensures that all reaction wells including the blank well receive the same amount of steroid-HRP conjugate. To maintain a homogeneous conjugate solution in the multi-analyte assay a mixture of the appropriate concentration of the two HRP-conjugates was used. The use of this mixture is only possible if neither of the antibodies show any cross-reaction with the other steroid being analysed or its HRP-conjugate. Any such cross-reaction would cause large errors and no differentiation between the concentrations of the two steroids as separate species would be possible.

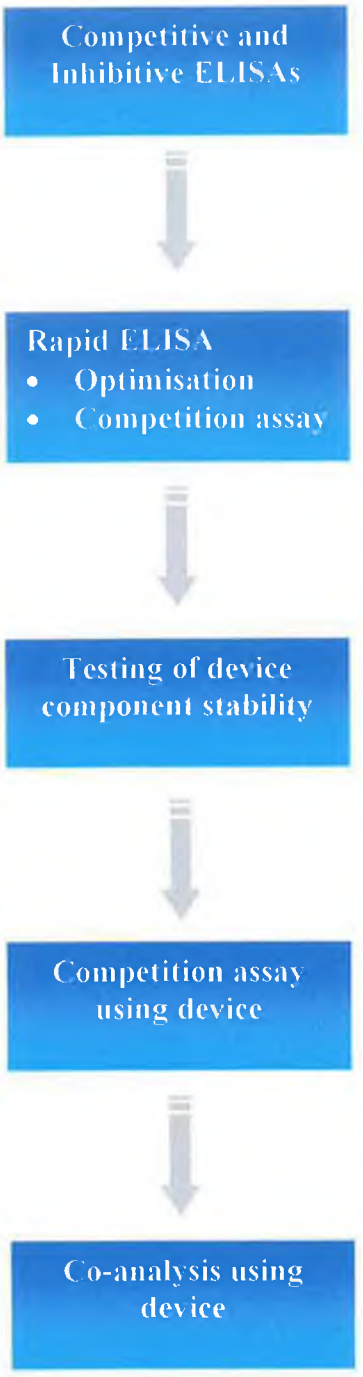


***Figure 5.3** : Development of rapid ELISA and prototype assay for device.*

5.2 Results

5.2.1 Antibodies used in rapid assay development

Polyclonal antibodies against two steroids were used in the development of the rapid ELISA protocol that shortens both the reaction and substrate development incubation times to assay their suitability for use with the rapid ELISA device. The production and characterisation of the anti-trenbolone antibodies is described in Chapter 3. Polyclonal antibodies were also raised against mono-carboxypropyl DES coupled to OVA (DES-CP-OVA) as described in section 2.4.4.1. This immunogen was produced as per section 2.4.1.2 and 2.4.2.2 and the confirmatory mass spectrometry results are contained in appendix B. They were purified by saturated ammonium sulphate precipitation and Protein G affinity chromatography and the purity of the resulting antibody-containing solutions were tested in SDS-PAGE (Figure 5.4).

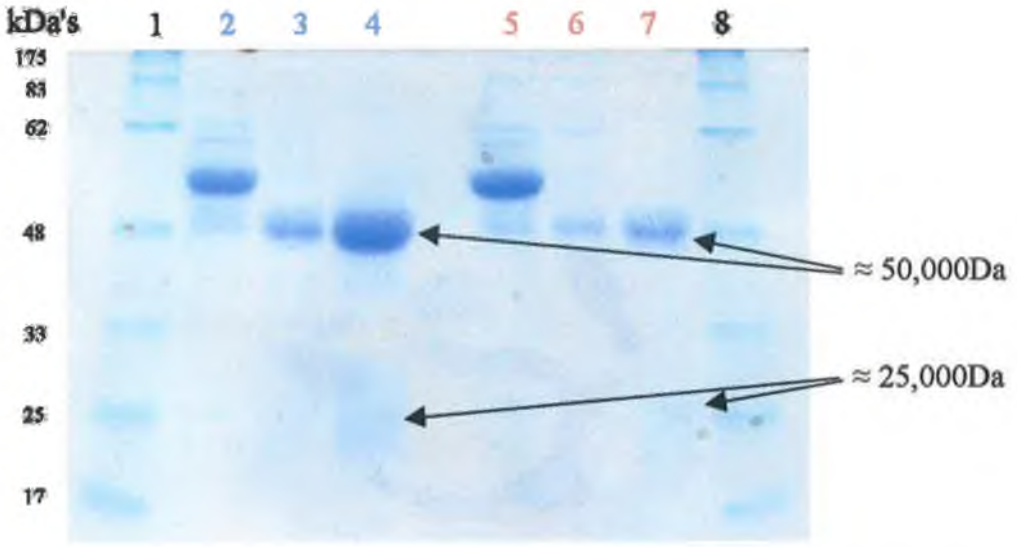


***Figure 5.4** : Coomassie stained SDS-PAGE gel with protein markers in lane 1 and 8. Lane 2: serum from rabbit 1; lane 3: dialysed SAS cut of rabbit 1 serum; lane 4: protein G affinity-purified IgG fraction (DESAb1); lane 5: serum from rabbit 2; lane 7: dialysed SAS cut of rabbit 2 serum and lane 8: protein G affinity-purified IgG fraction (DESAb2).*

5.2.2 Development of Competitive and Inhibitive ELISA for the detection of DES using DESAb1 Antibody

The optimisation of the ELISAs was carried out as described in chapters 3 and 4. Table 5.1 lists the concentrations of conjugates and antibody that were used in the standard length competitive and inhibitive ELISA formats.

Table 5.1 : Optimal coating and competing concentrations used in for the competitive and inhibitive standard length ELISA using DESAb1 as the specific antibody.

Assay	Coating Solution	Competing Solution
Inhibitive	1.38 μg ml^{-1} DES-CP-BSA	6.75 μg ml^{-1} DESAb1
Competitive	0.68 μg ml^{-1} DESAb1	0.31 μg ml^{-1} DES-CP-HRP

A range of standards of DES was prepared as described in section 2.4.7.1. In the inhibitive format the standards were added to a DES-CP-BSA-coated plate at 50 μl per well together with DESAb1 at the required concentration. After the incubation and washing steps had been completed HRP-labelled anti-rabbit pab was added to the plates. In the competitive ELISA format the standards and the appropriate DES-CP-HRP dilution were added to a DESAb1-coated plate. After washing, the bound HRP conjugate was detected in both assays as specified previously, (section 2.4.7).

For intra-day assay variation studies each concentration standard was assayed 5 times on a single day and the mean absorbance was plotted against the DES concentration. Figures 5.5 and 5.7 show the intra-day assay results for the two assay formats. The accompanying tables 5.2 and 5.4 show the means, the standard deviations, the coefficients of variation (C.V.'s) and the percentage accuracy of the standard curve using 4-parameter curve fitting.

The inter-day assay variation was determined by carrying out the assay over 5 separate days. The absorbance of each of the standards was normalised by dividing the mean absorbance value for each DES concentration by the mean value for the day's zero concentration value, (A/A_0). The A/A_0 value was compared across the 5 days. Figures 5.6 and 5.8 show the inter-day assays for both assay formats. The accompanying tables 5.4 and 5.6 show the means, the

standard deviations, the coefficients of variation (C.V.'s) and the percentage accuracy of the standard curve using 4-parameter curve fitting.

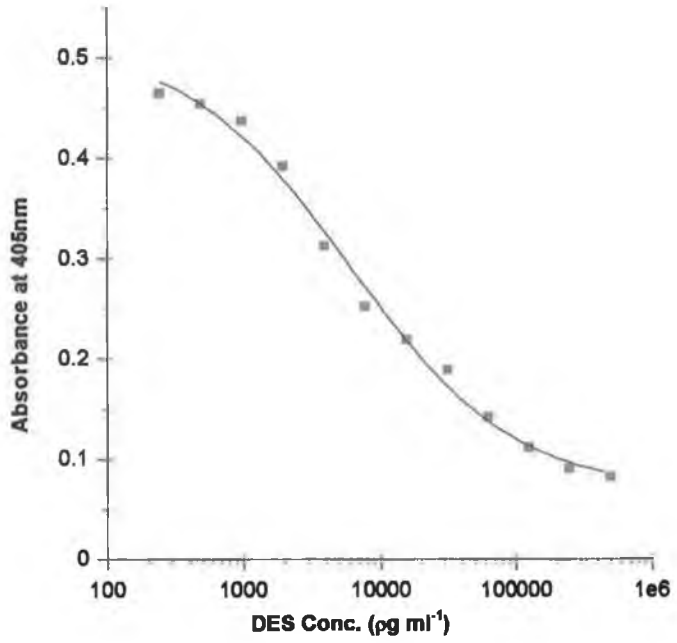


***Figure 5.5** : Intra-day studies on inhibitive ELISAs using the anti-DES Pab, DESAb1 for the detection of free DES in PBS/2% (v/v) EtOH. The inhibition assay uses DES-CP-BSA conjugate coated at 1.38 μg ml^{-1} and the antibody at a concentration of 6.75 μg ml^{-1}.*

Table 5.2 : Intra-day CV's and accuracies for DESAb1 inhibition ELISA in PBS/2% (v/v) EtOH. Five sets of twelve standards were analysed on the same day and the CV's were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

DES Concentration (pg ml^{-1})	Calculated Mean ± SD, (Abs)	Coefficients of variation (CV's), (%)	Accuracy, (%)
500000.00	0.083 ± 0.011	13.50	129.41
250000.00	0.092 ± 0.010	11.01	133.26
125000.00	0.113 ± 0.006	4.93	102.31
62500.00	0.143 ± 0.014	9.64	90.06
31250.00	0.189 ± 0.014	7.36	76.28
15625.00	0.220 ± 0.017	7.86	97.61
7812.50	0.253 ± 0.018	7.04	126.70
3906.25	0.313 ± 0.021	6.80	120.07
1953.13	0.393 ± 0.025	6.40	81.94
976.56	0.438 ± 0.030	6.86	73.89
488.28	0.455 ± 0.023	5.00	99.56
244.14	0.466 ± 0.028	5.91	145.65

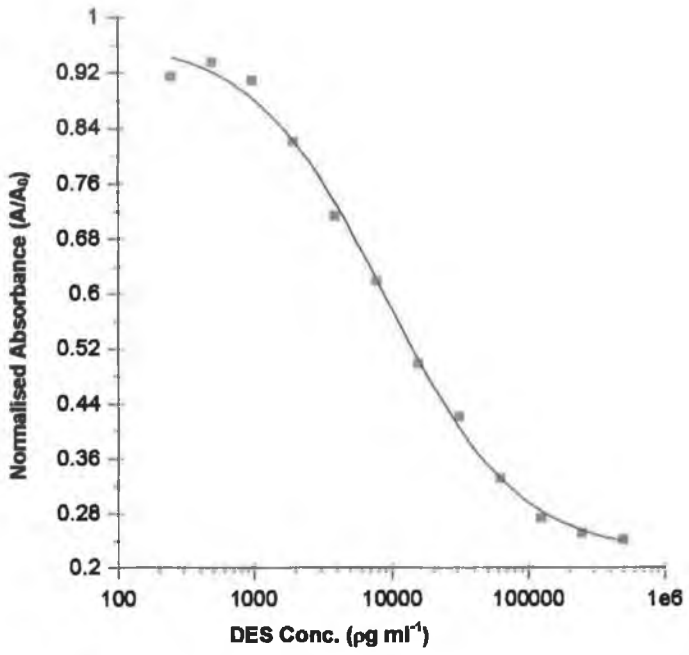


***Figure 5.6** : Inter-day studies on inhibitive ELISAs using the anti-DES Pab, DESAb1 for the detection of free DES in PBS/2% (v/v) EtOH. The inhibition assay uses DES-CP-BSA conjugate coated at 1.38 μg ml^{-1} and the antibody at a concentration of 6.75 μg ml^{-1}.*

Table 5.3 : Inter-day CV's and accuracies for DESAb1 inhibitive ELISA in PBS/2% (v/v) EtOH. Five sets of twelve standards were analysed over five different days and the CV's were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

DES Concentration (pg ml^{-1})	Calculated Mean ± SD, (A/A_0)	Coefficients of variation (CV's), (%)	Accuracy, (%)
500000.00	0.242 ± 0.065	26.97	85.40
250000.00	0.252 ± 0.050	19.92	111.91
125000.00	0.275 ± 0.032	11.65	119.44
62500.00	0.333 ± 0.043	12.98	97.74
31250.00	0.423 ± 0.050	11.71	86.46
15625.00	0.499 ± 0.089	17.77	103.07
7812.50	0.618 ± 0.065	10.49	101.00
3906.25	0.714 ± 0.094	13.19	112.82
1953.13	0.822 ± 0.091	11.03	99.48
976.56	0.909 ± 0.104	11.39	65.09
488.28	0.937 ± 0.082	8.73	63.46
244.14	0.917 ± 0.103	11.25	223.15

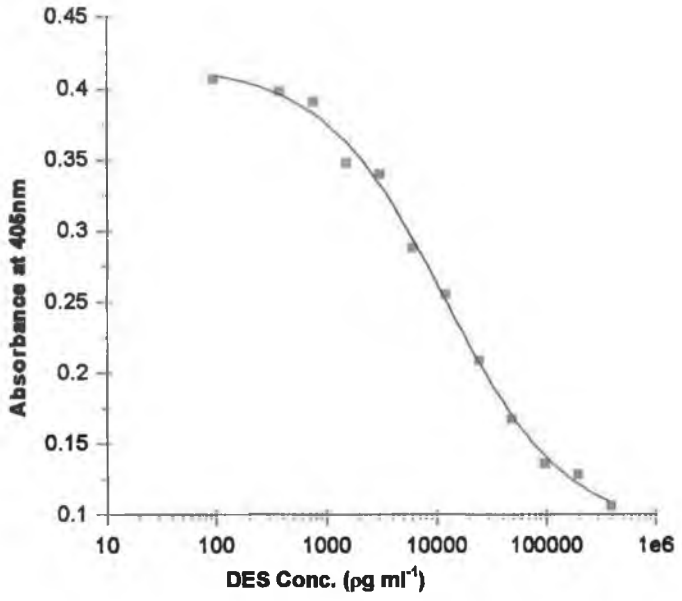


***Figure 5.7** : Intra-day studies on competitive ELISAs using the anti-DES Pab, DESAb1 for the detection of free DES in PBS/2% (v/v) EtOH. The plates were coated with 0.68 μg ml^{-1} solution of DESAb1 and the conjugate DES-CP-HRP was used at 0.31 μg ml^{-1}.*

Table 5.4 : Intra-day CV's and accuracies for DESAb1 competition ELISA in PBS/2% (v/v) EtOH. Five sets of thirteen standards were analysed on the same day and the CV's were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

DES Concentration (pg ml^{-1})	Calculated Mean ± SD, (Abs)	Coefficients of variation (CV's), (%)	Accuracy, (%)
390625.00	0.107 ± 0.010	9.00	117.58
195312.50	0.129 ± 0.006	4.68	75.86
97656.25	0.136 ± 0.017	12.70	117.58
48828.13	0.168 ± 0.013	7.54	104.62
24414.06	0.209 ± 0.011	5.37	97.62
12207.03	0.256 ± 0.001	0.55	92.48
6103.52	0.289 ± 0.005	1.82	110.68
3051.76	0.340 ± 0.019	5.53	86.12
1525.88	0.349 ± 0.028	8.10	142.25
762.94	0.391 ± 0.023	5.94	67.47
381.47	0.398 ± 0.052	12.99	89.12
95.37	0.407 ± 0.010	2.54	156.89

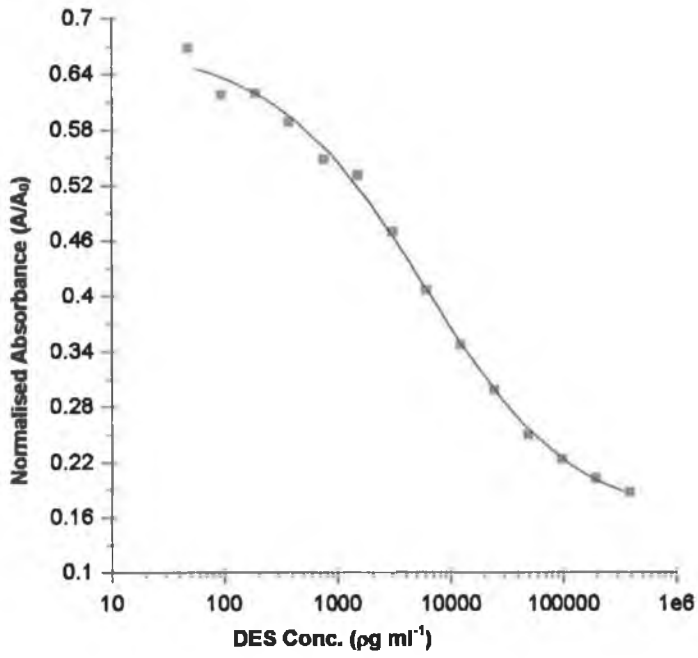


***Figure 5.8** : Inter-day studies on competitive ELISAs using the anti-DES Pab, DESAb1 for the detection of free DES in PBS/2% (v/v) EtOH. . The plates were coated with 0.68 μg ml^{-1} solution of DESAb1 and the conjugate DES-CP-HRP was used at 0.31 μg ml^{-1}.*

Table 5.5 : Inter-day CV's and accuracies for DESAb1 inhibitive ELISA in PBS/2% (v/v) EtOH. Five sets of twelve standards were analysed over five different days and the CV's were calculated. A four-parameter equation was used to fit a curve to the data using BIAevaluation software and its percentage accuracy was calculated.

