Analysis of some β-Adrenergic Agonists in Biological Matrices after Evaluation of Various Extraction Methodologies and Determination Procedures.

by

Damien Thomas Boyd B.Sc.

A thesis submitted for the Degree of

Doctor of Philosophy

Dublin City University September 1994

Declaration

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

Signed L Boyd Date 5/10/94

Candidate

Dedicated to my parents, Cathal and Bridgeen

Acknowledgements

During my three years research period I was fortunate to be the recipient of a great deal of help and encouragement from a number of people.

Firstly, my industrial supervisor at the National Food Centre, Dr. Michael O'Keeffe, for his excellent guidance and for directing me "through the deep and intrepid waters of residue analysis":

The people in the lab at the NFC especially Sean, Paula, Paddy, Audrey, Joan, Aodhmar, Sean, Liz and Greta:

The people in "Electroanalisis", Oviedo, especially Dr. Jose Ramon Barreira Rodriquez and Prof. Paulino Tunon Blanco, for making my days in Spain so memorable:

Teagasc, the Erasmus programme, the FLAIR programme and the School of Chemical Sciences for their financial support:

Karen Williams and Prof. Dai Games at the University of Swansea (re FLAIR project), Central Veterinary Laboratories (UK) (re supplying treated liver);

My family for their help and constant encouragement;

My fellow postgrads at DCU for keeping me in mind:

And finally to Prof. Malcolm Smyth for the constant support, advice and supervision and for giving me the opportunity to travel.

TABLE OF CONTENTS

ABSTRACT

Biological sample clean-up procedures were evaluated for the extraction of a range of β-agonists. Once extracted the compounds were determined by immunoassay, electrochemical techniques and supercritical fluid chromatography (SFC).

Clenbuterol was isolated from liver tissue using matrix solid phase dispersion (MSPD). The technique was optimised for extract clean-up and recovery by evaluation of various wash and elution solvents using radiolabelled clenbuterol. Recovery of clenbuterol was > 90 % at three levels tested (1, 2 and 5 ng/g). MSPD was then applied to the extraction of other compounds in this class like salbutamol, mabuterol, cimaterol and terbutaline in the low ng/g range. For residues which occur as conjugates, an enzyme hydrolysis procedure was used. Sample extracts were assayed by radioimmunoassay (RIA) and enzyme immunoassay (EIA).

Cyclic voltammetry (CV) was used to study the electrooxidation of salbutamol, fenoterol and metaproterenol at unmodified and Nafion-modified carbon paste electrodes (CPE's). All compounds were oxidised irreversibly at high positive potentials at the CPE. The Nafion-modified electrode allowed the accumulation of all compounds with time, resulting in an enhanced sensitivity. The application of the Nafion-modified electrode to the analysis of fenoterol in human urine and serum extracts was demonstrated at the 10^{-7} and 10^{-6} M level, respectively. In this case the more sensitive differential pulse voltammetric (DPV) mode of detection was chosen.

Finally, the application of supercritical fluid extraction (SFE) for the isolation of β-agonists was investigated. Extracts of pure standards and liver samples, dispersed on support media (Celite and C_{18} material), were assayed by supercritical fluid chromatography (SFC) with UV detection.

CHAPTER 1

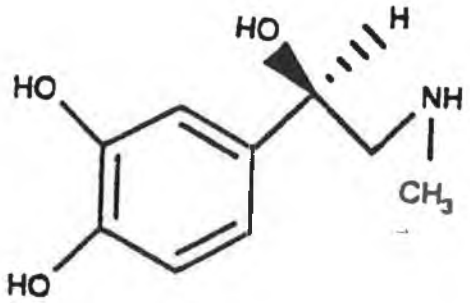
A REVIEW OF METHODS FOR THE ANALYSIS OF β-AGONISTS IN BIOLOGICAL MATRICES

1.1 INTRODUCTION

This chapter focusses on analytical procedures which are used for the determination of the class of compounds known as β_2-agonists. β_2-agonists are synthetic derivatives of naturally occurring molecules (catecholamines) and, as the name suggests, they bind to β_2-receptors on nerve cells to produce directly observable physiological effects (e.g. increase in heart rate). Their mechanism of action follows that of norepinephrine and epinephrine which, when released at nerve endings into the bloodstream, represent an important aspect of the functions of the autonomic (or involuntary) nervous system. This system controls many organs (cardiovascular, gastrointestinal etc) and metabolic processes. The autonomic functions are divided into two main components; the parasympathetic and sympathetic systems. The latter system is responsible for the "fight or flight" reaction. In such situations, energy is required immediately, and heart rate and blood flow through coronary vessels and skeletal muscles increase. These reactions are mediated by the catecholamines, epinephrine and norepinephrine. The structures of these two physiologically important compounds are shown in Figure 1.1