DES Concentration (pg ml^{-1})	Calculated Mean ± SD, (A/A_0)	Coefficients of variation (CV's), (%)	Accuracy, (%)
390625.00	0.189 ± 0.018	9.27	86.26
195312.50	0.203 ± 0.032	15.54	97.11
97656.25	0.225 ± 0.031	13.88	100.47
48828.13	0.251 ± 0.055	21.96	113.67
24414.06	0.299 ± 0.058	19.29	100.68
12207.03	0.348 ± 0.094	26.98	103.92
6103.52	0.408 ± 0.105	25.76	99.95
3051.76	0.471 ± 0.121	25.72	93.40
1525.88	0.532 ± 0.141	26.49	80.76
762.94	0.549 ± 0.127	23.14	123.36
381.47	0.589 ± 0.149	25.33	115.34
190.73	0.620 ± 0.160	25.84	98.06
95.37	0.619 ± 0.152	24.58	205.70
47.68	0.669 ± 0.168	25.15	0.36

5.2.3. Cross Reactivities of DESAb1 Antibody

The antibody was tested for its ability to recognise other related steroid compounds. The assays were carried out as described in section 2.4.7.8. Table 5.6 shows the percentage cross reactivities that were found. DESAb1 shows very low levels of cross-reaction with the related compounds tested.

Table 5.6 : Cross reactivity of the polyclonal antisera to DES and other structurally related steroid hormones and cross-reacting compounds in inhibition ELISAs. [a]Percentage cross reactivity was calculated as (IC_{50}[DES]/IC_{50}[test compound])*100.

Compounds Tested	DESAb1 % Cross Reactivity[a]
DES	100.00
Ethynyl estradiol	<0.05
Diethylstilbestrol	<0.05
α-Estradiol	<0.05
β-Estradiol	<0.05
Estrone	<0.05
Norgestrol	<0.05
19-Nortestosterone	<0.05
Methyl Testosterone	<0.05
Zeranol	<0.05
Norethisterone	<0.05

5.2.4 Optimisation of coating for rapid ELISA in a 96 well format

The assay format was first developed on 96 well immunoassay plates to test the rapid ELISA system. The format used was identical to that of the competitive assay described in section 3.1.4. The differences arise in the length of the incubation steps. The coating and blocking stages of the assay were carried out with either overnight incubation at 4°C or for one hour at 37°C. The drug-HRP conjugate and the standards are left in the wells for approximately 10min before washing. This simulates the reaction time allowed in the prototype rapid assay device. The OPD substrate is then added and the absorbance is determined after 5 and 20min.

Two different coating procedures were assessed. The first used an anti-rabbit IgG antibody coated onto the plate, which acted as a capture antibody for the specific antibody. The specific antibody was then added and allowed to bind to the immobilised capture antibody. The second system involved the direct coating of the specific antibody onto the surface of the wells as seen in the competitive assays described in chapter 3 and 4. Various amounts of anti-rabbit IgG (from 1/500 to 1/16,000) were coated onto the plate. The anti-TR antibody (TRAb1) was added to the plate at various concentrations. The TRAb1 was also absorbed onto wells that had not been coated with the capture antibody. The results from this assay are graphed in Figure 5.9 and little difference is observed between wells that used the capture antibody and those that did not. The coating method that did not use the capture antibody was used in all subsequent assays. With this method less time was required to prepare the plates and fewer reagents were used.

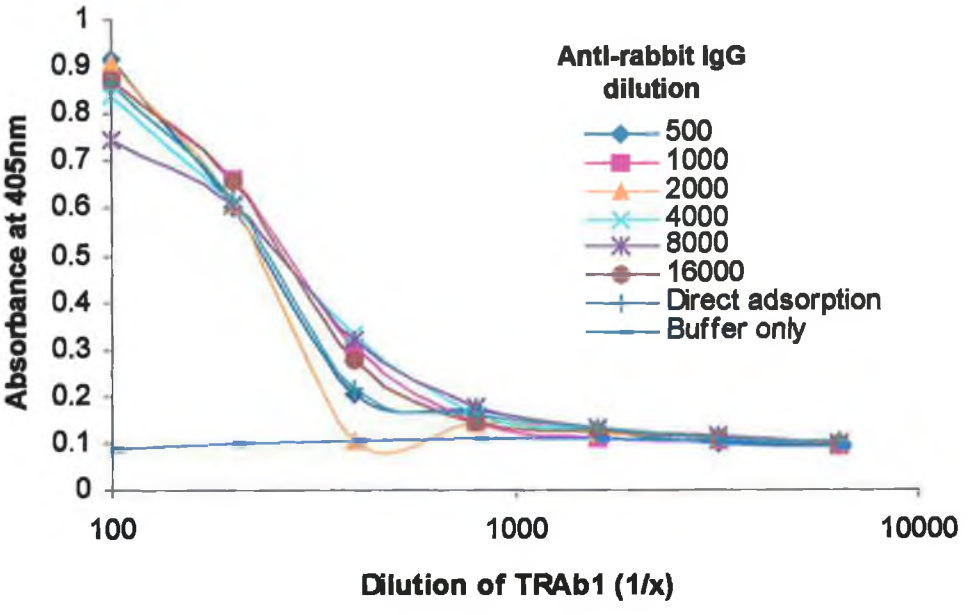


***Figure 5.9** : Effects in signal detected with different coating concentrations and procedures. The plates were coated with dilutions of anti-rabbit IgG antibody from 1/500 to 1/16,000. The specific anti-TR antibody, TRAb1, was added at dilutions between 1/100 to 1/6,400 to each of the pre-coated wells and uncoated wells. The TR-HRP conjugate was used at 0.5 μg ml^{-1} and left for 10mins on the wells. The substrate was added and the absorbance was determined at 405nm.*

5.2.5 Optimisation of the coating and labelled drug concentrations for the TR and DES rapid ELISA assay

A checkerboard ELISA was carried out to determine the optimum concentrations of both the antibody and the HRP conjugates. The method for this assay is described in detail in Section 2.4.7.3.

From the results (Figure 5.10) the optimum coating concentration for the antibody was 1/100 or 47.6 $\mu g\ ml^{-1}$ of TRAb1 and 1/10,000 or 0.5 $\mu g\ ml^{-1}$ of the TR-HRP conjugate. Similar experiments were carried out using DES-HRP and DESAb1 (Figure 5.11). 3.4 $\mu g\ ml^{-1}$ (1/400) was chosen as the optimal DESAb1 coating concentration and the DES-HRP was used at a dilution of 1/10,000 or 0.5 $\mu g\ ml^{-1}$.

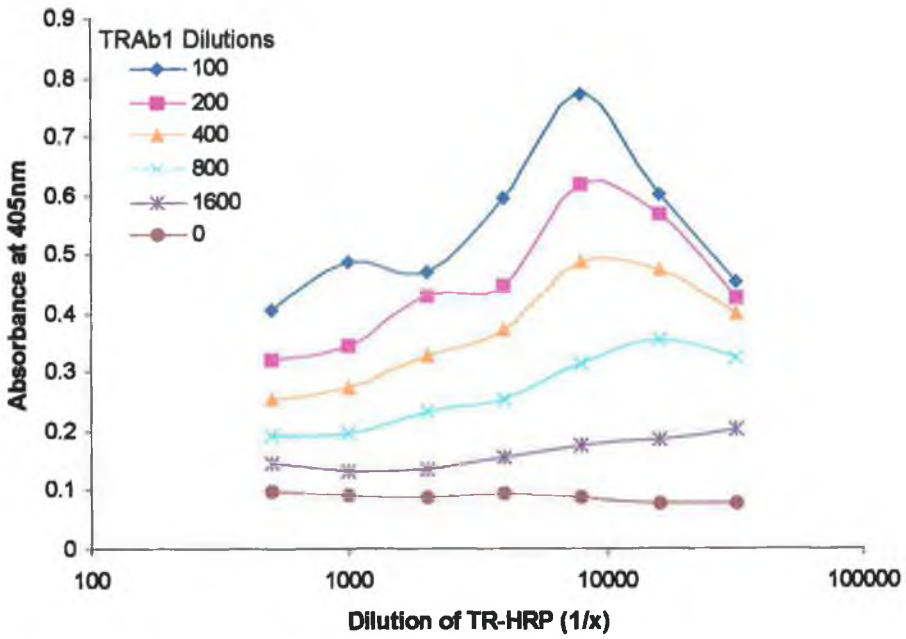


***Figure 5.10** : Non-competitive checkerboard ELISA to determine optimal TRAb1 antibody coating concentrations and TR-HRP dilution. From this assay the optimal coating concentration was determined to be 1/100 (47.6 μg ml^{-1}) of TRAb1 and the optimal TR-HRP dilution was 100 μl of 1/10,000, (0.5 μg ml^{-1}).*

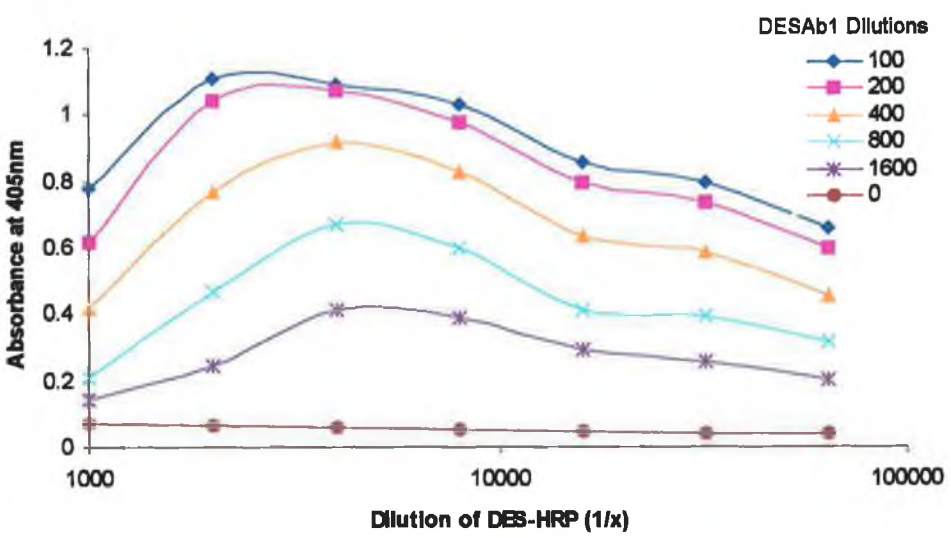


***Figure 5.11** : Non-competitive checkerboard ELISA to determine the optimal DESAb1 antibody-coating concentration and DES-HRP dilution. From this assay the optimal coating concentration of DESAb1 was determined to be 1/400 (3.4 μg ml^{-1}) and the optimal DES-HRP dilution was 100 μl of 1/10,000, (0.5 μg ml^{-1}).*

5.2.6 Competitive assay with a rapid ELISA format

The ability of the rapid ELISA format to detect free amounts of steroids was evaluated using the optimised conditions. Each of the assays was run with a broad range of standards, 0.39 - 100,000 ng ml^{-1}, prepared in PBS/2% (v/v) EtOH. Figure 5.12 shows the dose response curves obtained from these assays. They show quite clearly that the rapid protocol can be used to detect low concentrations of free drugs in buffer.

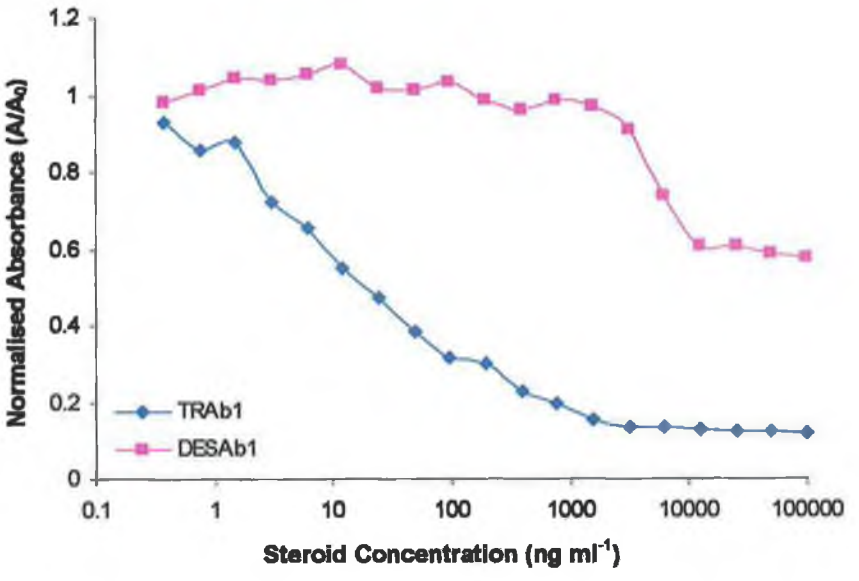


***Figure 5.12** : Dose response curves for the detection of free TR using TRAb1 and free DES using DESAb1 employing the rapid 96 well ELISA format. Both antibodies show a competitive assay with the ability to distinguish varying amounts of free steroid in a buffer matrix.*

5.2.7 Optimisation of the assay using the prototype rapid assay device

5.2.7.1 Stability Testing of Components for use with the rapid assay device

The next step was to determine if the reagents were compatible for use with the rapid assay device. The format and protocol for this assay are described in detail in section 5.1.3 and 2.4.8.3. The reaction in the wells is similar to that encountered in the competitive ELISAs already investigated (Chapter 3 & 4). The stability of the assay system was investigated by running a blank sample in the device at various times throughout a single day. It was expected that the results for the zero concentration would remain constant over the period but this was not the case (Figure 5.13). The machine reads the transmission values of each well and, over time, less colour is being developed using the same sample and reagents leading to an increase in the normalised transmission readings. To eliminate the source of this assay drift the stability of two assay components were enhanced, the conjugate solution and the coating on the wells.

There is an absolute requirement for the stability of the conjugate solution throughout the time the assay is running and it must also be stable in storage if the assay is to have any practical use. A commercial stabilising diluent was obtained, (Pierce Guardian Peroxidase Diluent) and used as the diluent for the conjugate and compared with results obtained when the conjugate was diluted with PBS. A slight improvement was seen but the increase in normalised transmission (decrease in amount of colour developed) was still evident (Figure 5.14).

The stability of the coating on the wells was then investigated. A number of wells were coated and blocked using the standard protocol. Half of the wells were stored dry and at room temperature while PBS was added to the other half and they were kept at 4°C in an attempt to maintain the activity of the antibody coated onto the surface of the well. The cold, wet wells were allowed to come to room temperature before use in the machine. The results clearly show that the wells are more stable when stored cold and wet with very little difference in the normalised transmission value over time (Figure 5.15).

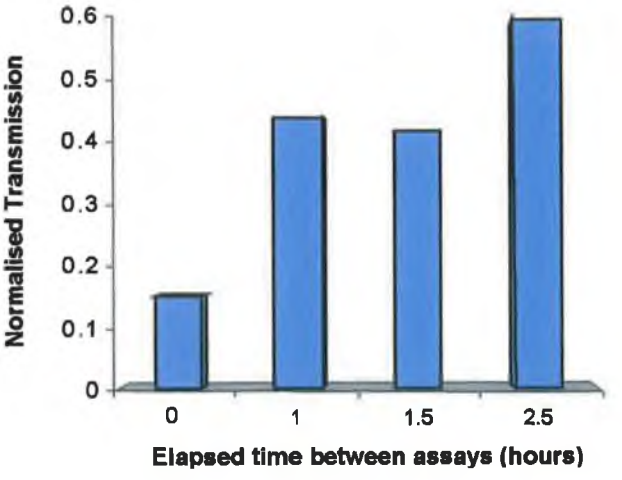


Figure 5.13 *: Increase in the value of the normalised transmission (average transmission reading for the four coated wells divided by the transmission recorded in the blank well) for the zero concentration standard (PBS/2% (v/v) EtOH) over time. The wells were stored dry and at room temperature until needed and the conjugate was diluted in PBS. The normalised transmission value increases over time.*

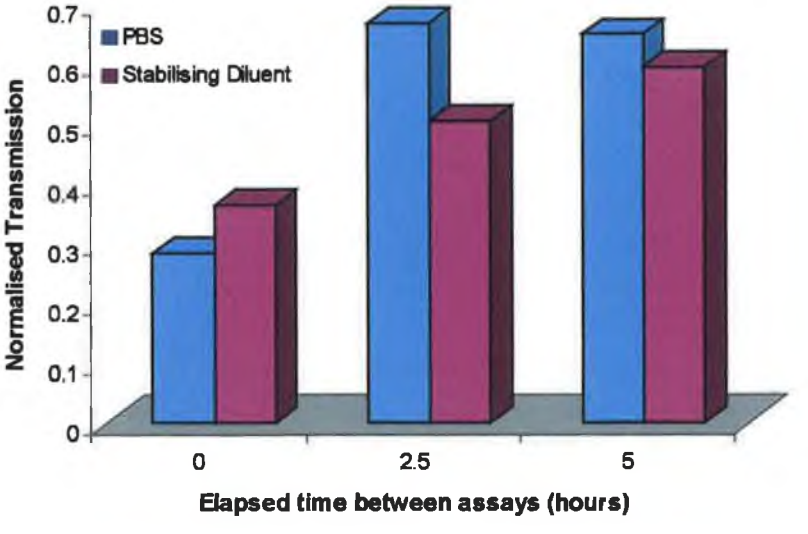


Figure 5.14 *: Increase in the value of the normalised transmission for the zero concentration standard (PBS/2% (v/v) EtOH) over time. The wells were stored dry and at room temperature until needed and the conjugate was diluted in either PBS or peroxidase stabilising diluent. The stabilising diluent did not eliminate the drift on the zero concentration value over time.*

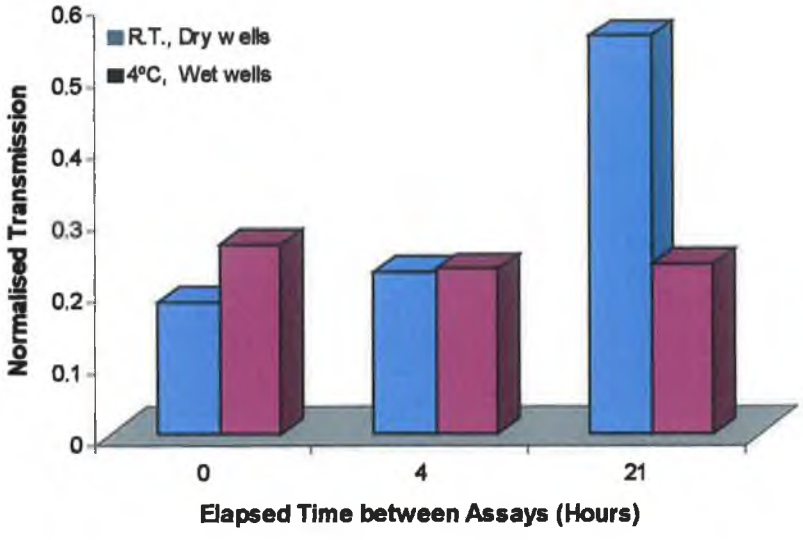


***Figure 5.15** : The value of normalised transmission for the zero concentration standard (PBS/2% (v/v) EtOH) over time. The wells were stored dry at room temperature (RT) or wet at 4°C until needed and the conjugate was diluted in peroxidase stabilising diluent. The dry, R.T. wells showed the increase in transmission values over the period of the experiment, whereas the cold, wet wells showed consistent results. This indicates the use of the cold, wet wells in future assays.*

5.2.8 Competitive assay using the prototype device

Using the optimised conditions (conjugate diluted in stabilising diluent and coated wells stored in PBS at 4°C) a competitive assay was run on the rapid assay device. Each sample was run in quadruplicate (four antibody-coated wells) with a fifth uncoated well as a control. The results for each of the wells are given as a ratio of the transmission before the 5min incubation and the transmission after it. The results are averaged and normalised by dividing them by the ratio for the blank well. The results for the assays are given in Figures 5.16 and 5.17. The dose response curves are not smooth but a clear trend can be seen and the assay is able to distinguish between 0 and 1 ng ml^{-1} of free TR. The DES assay is not as sensitive and is only able to detect levels of drug greater than 50 ng ml^{-1}.

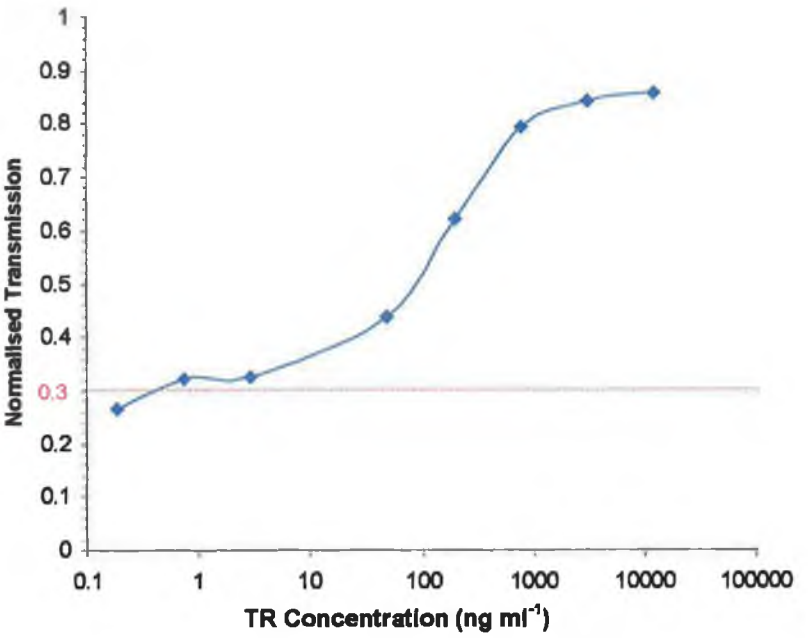


***Figure 5.16** : Dose response curve for the detection of free TR in PBS/2% (v/v) EtOH using the rapid assay device, TRAb1-coated wells (47.6 μg ml^{-1}) and TR-HRP conjugate (0.5 μg ml^{-1}). The normalised transmission for the zero concentration was 0.25 and the cut-off was chosen as 0.3 (red dotted line). Everything above this indicates the presence of free drug and below this the results are negative.*

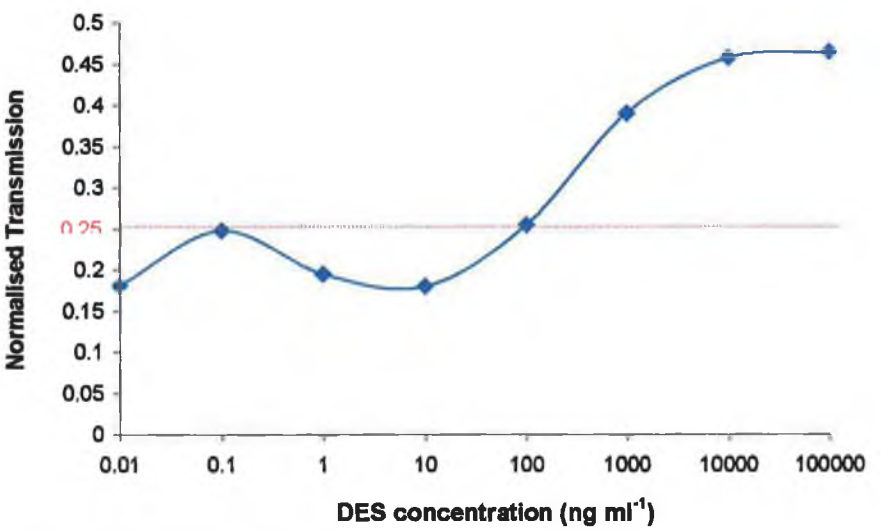


Figure 5.17 : Dose response curve for the detection of free DES in PBS/2% (v/v) EtOH using the rapid assay device, DESAb1 coated wells (3.4 μg ml^{-1}) and DES-HRP conjugate (0.5 μg ml^{-1}). The normalised transmission for the zero concentration was 0.2 and the cut-off was chosen as 0.25 (red dotted line). Everything above this indicates the presence of free drug and below this the results are negative.

5.2.9 Co-analysis of two steroids using the rapid ELISA device

The rapid assay device is capable of detecting low nanogram quantities of the steroid hormones in a buffer matrix. To increase the usefulness of this system, the design was altered slightly to allow the co-analysis of samples for the presence of two steroids. Five wells were still used, two coated with specific antibody against TR, TRAb1 and two coated with DESAb1. No antibody was absorbed onto the surface of the fifth well. The conjugate solution contained the correct dilution of both HRP conjugates, 1 $\mu g\ ml^{-1}$ of TR-HRP and 1 $\mu g\ ml^{-1}$ of DES-HRP.

The results in Figure 5.18 show the dose response curves for the co-analysis of both TR and DES. The assay is able to detect TR when on its own and in a mixture with DES. The magnitude of the response is similar for both single and the mixed samples. In both cases, where it is measured on its own and in combination with DES, the assay gives a clear distinction between the zero value (normalised transmission of 1) and a 10 $ng\ ml^{-1}$ sample. The results for the DES detection are not as clear. They show little, if any recognition of the free DES in solution.

Neither antibody shows any recognition of the other drug conjugate. The lack of cross reactivity between these antibodies and the co-analysed steroid gives the rapid assay device the ability to use a mixture of DES-HRP and TR-HRP conjugates to provide the competing conjugate molecules of the assay.

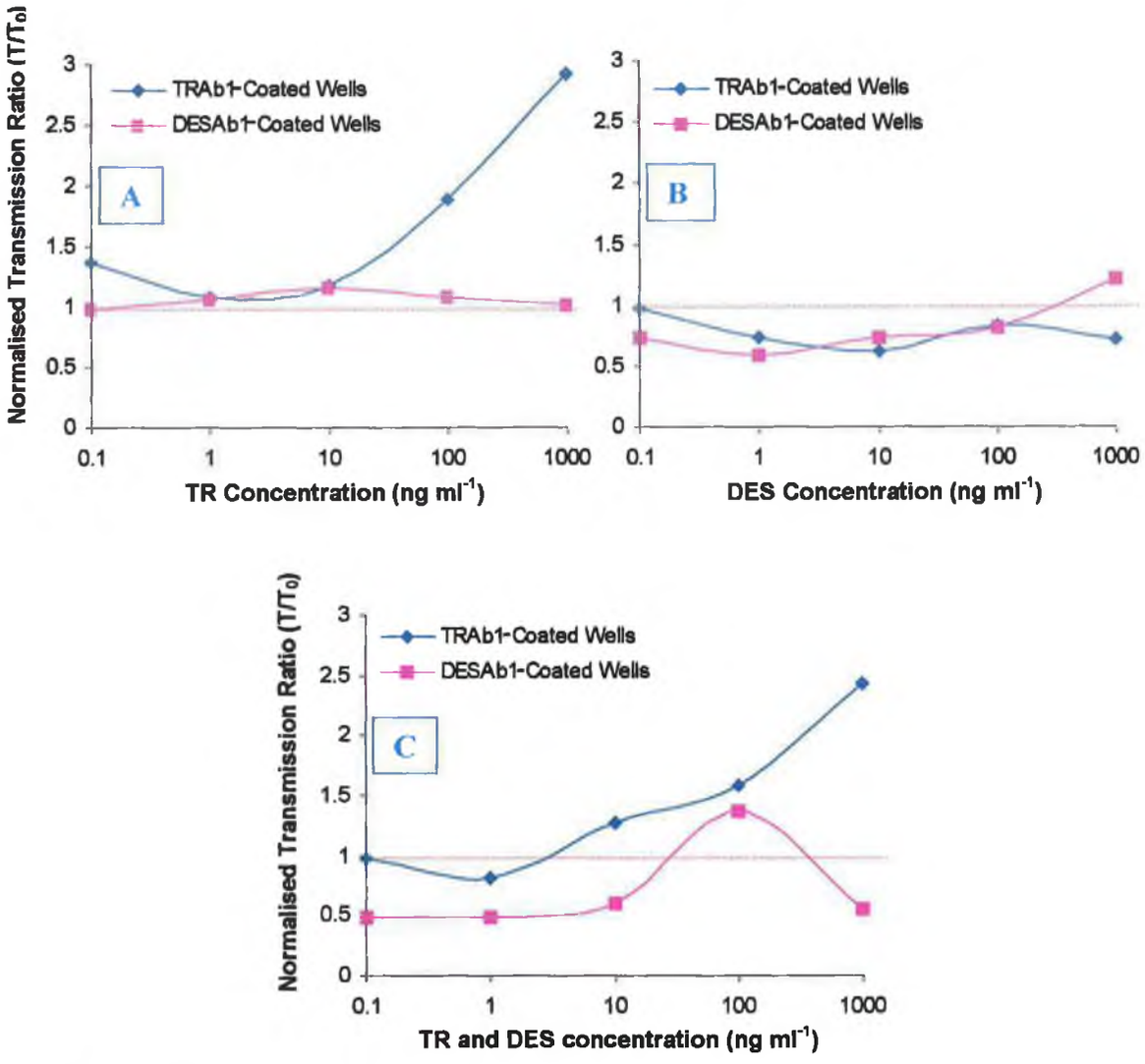


***Figure 5.18** : Dose response curves for the detection of both TR and DES containing samples on TR- and DES-coated wells. Each line represents the response seen for the one set of coated wells with a particular steroid standard. Part A of the figure indicates the responses for both the coated wells when the TR standard is run. Part B shows the results for the wells with the DES standard and Part C shows the response of the wells when samples containing both TR and DES are used. All results have been normalised so that the zero concentration for each set of results has a value of 1. Values above 1 are considered positive and those below are negative.*

5.3. Discussion

An anti-DES polyclonal antibody was produced, purified and characterised. It recognised free DES molecules in buffer solution and a very sensitive ELISA for DES was developed, which was able to detect less then 100pg ml^{-1} of DES. The antibody, termed DESAb1, did not show cross-reactivity with the related molecules tested, some of which may be found in biological samples, as shown in Table 5.6. This antibody preparation and TRAb1 were used as the antibody reagents for the investigation of the utility of the rapid ELISA device.

The number of wells for use on the prototype device were limited so the majority of the optimisation work for this assay was carried out on 96 well immunoplates. The wells were specially developed for use with this device and were manufactured by Nunc. Nunc were unable to supply the wells for a substantial period due to plant and machinery problems.

Two coating procedures for these wells were investigated. The first used an anti-rabbit IgG antibody coated onto the well followed by the specific antibody, while in the other system the antibodies were coated directly onto the plate. Figure 5.9 represents the results obtained. The difference in signal observed between the different amounts of anti-rabbit IgG coated onto the plate and the direct adsorption method was minimal. The direct method uses fewer reagents (one antibody instead of two) and it takes less time (single incubation) so this protocol was used for all subsequent assays. It has also been shown that reproducible ELISAs can be achieved using this method of well coating (Chapters 3 and 4).

The reaction incubation time on the prototype device is 4min at 35°C with a constant fast vibration to allow for a faster reaction. The assay will only be viable if the kinetics of the antibodies allow them to react sufficiently in the short incubation period available. The rapid ELISA cannot reproduce these conditions exactly. The plates are incubated at 37°C but no method was available to shake the immunoplates while in this incubator. To aid the reaction the plates were shaken by hand for 10s on 3 occasions during the reaction incubation. The plates were washed as normal and OPD substrate was used. The device uses a different chromogen tetramethylbenzidine, TMB, as it turns blue and this is the wavelength interrogated by the optical system in the device. OPD produces a yellow/orange colour. The colour developed on the plates was recorded after

5min incubation at 37°C using a plate reader. The colour intensity at 5min was usually adequate for the analysis of the plate but a further reading may have been taken at 10min if further colour development was required. The plate readers available detected the absorbance of the substrate, whereas the device measures transmission. As transmission and absorbance are related the absorbance was used directly without any alteration.

The rapid ELISA was carried out in a non-competitive format to optimise the coating concentration of the antibodies and the amount of competing HRP-labelled drug to be used in the assay. The plates were coated with varying concentrations of antibody and blocked as described (section 2.4.8.1) and varying concentrations of the steroid-HRP conjugate were added. The results in Figures 5.10 and 5.11 show that with both assays the signal increases as the amount of tracer present decreases at the higher concentrations of the steroid-HRP conjugate.

This is not an expected result but it is seen with the assay systems for both TR and DES. It may be an effect seen when a high antibody concentration is coated onto the well and the binding sites of the antibodies are at close to full occupancy with the steroid-HRP conjugates. Ordinarily, the curve would be expected to show saturation and not a fall in absorbance signal. This implies that some aspect of the high concentration of coating antibody and the high concentration of the steroid-HRP conjugate is interferring with the ability of the HRP to act on the substrate. As the tracer concentration is reduced the response curve returns to a more normal exponential decay pattern. From these graphs an antibody coating concentration that gave reasonable signals was chosen along with a steroid-HRP concentration that fell on the downward slope of the response curve, (Table 5.7). This ensured that the amount of antibody coated onto the surface of the well was the limiting factor for the assay not the conjugate concentration

Table 5.7 : The optimal coating and competing concentrations for use in the 96 well format rapid ELISA.

Assay for	Antibody Coating Conc. ($\mu g\ ml^{-1}$)	Steroid-HRP Conc. ($\mu g\ ml^{-1}$)	Approx. Detection Range ($ng\ ml^{-1}$)
TR	47.6 TRAb1	0.5	0.38 - 1,000
DES	3.4 DESAb1	0.5	48 - 100,000

Using the optimised conditions the competitive assays were carried out on the 96 well plates. These used the rapid ELISA protocol and the samples were diluted in PBS/2% (v/v) EtOH. A wide range of standards was run to determine the detection range of the assays, Figure 5.12. Both assays show competition with free steroids. Using these parameters the TR assay is far more sensitive than the DES assay. Attempts were made to lower the limit of detection for the DES assay by altering both the antibody-coating concentration and/or the competing HRP-conjugate. An increase in the sensitivity of the assay was not achieved. This implies that the kinetics of this antibody-antigen interaction are not as suitable to deliver a sensitive assay with this rapid assay format as the TRAb1 antibody is.

Once the assays were proven as viable using the 96 well format they were then transferred to the prototype device. The success of the rapid ELISA indicates that the reagents can be used in an assay that uses shorter reaction times than a normal assay. The TR antibody has a more sensitive response in this rapid format than DESAb1. This implies that it has faster reaction kinetics allowing it to reach equilibrium quickly during the short incubation time used in the rapid ELISA. The DESAb1 antibody may require a slightly longer incubation for detection levels similar to those shown with the standard length ELISAs.

With the rapid ELISA format each of the reagents are used at the same time, none of them are prepared and used over the course of a day. This avoids any problem of instability of the components if they have to be stored and this allows the solutions or solid phase to provide consistent results and show no bias with respect to any instability problems. The rapid assay device, however, runs assays sequentially and so reagents and components must be stored until needed. This can lead to problems if the reagent solutions are not stable or the antibody-coated wells lose activity over time.

A drift in the percentage transmission value was noticed with the device when zero concentrations were run throughout an assay procedure. The values for the normalised transmission in the coated wells were increasing over time, that is less colour was being produced in these wells. The standard itself (PBS/2% (v/v) EtOH) is stable at room temperature over many weeks and has shown the same results in previous ELISA studies. Other components that may have been unstable were the conjugate and the antibody-coated wells.

To counter the possible instability of the conjugate solution a commercial stabilising diluent was obtained. The conjugate was diluted in both the stabilising diluent and PBS and the results were compared. The drift was not removed by using the improved diluent and the normalised transmission values continued to increase after a number of hours (Figure 5.14).

The wells for the device, up until this point, had been stored dry at room temperature after they were blocked until they were required. All wells were used on the same day that they were blocked. To assess the stability of the wells they were coated and blocked as normal and divided in two lots, the first group were left dry, at room temperature, while the second set were filled with 200 µl of PBS and kept at 4°C. Antibodies are stored at 4°C to maintain their activity and 200 µl of PBS was added to each well to avoid the loss of activity when the antibodies are allowed to dry out on the surface of the well. An alternative solution to the effect of drying on the antibody-coated wells would be to add a layer of a sugar, trehalose, over the antibody coating. The trehalose maintains some moisture in the wells and prevents the loss of activity of the antibodies if the wells are dried. The cold wells were emptied and allowed to come to room temperature before use in the machine.

The results show clearly that the cold, wet wells are more stable and produce more reproducible results for the zero concentration standard (Figure 5.15). After 21hr of storage the result for the dry, R.T. wells had risen considerably with respect to the initial reading implying that the coated antibody had lost activity and was no longer binding the same amount of the steroid-HRP conjugate. This would lead to large inaccuracies in the assay. The cold, wet wells, however, had maintained their response and therefore their binding capacity after the 21h of storage.