How a β_2-agonist, whether synthetic (e.g. clenbuterol) or natural (e.g. epinephrine), actually brings about a response is dependent on a series of biochemical reactions at the β_2-receptor in the cell membrane. The β_2-adrenergic receptor model, shown in Figure 1.2, shows how the response is

(a)



(b)

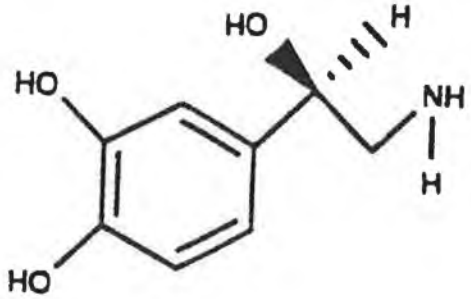


Figure 1.1 Chemical structures of (a) epinephrine and (b) norepinephrine

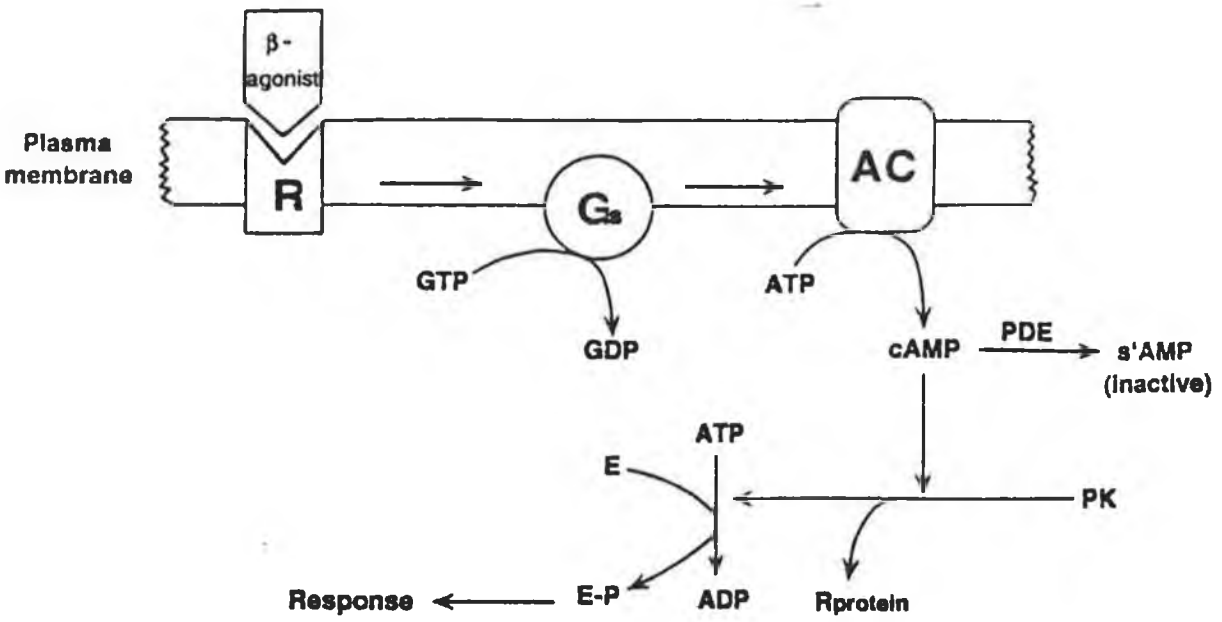


Figure 1.2 β-adrenergic receptor model

achieved. The β_2-receptor (R) is coupled via a protein (guanine-G_s) to the enzyme, adenyl cyclase (AC). Upon β-agonist-receptor binding, the G protein stimulates AC, which in turn stimulates the formation of a growth factor, cyclic adenosine 3, 5-monophosphate (cAMP). cAMP stimulates protein kinase (PK) activity, or it can itself be inactivated by phosphodiesterase (PDE). A regulatory protein (R protein) is split off and active PK stimulates phosphorylation of enzymes which can trigger the response. β-receptor activity results in the following effects; heart-rate increase, relaxation of muscle tissues in bronchi, uterus and intestinal wall, and the stimulation of insulin release and glycogen breakdown. The major clinical indications for use of β_2-agonists are respiratory diseases and tocolysis. In recent years it has been established that certain synthetic β_2-agonists, when administered to farm animals at multiples of the therapeutic dose, cause a shift in the flow of nutrients away from adipose tissue towards muscle tissue (commonly called a repartitioning effect) **[1]**. The net result of such practices is the production of a leaner carcass. The toxicological and pharmacological implications of residues present in edible tissues has led to the banning of these compounds as growth enhancing agents within the EU.