Once the drift had been eliminated the competitive assays were applied to the device. Figure 5.16 and 5.17 show the results obtained for each of the assays for the detection of TR and DES. Again it can be seen that the TR assay is more sensitive than the DES assay. The dose response curves are not smooth and become unreliable at low concentrations and this may lead to inconsistencies in results. The DES assay shows variations in the results obtained for the lower standard concentrations. In this case it would be prudent to set the detection limit

of this assay at above 0.25 to avoid false positive readings. By adding 0.05 onto the negative value for the EE assay would also limit the number of false positives in this assay leaving only concentrations above 1 ng ml^{-1} to give a positive result. Further validation of these assays is required before they could be used as screening tools. Unfortunately the supply of wells for the machine was extremely limited and the work here could not be continued.

This system has limited usefulness if it assays one sample for the presence of one steroid in a 20min period, (time taken to run each sample). The measuring of the sample in quadruplicate is not required for an initial screening test and so a system was developed whereby the presence of two drugs could be detected. Two wells coated with the TRAb1, two wells coated with DESAb1 and an uncoated well were used. The conjugate solution contained the correct concentration of both the TR-HRP and the DES-HRP conjugates. The cross reactivities of these antibodies, Tables 5.6 and 3.10, show that neither antibody has any specificity for the other drug and so the conjugate solution can be used in both wells without interference.

The results of this co-analysis assay are shown in Figures 5.18 and 5.19. The TR assay seems to work quite well with the assay being able to measure free TR when in buffer on its own and in combination with DES. The DES-coated wells did not show any recognition of the TR steroid. The DES assay, however, did not produce the expected results. Most of the responses seen to DES were below that of the zero concentration standard. When the DES standard is run the assay seems to begin to detect the presence of the steroid at 1000 ng ml^{-1}. This is far too high for use as a screening tool but a different antibody or even a change in the assay format may lower this detection limit to one that could be useful. The response of the wells when samples containing both TR and DES shows the assay easily detects the presence of TR but again the DES assay shows large inconsistencies that can not be explained by the assay itself and may be due to the device or the plastic in the wells. This implies that the assay is not optimised fully.

This experiment does show the potential of this device for the co-analysis of two steroids. With further optimisation it may be possible to use this device to screen for up to 4 different compounds in the one assay. Again the lack of availability of

the special wells required to use the rapid ELISA device has curtailed the work into this area.

In conclusion, 96 well rapid ELISA was developed to mirror the assay protocol of the prototype device. Using this assay system the parameters for the development of the rapid assay were investigated. The range of detection for these assays was defined. The assay was applied to the rapid assay device and a drift was seen in the results. This was corrected by stabilising the coating of the wells while being stored. The assays showed the ability to detect nanogram quantities of free steroids. The assay for TR showed a clear measurable difference between 0 and 1 ng ml^{-1} and DES could be distinguished at 50 ng ml^{-1}. The system was then altered to detect two steroids in the same sample. The principle of this assay is shown and with some further work the assay would become a viable screening method.

CHAPTER 6

PRODUCTION OF ANTI-STEROID SCFV USING RECOMBINANT METHODS

6.1 Introduction

6.1.1 Recombinant antibodies

The three main methods of antibody production are laid out in section 1.2. Polyclonal antibodies are the oldest and probably most widely used antibody type although monoclonal antibodies are also very popular due to their inherent advantages. It is thought that it soon may be possible to replace both these antibodies with recombinant antibodies that would retain or mimic many of the positive assets of these antibody types (Chiswell and M^{c}Cafferty, 1992). Even though some library-isolated scFv can be extremely sensitive and specific, many isolated are, at best, only as sensitive as their polyclonal and monoclonal counterparts. Phage display does, however, offer a cheaper and more rapid production of antibody fragments (M^{c}Elhiney *et al.,* 2000).

Recombinant antibodies are the resulting proteins when antibody genes have been cloned and expressed in culture. They are monoclonal in nature in that a single antibody producing clone can be isolated and grown to produce a mono-specific antibody or antibody fragment. Recombinant antibodies can be produced as either full antibodies or as antibody fragments, such as single chain variable fragments (scFv) or antibody binding fragments (Fab) (Le Calvez *et al.,* 1995). With this technique the genetic sequence of the antibody has been cloned into a vector and so the manipulation of the antibody fragment is facilitated (Krebs *et al.,* 2001).

6.1.2. Phage Display Technology

The production of recombinant antibodies using phage display technology is presently the most popular technique and it is sometimes considered a superior method to monoclonal antibody production using hybridoma technology (Berdichevsky *et al.,* 1999; Li *et al.,* 2000). This technique is described in detail in section 1.2.4 and it allows a fragment of the antibody, containing it's binding site, to be displayed on the surface of a bacteriophage, which can then be used to select the required affinities from a large number of antibody sequences. Once the antibody fragment is expressed on the surface of the phage it facilitates selection and screening of the clones (Andris-Windhopf *et al.,* 2000; M^{c}Elhiney *et al.,* 2000). This selection can be carried in a number of ways, using

immunotubes, immunoplates and biosensors. It is also very simple and cost-effective to amplify these phage particles (Casey *et al.,* 1995).

6.1.3. The Krebber Phage Display Library System

The method used in this library production is described by Krebber *et al.* (1997). This method allows for the amplification of a vast number of murine antibody variable fragments, both heavy and light, once they are present in the initial mRNA sample. It involves the use of 23 primers for the light chain variable regions and another 23 for the heavy chains. This large number of primers is required so that the greatest number of variable fragments will be amplified and hence amplifying the sequences of interest.

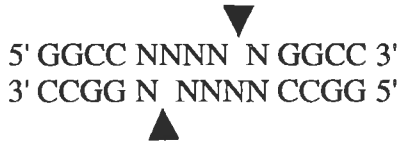
The primers listed (section 2.4.10.3.1) cover all the primers sets from previous studies (Kettleborough *et al.,* 1993; Ørum *et al.,* 1993 and Zhou *et al.,* 1994) and they also cover the sequences contained in Kabat database (Kabat *et al.,* 1991). Thus these primers are capable of amplifying all the expressed murine antibody sequences. This holds true regardless of whether or not the mouse has been exposed to antigen. These primers can be used to create a naive library, a pre-immunised library or a library from the genes of a hybridoma cell line. A naive library is one which is non-specific and contains a large number of binders with different specificities. With this type of library it is hoped that antibody-binding fragments can be selected against numerous different antigens. A pre-immunised library primes the immune system of the animal involved with the antigen of interest to increase the number of B-cells producing antigen-specific antibodies. These cells are extracted and the mRNA isolated. In theory, this library will have a higher proportion of binding fragments directed against the antigen of interest.

Once the heavy and light chains have been amplified this system then permits the combination of the two pieces in one reaction using splice by overlap extension PCR (Horton *et al.,* 1989). The V_H and the V_L fragments recombine through the sequence homology of the linker region that was built onto the 3' end of V_L and the 5' end of the V_H sequences by the primers used in the amplification steps. The overlap is 15bp long and it codes for one repeat of the $(Gly_4Ser)_4$ linker section. The sequence is repeated twice on the end of the V_H fragment and three times at the start of the V_L fragment. The linker region does not use the same codons to code for the glycine and serine residues in each of the repeats. By altering the

codons in the repeats on either side of the zone of homology it is possible to prevent incorrect overlapping during the assembly PCR. The linker region in this construct is 20 amino acids long. This is longer than the frequently used 15aa linker, and it will reduce the tendency of the scFv fragments to aggregate.

The primers used in this step also introduce a FLAG tag onto the N-terminus of the V_L chain. FLAG is a 4 amino acid peptide sequence that can be bound by commercially available antibodies, allowing for one simple method of detection and purification of the final antibody fragments. At the end of this step the sequences have combined to form 800bp V_{HL} fragments that contain many different specificities. These will eventually be expressed as functional scFv.

A single rare cutting restriction enzyme is used for the directional cloning of this fragment into the phagemid vector. The enzyme, *Sfi*1, has a recognition site of 4bp located on either side of a non-recognised sequence of 5 nucleotide bases. It is within these 5 unrecognised bases that the enzyme cuts.



The enzyme is used to cut the V_{HL} fragment at either end using the sites that have been engineered into the sequence. The site at the start of the construct was introduced in the heavy forward primers and the site at the end was added within the SOE-PCR back primer. The non-recognised bases have been designed so that they are not homologous and this ensures that the fragment will not self-ligate. CCCGA at one end of the sequence will not ligate with CAGCC at the opposite end. The phagemid vector, pAK100, contains two *Sfi*1 sites to allow for the ligation of the construct. The vector contains the complementary sequences to allow the directional ligation of the construct into the vector.

This is a simple system as opposed to some methods which use 2 or even 4 enzymes to clone the gene fragments. These systems have a higher chance of cutting within the antibody sequences so providing incorrect sequences for cloning.

The design of the pAK100 phagemid vector also includes a number of improvements from previous systems. This vector contains a truncated form of

the gIII surface protein to which the scFv is fused. The particular truncated form, (residues 250-406) eliminates a Gly/Ser linker stretch and an unpaired Cys residue that are included in the protein used in some systems. This increases the stability and the folding yield of the fusion protein.

A terminator sequence is included between the *lacI* gene and the *lac* promoter region and this, along with the glucose repression of the *lac* promoter, removes the background expression of the scFv-gIII fusion protein before induction. Even a small amount of this background expression can confer immunity from super-infection with helper phage to the cell, which is vital for the production of the phage particles carrying the altered surface protein.

One other great advantage with the Krebber system is the series of compatible vectors that are available. Each vector has different characteristics, which allows for great flexibility and ultimate usefulness for the selected scFv gene (Krebber *et al.,* 1997).

- pAK100 is used to display, select and possibly express the scFv protein
- pAK200 is used for producing greater amounts of protein displayed on the surface of the phage, although it may have a selective disadvantage compared to the lower level expression in pAK100
- pAK300 adds a hexa-histidine tag to the scFv allowing for detection and IMAC purification
- pAK400 retains the his tag from pAK300 but also includes a promoter with a stronger Shine Dalgarno sequence to enhance the level of expression
- pAK500 contains helices to allow for the dimerisation of the scFv
- pAK600 fuses the scFv to an alkaline phosphatase enzyme allowing for direct detection. It also produces dimers.

In this chapter the use of this system to construct libraries from the antibody repertoires of immunised mice is described. These libraries were then panned to select antibody fragments that bound to the drugs under study, namely EE, DES and TR.

A previously constructed library was also panned against the steroid hormones. This library was constructed from the spleens of naive mice. In theory, this library contained a wide variety of antibody specificities and can be used to select antibody fragments for a number of different compounds.

6.1.4 Human Naive Library

The construction of this library is described by Vaughan *et al.* in 1996. The rationale behind the construction of this library was to produce a resource from which a great many scFv's can be isolated against a number of different compounds. The library is derived from 43 non-immunised human donors and has a reported size of 1.4 x 10^{10} scFv fragments. This is a very large library and it should contain sequences specific for many compounds.

The donors provided peripheral blood lymphocytes, tonsil B-cells and bone marrow B-cells and from these mRNA was isolated and reverse transcribed to cDNA. The heavy chains were amplified separately from each of the sources of cDNA and they were re-amplified with tagged primers containing the restriction sites, *Sfi*1 and *Xho*1. The segments were then pooled, restricted and ligated into the pCANTAB 6 vector. The vector was transformed into *E. coli* TG1 cells giving a V_H repertoire of 1 x 10^8 individual recombinants.

The V_L kappa and lambda sequences were amplified separately from the tonsil B-cell samples only. These were re-amplified and restriction sites, *Apa*LI and *Not*1, were introduced. Two pools of light chains (V_κ and V_λ) were produced. Both pools were restricted and ligated into the phagemid vector, pCANTAB 3*his*. They were electroporated into *E. coli* TG1 cells and the resulting repertoires were calculated to be 1.1 x 10^5 V_κ and 1.7 x 10^6 V_λ. To produce the library itself the V_κ and V_λ pools were combined and ligated to an upstream dummy V_H sequence and a gly/ser linker. This construct was electroporated into *E. coli* TG1 cells and a repertoire of 2 x 10^7 V_L recombinants with an upstream linker sequence was developed.

To construct the library both the V_H and the linker-V_L fragments were amplified and assembled on the J_H region. The constructs were digested with *Sfi*1 and *Not*1 and inserted into pCANTAB 6 giving a final repertoire of 1.4 x 10^{10} individual recombinants (Vaughan *et al.*, 1996).

This study will attempt to select scFv clones that recognise each of the 3 steroid drugs, EE, DES and TR.

6.1.5 Panning

Once a library has been produced, regardless of its method of construction, the next step is to isolate a functional binder from it. This usually takes the form of selection as opposed to screening, which would be the usual method in hybridoma technology. If, for example, the Vaughan library of 1.4 x 10^{10} clones was to be screened it would take a vast amount of time and expense. If, on the other hand, the specific binders could be selected and enriched within the library, screening would become a much easier task (Wind *et al.,* 1997).

Panning can be defined as the selection of fusion proteins that bind to a specific ligand (Choudary *et al.,* 1995). There are a number of methods of panning using various forms of immunotechnology. Immunoplates, petri dishes, polystyrene and magnetic beads and immunotubes can all be used as solid matrices to recover selectively bound phage. Selection can be driven by competitive elution, differential panning or adjusting the conditions to allow panning with antigens that are otherwise unstable under physiological conditions (Krebs *et al.,* 2001).

In this study immunotubes were employed as the method of choice in the panning procedure for the selection of specific binders. This method requires the linking or absorbing of a ligand to the plastic surface of an immunotube by incubating a solution of the ligand or hapten-conjugate in the tube. The proteins are passively absorbed to the surface of the tube. The haptens are usually too small to be absorbed so use is made of a protein conjugate. Once the protein is absorbed the remaining areas on the plastic must be filled or blocked with a non-specific protein. The reasons for this are similar to the reasons for the same step in ELISA procedures (section 3.1.4).

There is a significant problem with the use of blocking proteins and carrier proteins during panning steps. Once they are present in the selection process any antibody fragments specific for them will bind in the immunotube along with those specific for the target (Lu and Sloan, 1999). If a non-specific method of elution is used then all binders will be eluted and amplified. To minimise the amount of interference this may cause to the actual selection of specific binders, some simple steps may be undertaken. To prevent the binding of recombinant antibodies to the blocking protein it may be included in the diluent used. In this way the antibodies will tend to bind to the saturating concentration of the protein

in solution and not to the surface of the tube. The solution-bound fragments will be removed by simply washing the tube. This may also help for the carrier proteins.

It is also possible to select for particular epitopes on a target antigen (Parsons *et al.,* 1996). By pre-incubating a phage repertoire with a cross-reacting protein or hapten the clones that recognise these common epitopes can be eliminated. In this way antibody fragments can be isolated that only recognise epitopes specific for the target and are not present on similar molecules.

Another suggested approach for the elimination of the carrier-specific binders is the use of different carrier molecules or even different selection methods in alternate rounds of panning (Lu and Sloan, 1999). This may limit the carryover of contaminating protein-specific binders from round to round. Phage that are specific for the carrier used in the first round will have no target in the second round if the carrier protein has been changed and will, therefore, be removed from the selection process. A non-protein carrier, such as dextran, may also be used. This is a repeating sugar molecule and has resisted attempts to produce recombinant antibody specific for it (Xiao *et al.,* 1995). Using this as the carrier far fewer carrier-specific binders may be amplified and carried through to the next panning stage.

The method of elution can be optimised to allow for greater specificity in the binders that are selected. One of the most common procedures for the elution of the bound particles is the use of low pH (Krebber *et al.,* 1997). This disrupts the binding between the immobilised target and the antibody fragment, releasing the fragment into the solution where it can be retrieved. This is a highly non-specific method as it releases all the bound particles regardless of their target. Other methods include the use of competing antigen to release the binders from the tube, the use of a monoclonal antibody recognising the target epitope, passive elution using male bacteria or the use of dithiothreitol (Wind *et al.,* 1997). Some of these methods are quite mild and some are specific but not all can be used with each constructed library. The choice of elution method must be made carefully and be compatible with the scFv system.

6.1.6 Drugs under study

The libraries are to be screened against three compounds with steroidal properties, EE, DES and TR. For more information on EE see section 4.1.1 and for TR, section 3.1.1. This section will deal with the synthetic estrogenic compound diethylstilbestrol, M.W. 268 (DES). DES has structural features at either end of the molecule that mimics the A-ring of estrogens, (Figure 6.1).

DES is a pharmaceutical estrogen with a molecular weight of 268.34 and it was administered to pregnant women in the 1950's and 1960's to prevent spontaneous abortion. It was later seen that children of the treated women suffered from reproductive organ dysfunction (Berg *et al.,* 1999; Strohsnitter *et al.,* 2001). The use of DES in both medicine and as a growth promoter has been phased out due to its numerous side effects and its use in livestock was banned completely in the late 1970's (Sawaya *et al.,* 1998b). It is now used only as a drug in certain cases of prostatic and breast cancer and some postmenopausal vaginal disorders (Marselos and Tomatis, 1993). Pharmaceutical estrogens have also been associated with increases in breast and vaginal cancer, endometriosis, testicular and prostate cancers and lower sperm counts (Folmar *et al.,* 2000). There is also evidence that the administration of DES to pregnant women can have detrimental health effects lasting into the third generation (Economou *et al.,* 1990)

In a study of numerous natural and synthetic estrogens, phytoestogens and industrial chemicals, EE and DES were shown to be most potent and usually have a higher estrogenic activity than 17β-estradiol (Gutendorf and Westendorf, 2001). Other studies using different testing criteria have concluded that DES is less estrogenic than 17β-estradiol but all studies have shown the ability of DES to act as a strong estrogen (Berg *et al.,* 1999; Folmar *et al.,* 2000).