β_2-agonists are generally divided into two main groups, the substituted anilines which include clenbuterol and cimaterol, and the substituted phenols which include salbutamol and terbutaline. Figure 1.3 gives the chemical structure of the β_2-agonists studied, representing compounds from both groups.

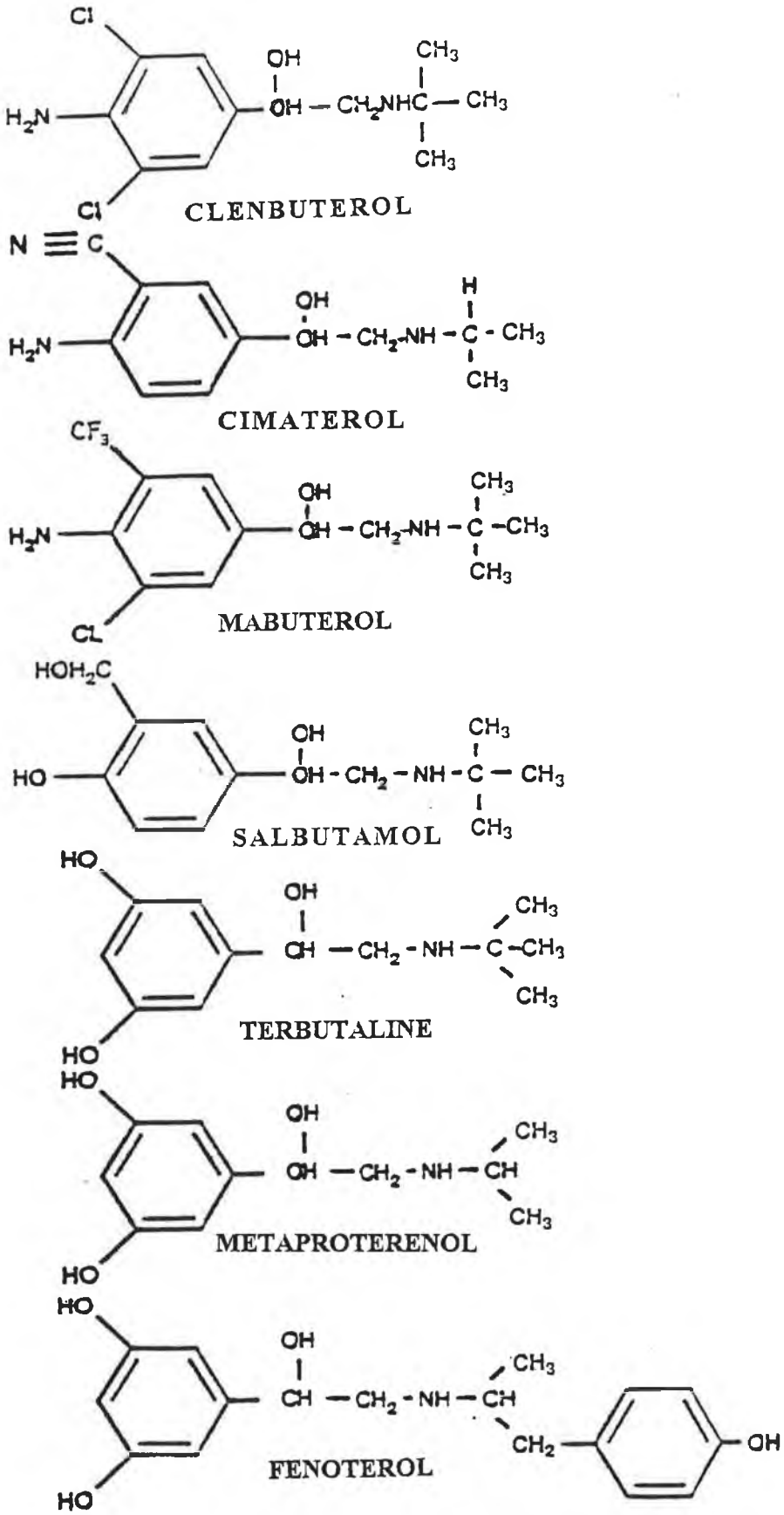


Figure 1.3 Chemical structures of the β-agonists studied

The compounds possess a common β-hydroxyamino group on the side chain, but are differentiated from each other by varied substituents on the aryl moiety and the terminal amino group. The hydroxyl group(s) on the aromatic ring of the substituted phenols is a target for glucuronide or sulphate conjugate formation **[2]**. Hence for determination of total residue concentration, a deconjugation stage must be incorporated into any assay procedure.