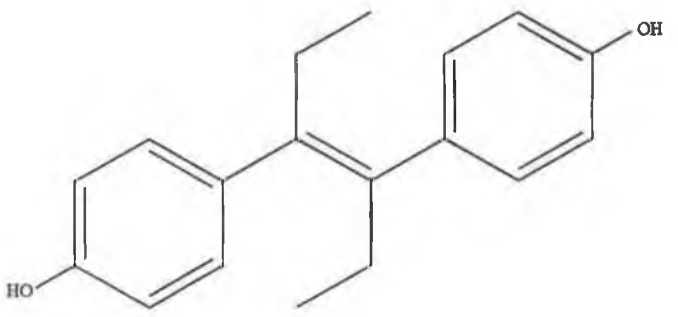


***Figure 6.1** : Structure of diethylstilbestrol, DES. This compound is a synthetic estrogen although it does not have a classical steroidal structure.*

6.1.7 Antibody Fragments Specific for Steroids

To test the utility of a naive human library described in Vaughan *et al.* (1996) antibodies against a number of compounds were isolated, including fluorescein, diethylenetriamine pentaacetic acid (DTPA), doxorubicin and estradiol. Using a single estradiol conjugate (estradiol -BSA) they were able to isolate a scFv with a K_d of 3.7nM after 2 rounds of panning (Vaughan *et al.*, 1996).

Pope *et al.* (1996) isolated a scFv recognising estradiol from a human naive library. This had a high affinity (3.7nM) and was very specific for estradiol when compared to other steroids. The screening procedures used were designed to allow the selection of clones that bound favourably to free steroid. This antibody fragment was also able to detect differences in both ends of the estradiol molecule showing that it did not have the 'blind-spot' usually associated with monoclonal or polyclonal antibodies.

The availability of the genetic sequence of a scFv clone makes it possible to alter the specificity of the scFv to improve its affinity or even change its specificity. Chames and Baty (1998) have shown that it is possible to take a monoclonal antibody specific for cortisol and through antibody engineering and phage display create an antibody that binds estradiol. This exemplifies the usefulness and power of the recombinant antibody techniques.

6.2 Results

6.2.1 Conjugates used in this study

The conjugates used in this study for EE and TR are described in chapters 3 and 4.

To produce conjugates of DES it was necessary to derivatise it to mono-carboxy propyl-DES, M.W. 354 (Figure 6.2), as described in section 2.4.1.2. The success of the reaction was judged from analysis of the mass spectrums of the compound produced, (Appendix B). This derivative provided the chemical handle through which carrier molecules could be linked. BSA, OVA, THY, and DEX conjugates were all produced using either the mixed anhydride or NHS ester chemistry (section 2.4.2).

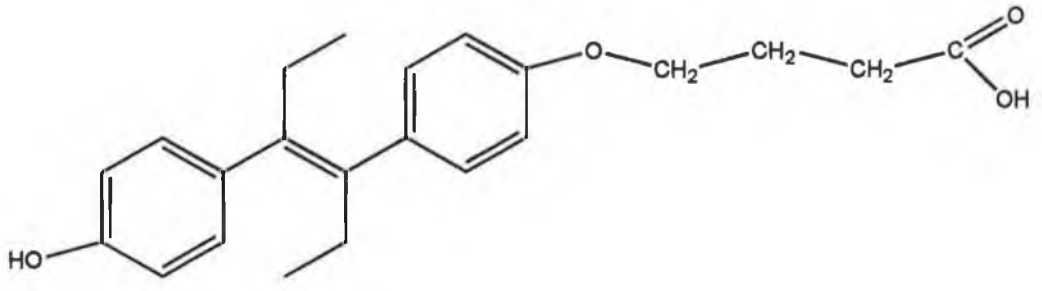


***Figure 6.2** : Structure of monocarboxypropyl-diethylstilbestrol, used to link carriers to diethylstilbestrol.*

6.2.2 Pre-immunised Library Production

6.2.2.1 mRNA extraction

Mice were immunised using the procedure described in section 2.4.4.3. The mice were sacrificed and the spleen was removed aseptically. The spleen was homogenised and subjected to a procedure to extract the mRNA present. The resulting mRNA was investigated spectrophotometrically at both 260 and 280 nm. The ratio between these two readings should be greater than 1.65 to allow for a good quality preparation with little protein contamination. Table 6.1 shows the results obtained for the preparations.

Table 6.1 : Purity and concentration estimations for spleen extracted mouse mRNA.

Immunogen	OD @ 260nm	OD @ 280nm	$\frac{260nm}{280nm}$	Estimated Conc. (mg ml^{-1})
EE	1.034	0.616	1.68	8.27
DES	0.729	0.428	1.70	5.83
TR	0.858	0.540	1.66	3.43

The values for the 260/280nm ratio are all above 1.65 giving them sufficient purity to continue with the library production. The concentration values are estimated from a standard reading of 1 absorbance unit at 260nm equating to 40 $\mu g\ ml^{-1}$. The absorbance values in Table 6.1 are for a 1/200 dilution of both EE and DES and a 1/100 dilution of TR.

6.2.2.2 cDNA synthesis

The synthesis of cDNA from the extracted mRNA was carried out as described in section 2.4.10.2. 8 µg of mRNA was used in each reaction. When synthesis occurs successfully three bands are visible within the cDNA smear when it is run on an agarose gel. These three bands correspond to the amplification of ribosomal RNA present in the RNA sample. These bands will only be seen after mRNA has been reverse transcribed into cDNA (Figure 6.3).

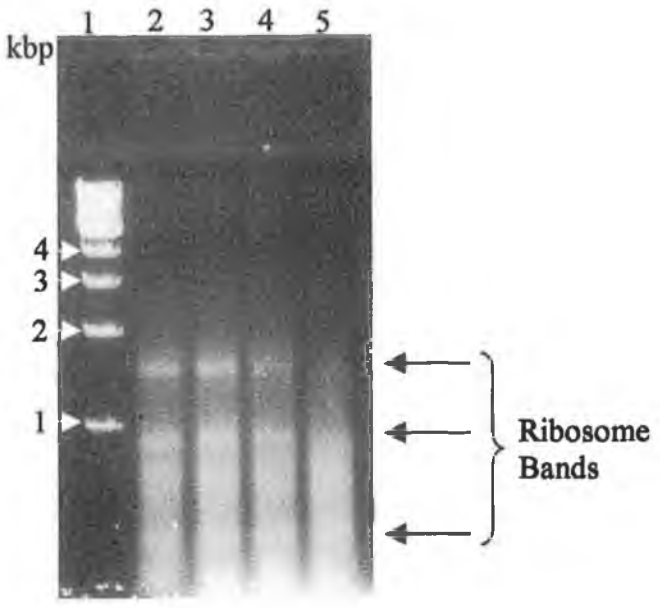


***Figure 6.3** : Synthesis of cDNA from mRNA template. Lane 1 is a 1kbp marker; Lanes 2-5 are results of the synthesis. The ribosome bands are clearly visible in the lanes.*

6.2.2.3 Heavy and Light Chain Amplification

The procedure for amplifying the variable heavy and light chain fragments contained in the cDNA is laid out in section 2.4.10.3. This procedure uses many different primers to amplify the majority of the individual chains. This is a very complex multiplex PCR and each component of the PCR must be optimised. A number of different buffers were used to select the right conditions for the amplification of the variable fragments. In these buffers the $MgCl_2$ concentration and the pH varied. By testing the reaction in a number of these buffers the optimal $MgCl_2$ concentration and pH could be determined (Figure 6.4). A $MgCl_2$ concentration of 2.5mM was used in subsequent heavy chain amplifications along with a buffer pH of 9.0.

The optimal amount of *Taq* enzyme and of each primer was also found by a simple concentration titration. The optimal amount of *Taq* was chosen to be 5 units per reaction, though results did not change significantly between 2 to 6 units. The primer concentration was titred to determine the optimum concentration for use in the reaction. Concentrations between 0.1nmol to 0.0125nmol of each primer per reaction were tested (Figure 6.5). The optimum primer concentration was found to be 0.025nmol per reaction.

The reactions were then run to prepare stocks of both the heavy (V_H) and light chain (V_L) variable fragments for each of the three drugs. These chains ran at approximately 400bp on an agarose gel and from this they were purified and their concentration was estimated.

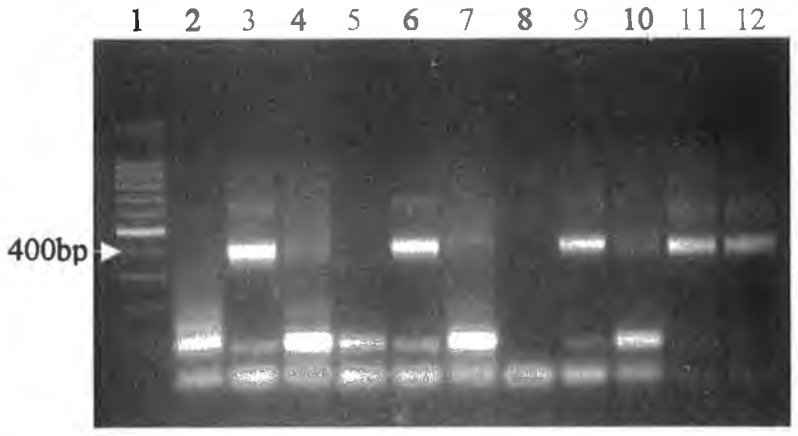


***Figure 6.4** : Effect of different $MgCl_2$ concentrations and pH on amplification of heavy and light chain variable fragments. Lane 1: 100bp marker, Lane 2-10: heavy chain amplification with various buffers, Lane 11-12: Light chain amplification with 2 buffers.*

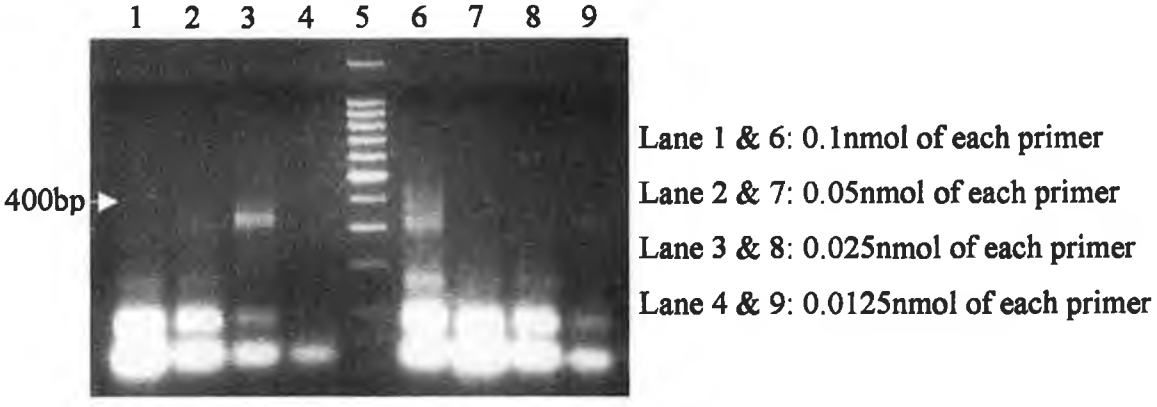


***Figure 6.5** : Effect of different primers concentration on the multiplex PCR for the amplification of variable heavy chains. Lanes 1-4: heavy chains primers with cDNA for EE library, Lane 5: 100bp marker, Lane 6-10: heavy chain primers with cDNA for DES library. Lanes 8 and 9 have faint bands at 400bp but they have not reproduced well. The optimal concentration was taken to be 0.025nmol per primer per reaction.*

6.2.2.4 Splice-by-Overlap Extension PCR

This technique allows the joining of the heavy and light chains together in one reaction. Each chain has an identical overlap that was introduced by the primers in the amplification stage. This allows the two chains to associate and so be amplified as one chain with a linker in the middle. The two 400bp fragments become an 800bp fragment after this step containing both the V_H and V_L chains. This reaction must be optimised as described previously for the multiplex PCR. The array of buffers was used to determine the optimum $MgCl_2$ concentration and pH. A reaction without the bands at 650bp and 1100bp, as seen in figure 6.6, would be the optimum reaction, but an amount of these bands were seen with all buffers. The optimum was found to be 2.5mM $MgCl_2$, pH 9.0. This was used to run all the SOE-PCR as it gave the least amount of contaminating bands.

6.2.2.5 Sfi*I Digestion*

Sites for the rare cutting enzyme, *Sfi*1, have been engineered at either end of the 800bp fragment. This allows the insert 800bp and the phagemid vector to be cut only using this enzyme. This restriction enzyme recognises 4bp either side of a random sequence of 5bp. This fact ensures that neither the insert nor the vector will self-ligate giving only intact phagemid containing the insert following ligation. The difference in size between the restricted and unrestricted insert is very slight with only a few base pairs being removed from the sequence. Figure 6.7 show both unrestricted and restricted inserts while Figure 6.8 shows the restriction of the pAK100 phagemid.

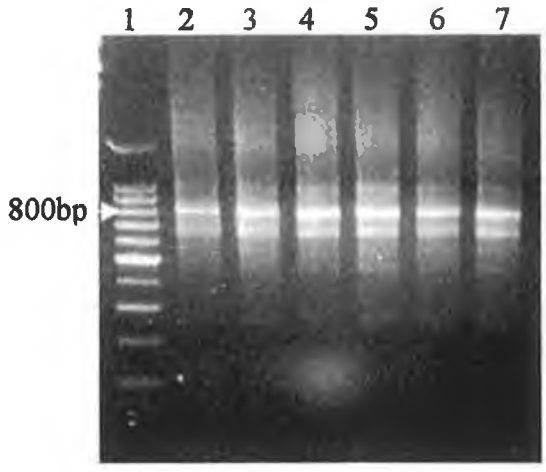


***Figure 6.6** : SOE-PCR on heavy and light chains for EE, DES and TR libraries. Lane 1: 100bp marker, Lane 2-3: EE, Lane 4-5: DES, Lane 6-7: TR.*

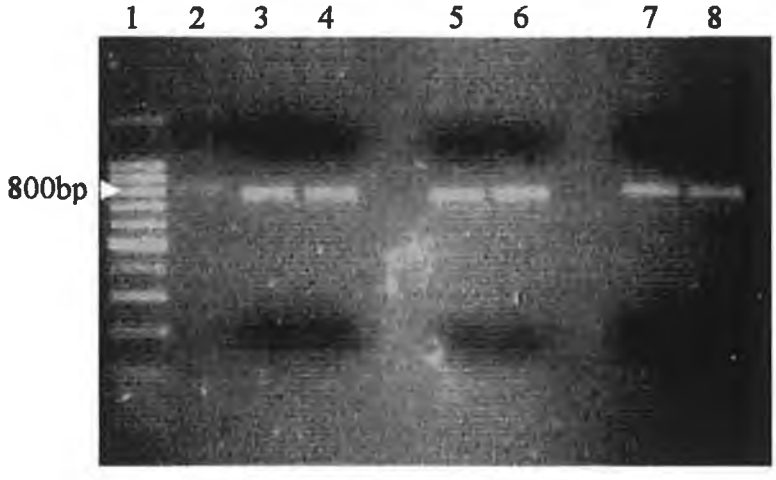


***Figure 6.7** : Sfi1 digestion of V_{HL} fragments. Lane 1: 100bp marker, Lane 2: unrestricted purified V_{HL} fragment, Lane 3-4: restricted EE V_{HL} fragment, Lane 5-6: restricted DES V_{HL} fragment. Lane 7-8: restricted TR V_{HL} fragment.*

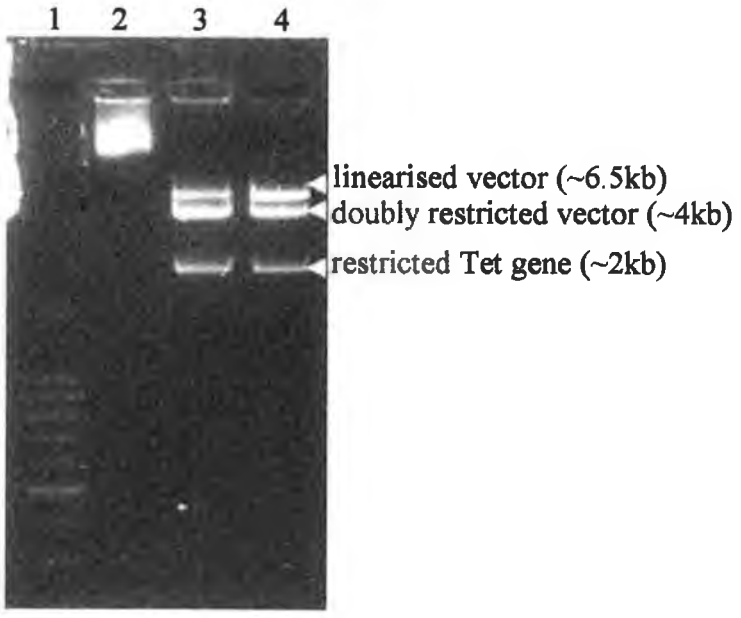


***Figure 6.8** : Restriction of pAK100 phagemid using* Sfi*I restriction enzyme. Lane 1: 100bp marker, Lane 2: Unrestricted pAK100, Lane 3-4: Restricted pAK100.*

6.2.2.6 Ligation of insert into phagemid vector

The ligation is described in section 2.4.10.9. The formula below was used to calculate the amount of DNA to add to each reaction. The concentrations were estimated using a quantifiable marker on an agarose gel.

$$\text{amount of insert (ng)} = \frac{\text{amount of vector (ng)} \times \text{size of insert (kb)}}{\text{size of vector (kb)}} \times \text{required ratio of } \frac{\text{insert}}{\text{vector}}$$

Using the ratio of 1.5 vectors for each insert increased the chances of each V_{HL} construct being incorporated into a vector. The 5 µl of the ligation mixture was visualised on an agarose gel to ensure that ligation had occurred before proceeding to the transformation step.

6.2.2.7 Transformation of Supercompetant XL1-Blue *E. coli* cells with ligated vector

The cells were transformed as per the manufacturers instructions and the resulting colonies were titred. The cells were either screened immediately or they were panned and then screened by phage ELISA depending on the size of the library. The sizes of the libraries constructed using this method are listed in Table 6.2. A number of different libraries were constructed. Not all of them were large enough to pan and some did not produce sufficient colonies to screen (Table 6.3). Figure 6.9 shows the results from a screening ELISA. The positives are fairly weak but they were all tested to see if they had specificity for the drug in question.

Testing was carried out on all positive clones from the non-competitive ELISA. All results showed that the results seen in the non-competitive ELISA were non-specific and the clones did not contain the genes for a scFv coding for the required drug specificity.

Table 6.2 : Number of colony forming units (cfu) in each of the constructed libraries produced using the Krebber method.

Library Size	Library 1	Library 2	Library 3	Library 4
EE	2×10^4 cfu	1.4×10^3cfu	0 cfu	4×10^3 cfu
DES	6.6×10^3 cfu	3×10^3 cfu	2×10^3cfu	7×10^3 cfu
TR	5×10^3 cfu	3×10^2 cfu	6×10^2 cfu	4×10^2 cfu

Table 6.3 : Panning and screening conjugates for each of the sets of constructed libraries

Library	Round	Panning Conjugate	Screening Conjugate
Library 1	1	THY	BSA
	2	BSA	BSA
	3	DEX	BSA
	4	OVA	BSA
Library 2	1	BSA	No binders eluted
Library 3		No pan	BSA
Library 4	1	DEX	BSA
	2	THY	BSA
	3	DEX	BSA
	4	DEX	BSA

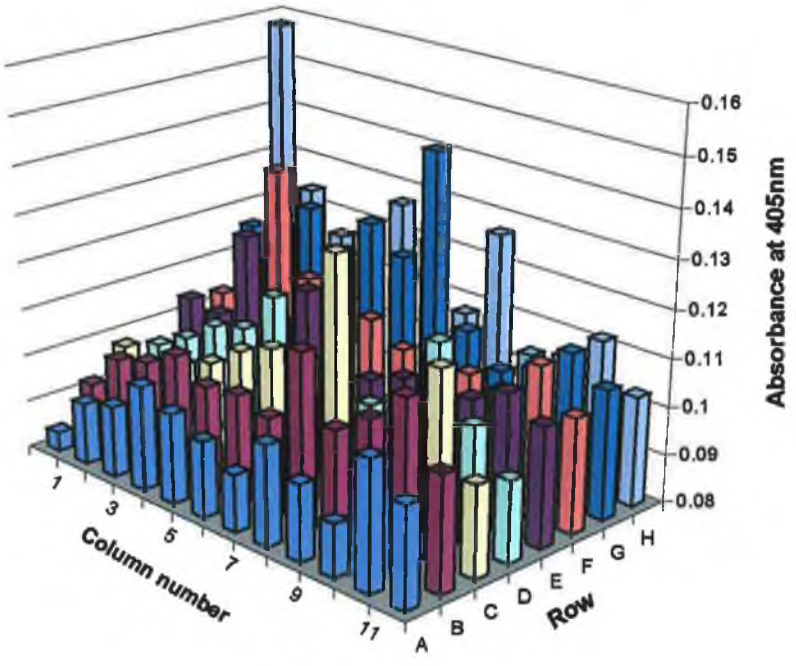


***Figure 6.9** : Absorbance values obtained after screening 95 clones in a non-competitive ELISA after pan 3 against EE with library 4. Four clones were tested further for EE specificity, H1, F3, G7 and C8. The negative control was H12. None of the clones tested showed competition in further analysis.*

6.2.3 Naive Krebber Library

This library was created from the spleens of naive mice that had not been exposed to any immunisation. In theory they will have the entire antibody repertoire present for amplification. In creating this library the diversity is the most important issue. It must be as diverse as possible as it will be used to isolate scFv's for many different antigens. In this study a naive library, Manning1, was panned against two of the drugs under investigation, EE and DES (Table 6.4).

Phage from rounds 2 and 3 were grown up in 96 well format for a phage ELISA. Round 2 was screened against BSA conjugates and did not produce any clones that gave a significant reaction in the phage ELISA. Round 3 was screened against DEX conjugates and some weak positives were detected. Figure 6.10 shows the results from the phage ELISA for the third round screening of the EE library.

These positives were grown up using different scale-up procedures and each was tested against conjugates, carrier proteins and blocking solutions. Figure 6.11 shows the results using one of the clones from the DES library. The only significant response was seen against THY. Clones chosen from the EE library gave comparable results. No clone from either the DES or EE libraries showed specificity for or competition with the required drug.

Table 6.4 : **Results from the panning of a naive library, Manning1, against EE and DES.**

Panning Round	Phage	EE Selection	DES Selection
Round 1	Input	1×10^{10}	1×10^{10}
Conj.: OVA	Output	8.48×10^{5}	1.06×10^{5}
Round 2	Input	1×10^{10}	1×10^{10}
Conj.: DEX	Output	3.4×10^{4}	1.04×10^{4}
Round 3	Input	1.23×10^{11}	1.41×10^{11}
Conj.: THY	Output	1.29×10^{4}	4.92×10^{2}

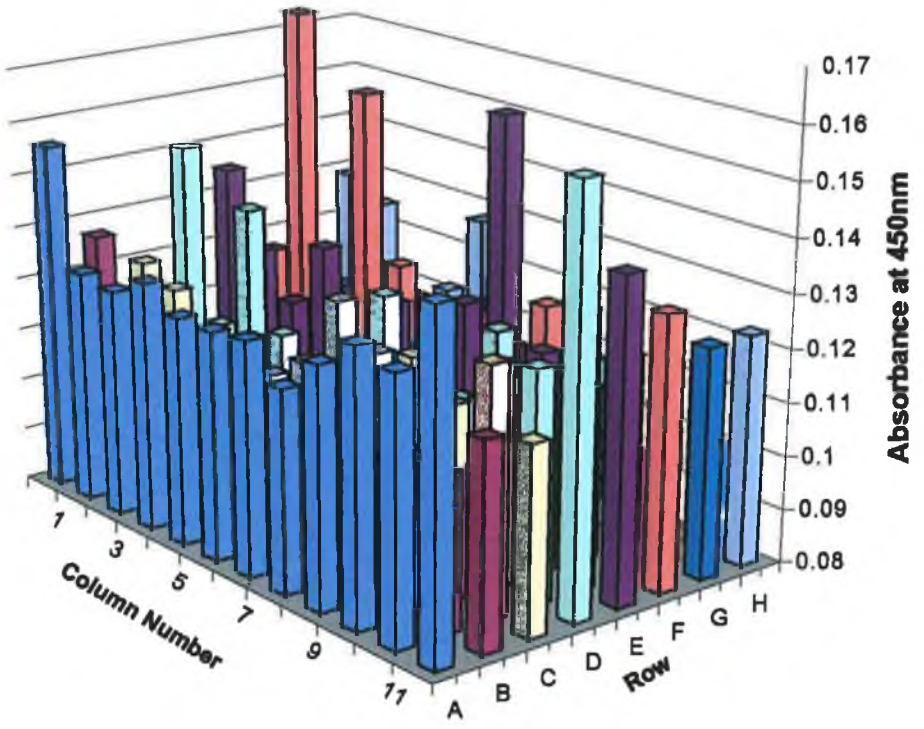


***Figure 6.10** : Absorbance values obtained from the screening of 95 clones after the third pan against EE. Three of the clones were chosen for further testing, F2, D12, E9. Clone D6 was grown and used as a negative control in further experiments. H12 was a blank well that was not inoculated with a clone from the library.*

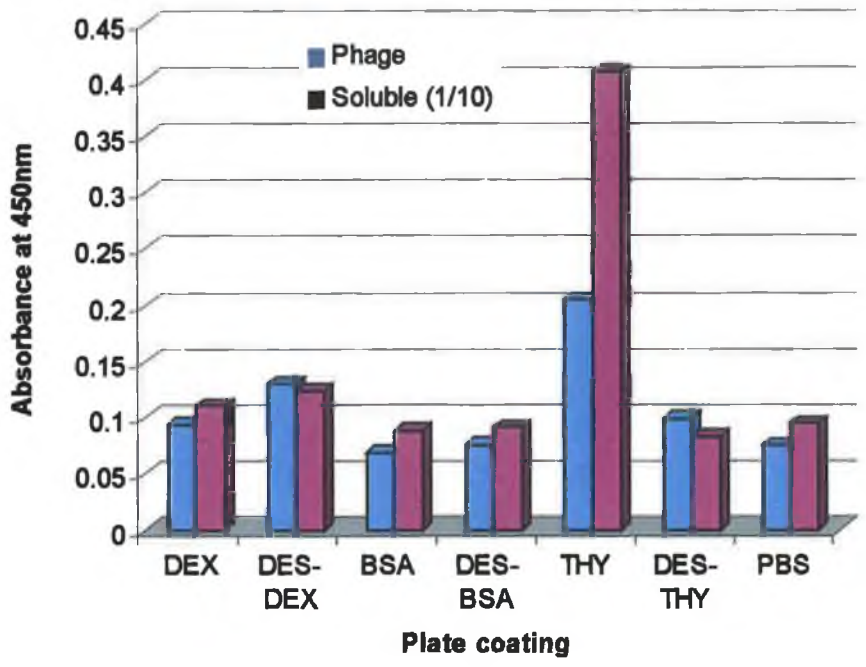


***Figure 6.11** : Specificity of anti-DES clone A6, as both a phage bound and soluble scFv, against a number of conjugates and hapten carriers. The clones did not recognise any of the conjugates or proteins apart from THY. The clone did not show any response to the DES steroid.*

6.2.4 Selection of scFv from the Vaughan naive library

The Vaughan library is a naive human library with a repertoire of 1.4 x 10^{10} scFv's displayed on the surface of phage (Vaughan *et al.*, 1996). This library was panned against EE, DES and TR (Figure 6.5).

The selected phage from rounds 3 and 4 were grown up in 96-well format for screening by phage ELISA. Both rounds showed positives in these ELISAs and it was decided to continue work with the positives from round 4, Figure 6.12.

Table 6.5 : Results from the panning of a naive library, Vaughan, against EE, DES and TR.

Panning Round	Phage	EE Selection	DES Selection	TR Selection
Round 1	Input	2.1 x 10^{11}	2.1 x 10^{11}	2.1 x 10^{11}
Conj.: BSA	Output	1.7 x 10^{6}	4.9 x 10^{6}	2.2 x 10^{6}
Round 2	Input	2.6 x 10^{9}	4.0 x 10^{9}	5.5 x 10^{9}
Conj.: DEX	Output	2.4 x 10^{2}	8.4 x 10^{3}	9.0 x 10^{2}
Round 3	Input	2.4 x 10^{10}	2.1 x 10^{10}	2.8 x 10^{10}
Conj.: OVA	Output	1.3 x 10^{6}	3.7 x 10^{5}	2.9 x 10^{7}
Round 4	Input	1.8 x 10^{10}	3.4 x 10^{10}	3.4 x 10^{10}
Conj.: BSA	Output	4.2 x 10^{7}	2.5 x 10^{6}	7.3 x 10^{6}

Clones were grown up as phage particles, soluble scFv and as periplasmic extracts. They were tested for their ability to recognise bound conjugate and for their ability to recognise competing drug. None of the clones tested showed any significant amount of recognition of the steroid over carriers or blocking solution (Figure 6.13).

In many cases the response seen in the ELISA analysis was due to the recognition of the blocking proteins by the phage or the soluble scFv's.

One clone was grown and scFv was expressed into the periplasm. This was purified using IMAC and analysed by SDS-PAGE, (Figure 6.14). The resulting fraction had a molecular weight of approximately 31kDa, the expected weight of a scFv protein. It did not, however, recognise the drug on which it was selected.

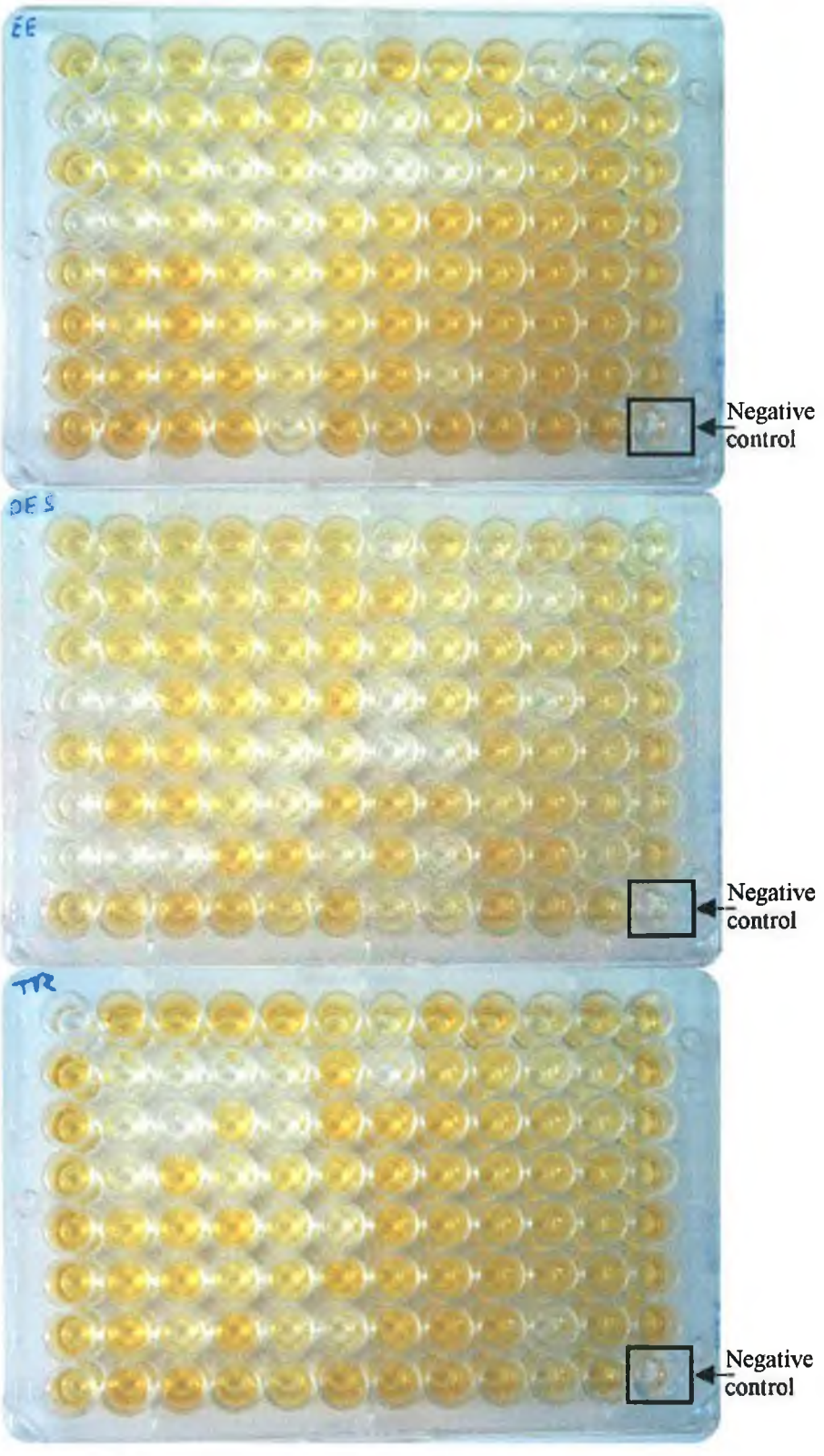


***Figure 6.12** : Results from phage ELISA after the 4th round of panning of the Vaughan library. H12 contains a negative control on each plate. Varying amounts of reaction are seen to the coated antigen and a number of these clones were tested further.*

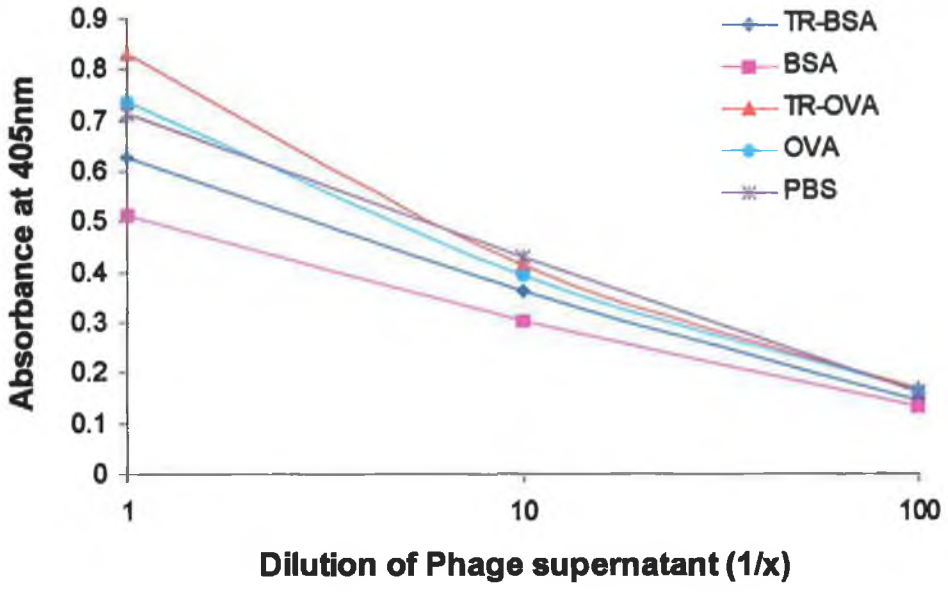


***Figure 6.13** : Response of phage particles from clone, A4, from the 4th round screening of the Vaughan library against TR conjugates and their carriers. There is no significant difference seen in response between the conjugates, the carriers and the blocked plastic. The other clones tested showed similar results.*

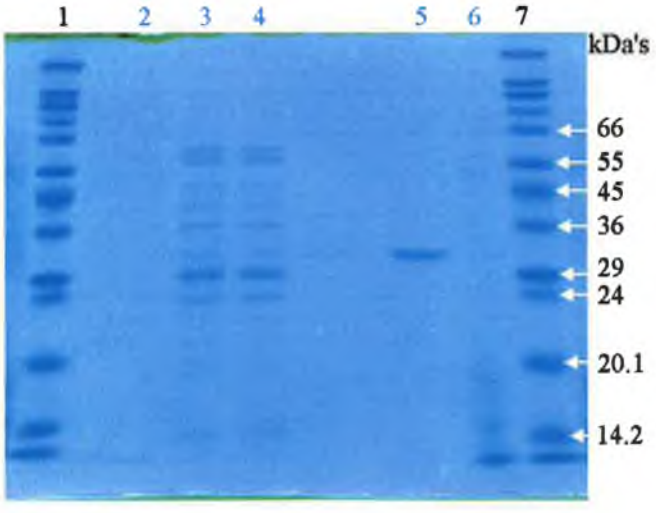


***Figure 6.14** : SDS-PAGE of scFv purification by IMAC. Lane 1 & 7: Molecular weight marker, Lane 2: Growth media supernatant after cells were removed, Lane 3: Periplasmic lysate, Lane 4: Non-binding fraction on IMAC column, Lane 5: IMAC-purified scFv fraction. Lane 6 contains some overflow from the marker in lane 7.*

6.3 Discussion

6.3.1 Pre-immunised Libraries

The pre-immunised libraries that were produced were quite small with only one library containing more than 10,000 clones. This is a very small library system when libraries containing 10^{10} clones are possible. The reason for continuing work with these small libraries was the fact that they were constructed from mRNA extracted from a pre-immunised mouse. This mouse had been immunised with a drug conjugate previously and had been sacrificed following the appearance of a suitable antibody response from its serum. Once it was clear that the mouse was producing antibodies for this steroid it is assumed that the number of antibody producing cells with specificity for this drug would be increased. The spleen was removed and the mRNA, representing the template for protein production, was isolated. This had to be done quickly so as to avoid degradation of the mRNA by the naturally present RNAses. Using a smaller library derived from this template is feasible as much of the antibody mRNA present codes for binding sites specific for the immunogen, in this case the steroids, EE, DES and TR.

Using this mRNA as a template, cDNA was produced and used as the starting material for the library construction. The extensive lists of primers that are used in this system were developed by Krebber *et al.* in 1997. They use a set of primers that cover all mouse V_H, V_λ and V_κ sequences found in the Kabat database and other previously described primer sets. Using this extensive set of primers it is proposed that the widest range of sequences possible will be amplified.

Each amplification is performed a number of times and the resulting chains are purified and combined. By amplifying the chains on a number of occasions the greatest diversity of sequences can be achieved. This also occurs for the SOE-PCR. This stage can be enhanced by repeated the PCR more than once and combining the results so increasing the amount of different V_{HL} constructs created.

The restriction in this particular library construction is quite ingenious as it only requires a single enzyme and yet still allows for directional cloning. The *Sfi*1 restriction enzyme is a cutting enzyme that recognises a sequence of 4 nucleotide

bases on either side of a random, non-recognised 5bp sequence. The insert itself can be ligated in the correct orientation by engineering this random sequence to be different on either side of the insertion point of the vector.

A commercially available preparation of supercompetent XL1-Blue *E. coli* cells was used to maximise the number of transformants. These cells enhanced the chances of all ligated phagemid vectors being incorporated into a host cell. It is possible to estimate the size (no. of phagemid clones) of the constructed library by calculating the number of cells containing the phagemid after transformation.

The constructed libraries were then tested to determine if they contained any clones that recognised the haptens of interest. The clones from libraries 1 and 4 were subjected to bio-panning before they were screened. In theory this step would enrich the clonal population to one that contained mainly V_{HL} sequences that would bind to the specific targets. Repeating this step a number of times and altering the selection conjugates should isolate specific antibody fragments. The scFv proteins displayed on the surface of phage were panned against a number of hormone conjugates including THY, BSA, DEX and OVA. One of the advantages of using DEX is that it has proven very difficult to raise scFv's against it. This makes it an ideal choice as a conjugate carrier as few, if any scFv's specific for it will be selected. This is borne out in the low amount of phage that are eluted after a DEX panning in contrast to the amounts seen with protein carriers as shown in Table 6.5.

Usually a phage ELISA was carried out on clones after the third or fourth rounds of panning. The second and third libraries were tested directly on phage ELISA due to their small size. A positive result was taken as any absorbance value that is significantly higher, 150%, than the background or negative control on the plate. The phage ELISAs were mostly carried out using a steroid-BSA conjugate-coated plate to which the phage supernatant was added. The bound phage was detected by an anti-fd bacteriophage polyclonal antibody followed by a HRP conjugated anti-rabbit IgG. This does not test for total specificity to the steroid but it is a useful guide as to which clones may bind to the target molecule.

Clones that showed a response on the phage ELISA were subjected to further testing, for example competitive ELISAs and soluble expression of the scFv. Competitive ELISAs using the phage particles or soluble scFv proteins were carried out. The clones tested were shown not to be competitive, that is specific

for the steroid molecules. The clones were grown in small culture volumes, 5-15 ml, and induced to produce phage or soluble scFv. These particles were tested in ELISA for their ability to bind to both an immobilised conjugate and to free steroid. In all cases this competition was not seen. This indicates that the response seen on the ELISA was due to non-specific binding of either the phage particles or one of the detection antibodies and not due to a specific recognition of the target molecule.

In addition, the clones did not produce absorbance readings much greater than the background absorbance of the plate in the original screening ELISA. Clones were considered to be positive if they had an absorbance of over 0.12 when the negative control was 0.08. It could be argued that these results were not due to specific interactions. It was decided to investigate the clones further to determine if the low signals seen were due to low phage concentrations or lack of specificity.

In our laboratory the Krebber method has been used successfully to produce scFv libraries containing specific clones for aflatoxins and morphine (Daly, 2002; Dillon, 2002). The size of the constructed libraries was reported to be $5x10^3$ clones and $5x10^3$, respectively. Both libraries have produced scFv and they have been expressed using a number of the pAK vectors. One of these vectors fuses the scFv fragment to an alkaline phosphatase enzyme. This has been used in ELISAs to produce assays that can be run quickly and without the need for any further enzyme labelling (Brennan, 2002).

Both the aflatoxin and morphine molecules are small and as such required conjugation before immunisation. They have also been shown to be very immunogenic in this form and useful polyclonal antibodies have been raised against these conjugates. This can also be said for the target molecules used here with specific and sensitive polyclonal antibodies having been produced using conjugates of TR (Chapter 3), EE (Chapter 4) and DES (Chapter 5). The lack of success in raising recombinant antibodies could be due to the loss of diversity in the initial stages of the library construction. If at this stage all the primers were not acting optimally, this could result in the loss of some diversity in the constructed library. One method of maximising the diversity may be to limit the number of primers used in each amplification reaction and then pool the results.

By limiting the number of primers each primer set may have a better chance of amplifying a sequence and so creating a larger genetic repertoire.

6.3.2 Naive Murine Library (Manning1)

A naive library was constructed previously using the same technique as that described for the production of a pre-immunised library. This was done using spleenomic mRNA from mice that had not been exposed to any antigens and therefore had the largest undifferentiated B-cell population. With this large population it should be possible to isolate all the possible variable sequences and amplify them to produce a large, broad scFv library. The advantages to this approach are outlined in section 1.2.4.

The Manning1 library was panned against two of the steroids under study, EE and DES. This library failed to provide scFv fragments specific for either drug. Both libraries showed weak positives on phage ELISA (Figure 6.10) but none of these showed competition or specificity for the steroids in further testing. Figure 6.11 shows the responses seen when one of the anti-DES clones was grown both as phage particles and as soluble phage. The clone does show a response but it is to THY, a carrier protein.

One of the rationales of panning with a number of different conjugates is to eliminate the number of clones that are specific to carrier proteins being selected. In this study an OVA pan was followed by a DEX pan and finally a THY pan. With this protocol the phage that may have bound to the OVA in the first round will have no target in the immunotube during the second round panning. In this way they should not be selected and only those phage that react with the elements common to both the first and the second round of panning, i.e. the steroid, should be amplified.

The results from these libraries show that the phage selected in rounds 1 and 2 contained many non-specific clones. The clones giving positive results on the phage ELISA after round 3 were shown to be specific for THY, the carrier used in the third pan. It was expected that some of the positive clones from the phage ELISA would have directed towards the steroid. The fact that only THY-specific clones were isolated implies that no specific clones were being selected throughout the panning procedure.

If it is the panning procedure itself that was inadequate a number of strategies can be applied to increase the likelihood of success. An alternative panning strategy or different elution methods could be utilised to increase the effectiveness of the panning procedure.

This library has been used previously to screen for scFv's against aflatoxins, morphine, chlorpyrifos, and *Listeria monocytogenes*. None of these studies were successful in producing specific clones. This evidence, coupled with the inability to select scFv capable of binding either of the two steroids tested, leads to the conclusion that this library does not contain enough diversity to be used to develop numerous binding specificities.

6.3.3 Naive Human Library

The Vaughan library is a naive human scFv library with a reported diversity of 1.4 x 10^{10} clones (Vaughan *et al.,* 1996). This library was panned against three different steroid hormones, EE, DES and TR with a sequence of BSA, DEX, OVA and BSA conjugates. This approach was designed to eliminate non-specific protein binders from being selected and passed from round to round.

One hapten for each of the steroids was used to produce all the conjugates required for his work, TR-17-hemisuccinate, EE-6-carboxymethyloxime and DES-monocarboxypropyl. This meant that throughout the panning process the phage were exposed to the same linker region along with the steroid structure itself. During the selection process phage specific for these linker or bridge regions could be amplified. To avoid the use of these clones careful examination of positive clones is required. A clone recognising the linker region would bind to a conjugate specifically but would not show competition for the free steroid. This pattern of binding was not shown with any positive clone tested from any of the library methods.

The number of phage that were eluted following the DEX panning step was quite low in relation to the amounts eluted when proteins were used. Dextran is a long repeating sugar and it has proved difficult to raise scFv against it, as mentioned previously. With the use of dextran conjugates it is usual to assume that a greater proportion of eluted phage are directed against the drugs in question.

After the third and the fourth round of panning phage ELISAs were carried out on 95 clones eluted from the immunotubes. The results from the fourth round are

shown in Figure 6.12. The negative control on these plates was contained in H12 and as can be seen the majority of the clones were positive, to different extents, against the drug in question. This result seemed promising as the number of clones seemed to suggest that the selection strategy had worked and there were a number of different clones recognising one of the components immobilised on the surface of the plate.

12 positive and 1 negative clones in total from each library were tested for specificity. The clones were grown as phage particles, soluble scFv released into the supernatant and soluble scFv retained in the periplasm. Each of these preparations was tested for their ability to recognise the steroid, both in the immobilised and free forms. Figure 6.13 shows the response of one such clone to a number of conjugates and proteins. The clone seems to recognise each of the presented coatings just as much as any of the others including uncoated wells. This implies that the clones are recognising the only proteins that are common to all the wells, which is the milk blocking solution.

It is difficult to reconcile this conclusion with the procedures used. The phage used in the panning procedure were pre-blocked in a solution containing large amounts of the blocking agent (2% (w/v) marvel milk powder). In this way it was hoped that any milk-binding phage would be kept in solution and not bind to the blocked immunotubes during the panning. This does not seem to have worked in this instance. Another factor that supports this conclusion was the reduction in signals obtained when milk was used in the diluent for the phage or scFv. A further ELISA was carried out eliminating the use of milk marvel as the blocking solution. The plates were blocked with 1% (v/v) foetal calf serum but this did not remove the absorbance signal (data not shown). Responses were seen with FCS-coated wells. This implies that the clones isolated are polyreactive and are not recognising any one epitope in particular.

To ensure that a scFv was actually being expressed, SDS-PAGE analysis was carried out on the purified fraction from one of the periplasmic expressed clones. Figure 6.14 shows the resulting fraction using the IMAC purification method. A single strong band is seen at approximately 31kDa, the estimated size for a scFv. A protein of approximately 31kDa with an affinity for a metal chelating column was produced in the periplasm of the cells. It is not specific for the steroids and it may suggest that an alternative panning strategy would be worth investigating.

To use the same biopanning procedure with a different blocking protein may not cause any change in the reactivity of the binders produced. A new method for preventing the non-specific binding of the phage particles to the uncoated sites of the plastic immunotubes may be required. An alternative method of eluting the specifically bound phage may also prove useful. A strategy that uses a form of specific elution, possibly with competing steroids, may provide a more efficient way of selecting the correct phage (Wind *et al.*, 1997).

This library was subjected to panning, in our laboratory, against xilpaterol, a β-agonist used as a bovine growth promoter (Dillon, 2002). The results from this study were similar to those found here. The clones giving positive results in the phage ELISA were tested further and they proved to bind non-specifically to all proteins they were tested against. This included proteins that had not been a part of the panning process. From this evidence it seems that this particular method of clone isolation may not be the optimal process. The library has been used to isolate a scFv specific for estradiol (Vaughan *et al.*, 1996) showing that scFv for steroids are present in this library. An alternative panning and selection strategy may provide a greater return of specific scFv from this library.

A scFv specific for halofugine has been isolated from this library (Duffy, 2002). It has shown to be competitive and specific. The library was also panned against a number of other compounds but non-specific poly-reactive clones were again isolated. This implies that the library has a high proportion of these poly-reactive clones and they are preferentially isolated with the selection process used.

6.3.4 Summary

Three different approaches to the selection of steroid-specific scFv proteins have been attempted. A number of pre-immunised libraries have been constructed and panned for the presence of specific steroid binders. This strategy did not produce any antibody fragments that were able to bind to the target molecules. A naive mouse scFv library was panned. This strategy provided phage and soluble scFv that recognised THY, the protein carrier used at the third round of panning, above other proteins and molecules tested. None of the clones tested showed any specificity towards the hormone of interest. Lastly, a naive human antibody fragment library was selected against the three drugs. This library also failed to

produce clones specific for the drugs under study. The clones that showed positively on the phage ELISA proved to be non-specific in further testing.

It seems that these strategies, as applied in this study, are not able to select for the specificity that is required. An approach that may be useful in altering the outcome of this study could be the application of alternative selection or panning strategies.

CHAPTER 7

CONCLUSIONS

7.1 Conclusions

Steroid drugs can be used to enhance the growth characteristics of animals destined for the food chain. The drugs increase the feed-to-muscle ratio of the animal resulting in greater amounts of leaner meat produced per animal. The use of these drugs is illegal within the European Union and, as such, testing systems must be in place to ensure that animal products tainted with steroid residues are not permitted to enter the food chain.

The use of immunochemical methods of analysis is one approach to the screening of animals for the presence of these compounds. The testing systems must be very sensitive and be able to detect as little as 2 ng ml^{-1} of these drugs in a sample. Using specific antibodies as reagents confers the advantages of a specific recognition system and a strong non-covalent binding onto any analytical system.

One steroid analogue used extensively as a growth promoter before the European wide ban, which is still legal in both the US and Canada, is trenbolone acetate. This compound is readily metabolised into 17β-OH trenbolone, the active form of this drug. The use of this steroid must be monitored, not only in meat produced in Europe, but also in meat imported from elsewhere.

A hapten of TR was constructed by converting the hydroxyl group at the 17 position to a hemisuccinate. This introduced a chemical group (COOH) that could be easily linked to carriers for both immunisations and analytical methods. Two anti-TR-17-HS antibodies were raised in rabbits, purified and characterised. These antibodies were applied to two competitive ELISA formats (designated 'competitive' for direct competitive assays and 'inhibitive' for indirect inhibitive assays). The assays showed excellent sensitivity and precision for the detection of free TR in both buffer and bile. The competitive assays showed the greatest sensitivity and were the more robust of the two ELISA formats. The best competitive assay used TRAb1 and it was able to detect less than 2 ng ml^{-1} of free TR in a bile sample. This is the recommended cut-off or action limit for the residue level of this compound. The precision and accuracy levels of this assay were well within the limits for an ELISA-based assay.

These ELISAs have a number of advantages over previously described assays by combining low levels of cross-reactivity to related compounds, minimal sample

pre-treatment, the use of enzymatic labels and taking hours instead of days to perform. With these attributes these assays could be useful in large scale screening programmes to avoid the incorporation of tainted meat into the food chain.

Two polyclonal antibodies were raised in rabbits against the constructed conjugate EE-6-CMO-OVA. The antibodies were purified and their ability to recognise free EE was established. These antibodies were applied to both competitive and inhibitive ELISA formats and a biosensor assay. The precision and accuracy of the assays was investigated and each assay was well within the tolerances for an assay of this type. The assays were then adapted to detect free EE in bile samples. The precision and accuracy of the assays was again determined and while being slightly less precise and accurate than the corresponding assays for the detection of free EE in buffer, they are still with acceptable limits (± 20%).

The competitive ELISA using EEAb1 was capable of detecting 12 ng ml^{-1} of free EE in bovine bile. The assays to detect EE have similar advantages over previous analytical systems as the TR assays. They combine a specific recognition system with a simple pre-treatment step, easy to detect enzyme labels, a high throughput system and a short assay time. The BIAcore assays are not as sensitive as the corresponding ELISAs but may find a use in on-line rapid screening of single samples.

The sensitivity of both assays systems must be improved to lower their detection limit so that they would be able to detect the low residual amounts of EE required by the 2 ng ml^{-1} action limit that would lead to the removal of meat from the food chain.

With the ELISA assays described, although being quick and simple to perform, there is a presumption that they would be carried out in a laboratory environment. A test system like this may benefit from the ability to be portable and even more rapid. To this end the reduction of the incubation times of the competitive ELISAs were investigated. It was shown that the TRAb1 antibody could produce a result in less than 20min. The DES antibodies were also tested and, whereas, they were not as sensitive as the TR antibodies they could also detect nanogram levels of free DES in buffer.

These antibodies were then applied to a rapid ELISA machine. This machine uses the same reagents as for a competitive ELISA but it carries out the steps required for the assay without the intervention of an operator. The device automatically aliqouts the sample, mixes it with the enzyme-conjugate solution, incubates the wells, washes them and, finally, detects the bound enzyme. Assays using both the DESAb1 and TRAb1 antibodies were adapted for use with this machine. An assay system that detected the presence of either of the two steroids, TR and DES, in a single sample was also developed. This system could distinguish and measure low nanogram amounts of TR in a single or mixed sample but due to the slower kinetics of the DES antibody it was not able to detect nanogram amounts of DES. If more wells had been available for this device this work could have been extended and completed to give a simple, user friendly and portable assay system for the detection of a number of steroid hormones in a single sample.

While polyclonal antibodies have been shown to be specific reagents and useful in the development of assays systems, they do have drawbacks. Polyclonal antibodies by their very nature have numerous specificities contained within their solutions and these may cause large problems in assays due to cross-reactivity with related or even unrelated compounds. There is also a finite supply of this reagent. Once a particular antibody has been consumed the assay based on it must be re-optimised for any other polyclonal antibody that may be substituted.

To alleviate this problem the development of recombinant antibodies with specificities for 3 steroid hormones was attempted. This would result in an infinite antibody source that was monoclonal in nature. Three approaches were tried for the construction and isolation of scFv antibody fragments. A naive murine library and a naive human library were panned against the three drugs in question, EE, DES and TR. ScFv specific for the hormones were not isolated from these libraries. It is proposed that the murine library may have been too limited to contain the scFv required. This library had been panned on previous occasions against other small haptens and proteins and no specific antibody fragments have been isolated from it.

The naive human library was a large library system with greater than 10^{10} individual clones and one of the compounds used to test the utility of the library was a steroid hormone. It was hoped that this library would contain the required

scFv clones. The library was panned against the three steroid drugs and the clones isolated were all non-specific. They each recognised a number of different proteins, some used in the panning procedure and some that were not. None of the clones tested showed any specificity for the drugs being studied.

The third approach consisted of pre-immunising a mouse with the hapten of interest so that the number of B-cells producing specific antibodies would be maximised. Libraries were then constructed from the mRNA isolated from the spleen of the immunised mice. These libraries should have contained a greater number of clones specific for the original immunogen. The libraries were all panned and tested for their ability to recognise both conjugate and free drug, but no specific clones were isolated.

This research has successfully generated a number of different working immunoassays for the detection of three steroid hormones, used as illegal growth promoters in meat.

CHAPTER 8

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Appendix A

Glossary of terms and definitions commonly employed in bioanalytical validation procedures

The terms listed below were referred to for validation purposes of certain procedures carried out in this thesis. The criteria which they can be defined under have been reviewed elsewhere (Findlay *et al.*, 2000).

Mean

Describes the average of replicate (x) measurements, (i.e. $\frac{\sum n_{1-x}}{x}$)

Precision

Is defined as the closeness of agreement, or variance between independent test results of multiple measurements of the same sample obtained under a set of specified analytical test conditions. It is normally expressed in terms of the relative standard deviation (% R.S.D.), or the coefficient of variation (% C.V.) of the determined concentration of a replicate number of assays. The degree of precision assessed between replicates (i.e. % C.V.) performed during a single assay batch, is commonly referred to as the intra-assay variation (also referred to as repeatability). The term inter-assay variation is (also referred to as reproducibility) is used to describe the precision between assays when related to multiple batches.

Limit of Detection (L.O.D)

Defines the lowest analyte concentration that the analytical technique can differentiate from background signals and is usually determined as background noise ± 3 standard deviations.

Robustness

Is a term used to describe the ability of an analytical techique to withstand fluctuations in the described analytical test conditions. For immunoassay

procedures the term could be used to describe changes in the ionic strength of the sample matrix, as well as pH and temperature changes.

Standard Curve

This describes the relationship between the measured analyte response (i.e. absorbance, response units) and the analyte concentration.

Non-Specific Binding

This describes matrix effects which affect the degree of binding of the antibody:antigen interaction, and can occur as a result of increased protein concentration and sample viscosity in the sample matrix (e.g. grain matrix), and also as a result of altered ionic composition (e.g. bile) of the sample matrix.

Coefficient of Variation

A quantitive measure of the precision of an analytical measurement expressed as a percent function of the mean value, also referred to as the Relative Standard Deviation (% R.S.D.)

$$\%CV = \frac{S.D.}{Mean} \times 100$$

Precision Profile

A quantitive measure of the variation between measurements, usually the coefficient of variation versus the nominal concentration of analyte in the sample.

Normalised Absorbance Values

The absorbance recorded (AU) at each particular antigen concentration divided by the absorbance recorded in the presence of zero antigen.

$$\text{Normalised Absorbance} = \frac{\text{Absorbance measured at particular antigen concentration}}{\text{Absorbance measured at zero antigen concentration}}$$

Appendix B

Mass spectrometry analysis of steroid derivatives

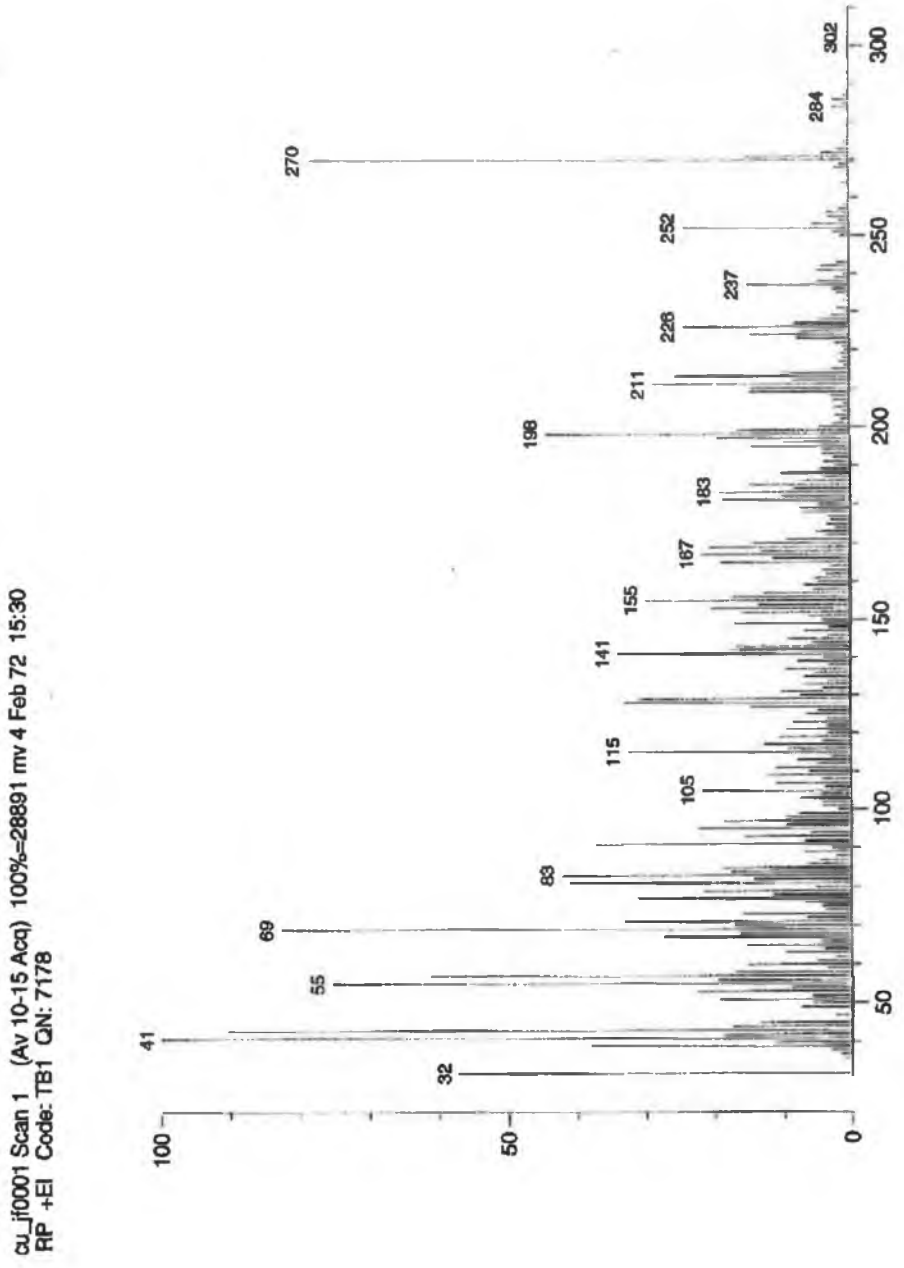

***Figure 1**: Nominal Mass Spectroscopy analysis of Trenbolone (MW 270).*

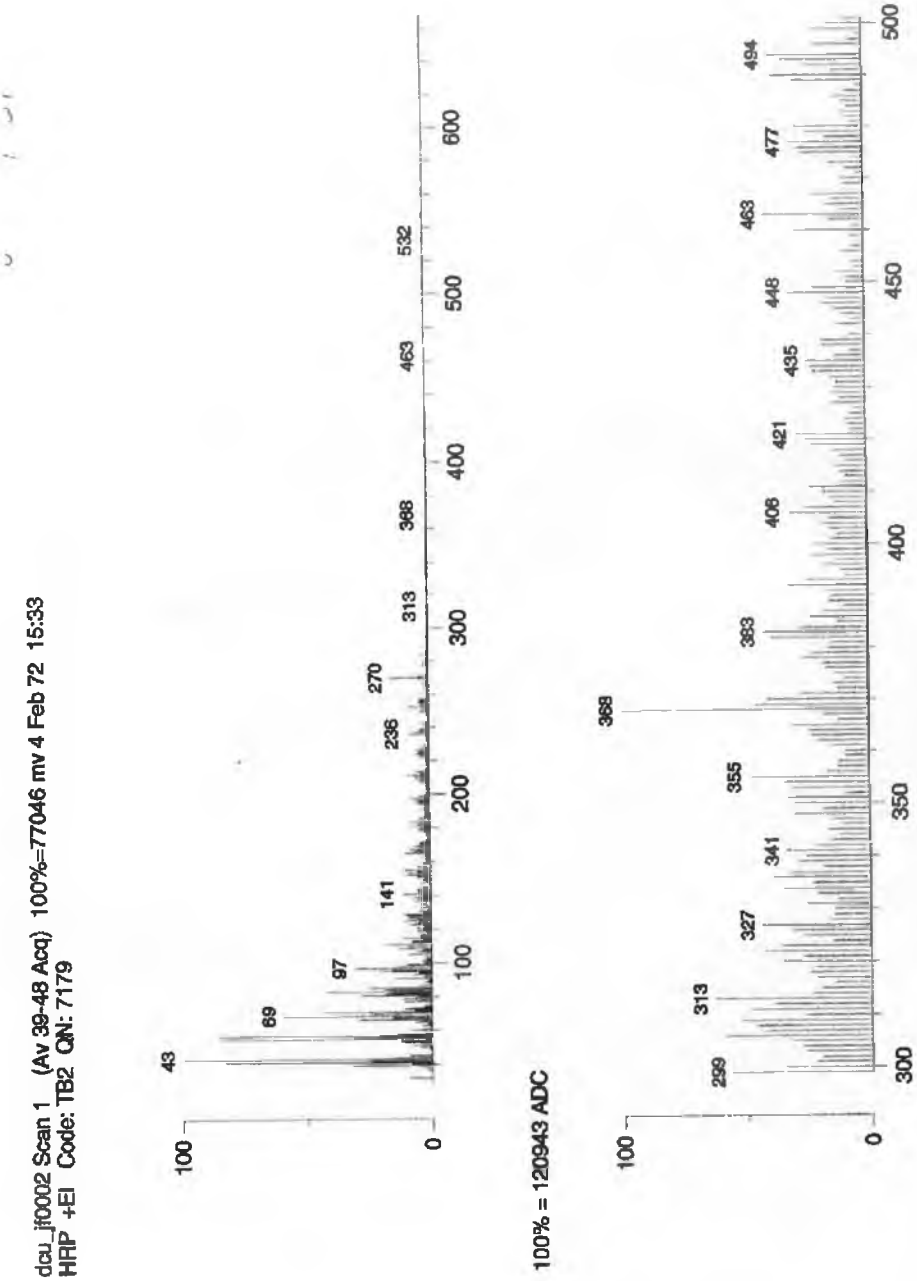

***Figure 2:** Nominal Mass Spectroscopy analysis of Trenbolone-17-hemisuccinate (alkalinated, MW 368)*

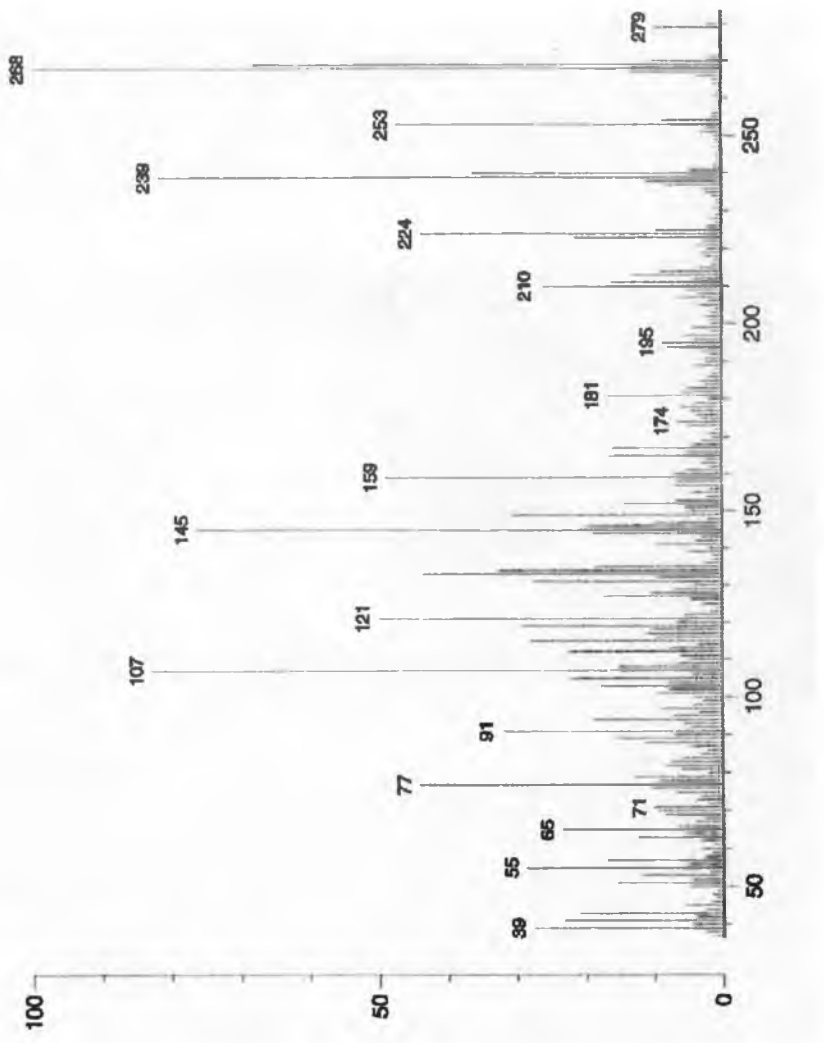

***Figure 3:** Nominal Mass Spectroscopy analysis of Diethylstilbestrol (MW 268)*

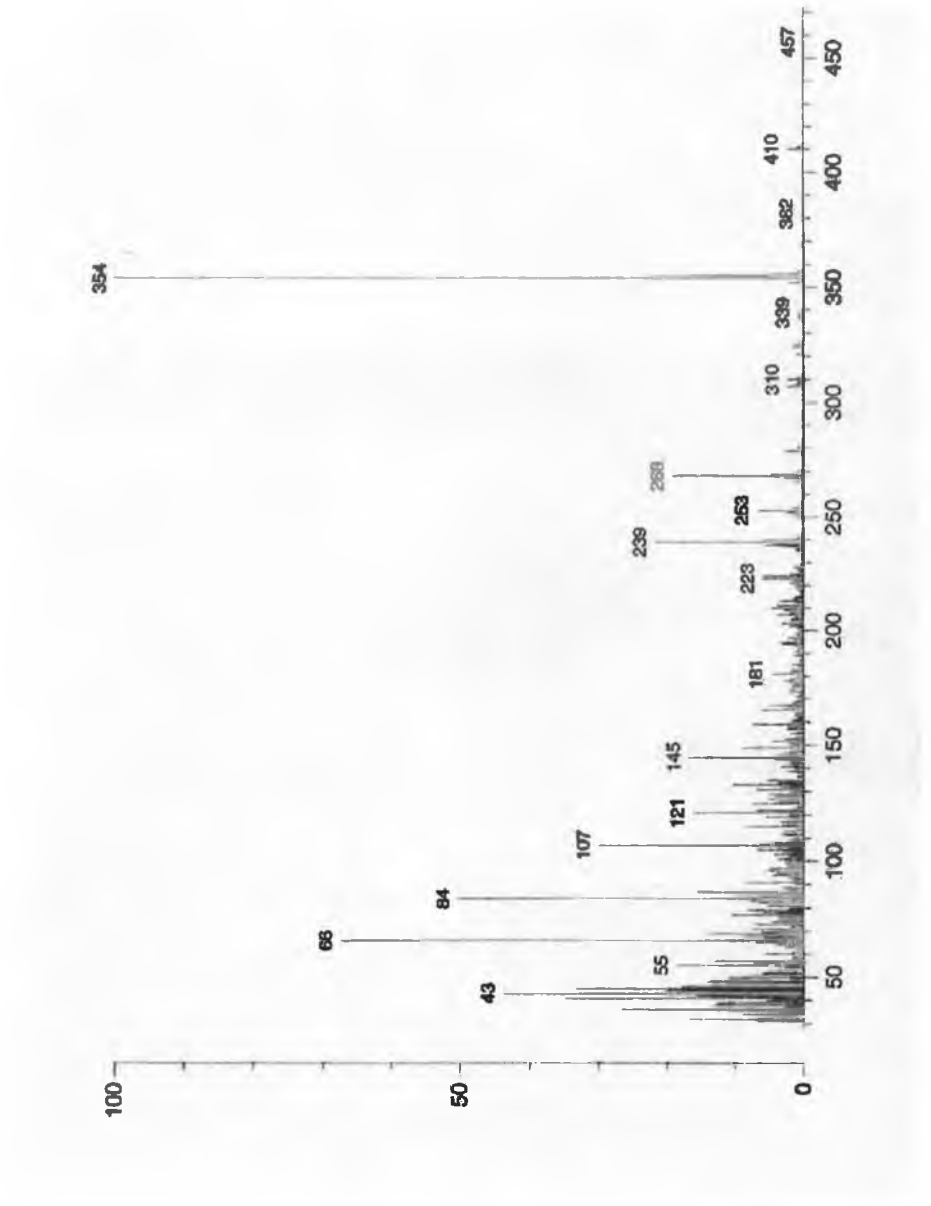

***Figure 4:** Nominal Mass Spectroscopy analysis of Mono-carboxypropyl Diethylstilbestrol (MW 354)*