Selection of the appropriate biological sample for analysis is an important criterion for this type of compound. Plasma and urine may be monitored, but the levels are usually low (ppt to low ppb) as the compounds have short half-lives and are cleared rapidly from the body. Organs which accumulate β_2-agonists are more suitable for monitoring usage. Work by Meyer and Rinke has shown that the liver contains detectable clenbuterol residues for up to two weeks after withdrawal of the drug from the animals diet **[3]**. More recently, several authors have reported that eye fluids and tissue fractions may attain concentrations an order of magnitude higher than in liver **[4, 5]**.

The ideal determination procedure for β-agonists would combine efficient clean-up of sample matrix with sensitive measurement of analyte(s). For sample clean-up, a wide range of approaches have been adopted. Classical solvent extraction procedures have resulted in good recoveries of the substituted anilines such as clenbuterol and mabuterol **[6]**, but are less effective for the more polar compounds, such as salbutamol **[7]**. Emphasis

has now been placed on safer and more rapid alternatives like solid phase extraction (SPE), matrix solid phase dispersion (MSPD) and immunoaffinity chromatography (IAC). Such techniques may be more suited, also, for a multiresidue approach to analysis. After sample clean-up has been achieved, the compound(s) may be detected by any one of a number of sensitive techniques. High performance liquid chromatography (HPLC) with fluoresence or electrochemical detection has proved sensitive for one or a combination of β-agonists **[7-9]**. Ultraviolet (UV) detection only gives optimum sensitivity after a post-column derivatisation procedure **[10]**. Immunoassay techniques, such as radioimmunoassay (RIA) and enzyme immunoassay (EIA) or radioreceptor assays are most sensitive detection systems for β-agonists. Procedures have been reported, for the analysis of a β-agonist of choice (i.e RIA) **[11]** or a number of β-agonist compounds (i.e. radioreceptor assay) **[12]** in this class. Mass spectrometry is the most popular confirmation technique used in this field. Methods based on gas chromatography-mass spectrometry (GC-MS) have been developed for many β-agonists **[13, 14]** and, more recently, clenbuterol has been determined using methods based on liquid chromatography-mass spectrometry (LC-MS) **[15]**.

This review provides an up-to-date evaluation of the methodologies developed to determine β-agonists in complex biological samples. Emphasis has been placed on sample purification procedures leading to extracts

containing one or a combination of β-agonists which can be identified and quantified by an appropriate detection system.

1.2 SAMPLE PREPARATION PROCEDURES

Before a clean-up procedure may be carried out the sample must be prepared in such a way that optimised conditions for the extraction of the analyte(s) may be used. This is particularly relevant for complex matrices like biological samples, the nature of which determines the kind of pretreatment step required. For liquid samples, which include urine and plasma, suspended matter may be removed by centrifugation **[13]** or filtration **[16]**. For residues which occur as conjugates, enzyme and acid hydrolysis procedures have been used, prior to sample clean-up. An enzyme hydrolysis procedure on clenbuterol-incurred urine was carried out by Hooijerink *et al.* but their results showed that this compound forms relatively low levels of glucuronide (5%) conjugates (sulphate conjugates not found) **[17]**. For the phenolic-type β-agonists conjugate formation has been reported **[2, 18]**. Enzyme hydrolysis of salbutamol was carried out in urine, after the sample was adjusted to pH 5.0, by incubation with a dilute solution of enzyme (glucuronidase/sulphatase) at 37°C for between 16-18 h **[13, 19]**. Acid hydrolysis has been reported for the deconjugation of O-sulphate esters of metaproterenol in plasma samples **[20]**. After protein precipitation using trichloroacetic acid, the plasma

supernatant was incubated with 0.2 ml 0.2 M hydrochloric acid at 65°C for 90 min and free metaproterenol was extracted using C_{18} SPE after pH adjustment to 10.0. Alternatively, the hydrolysis step can be omitted altogether; incurred residues of salbutamol may be extracted from urine by cation-exchange SPE and analysed as the conjugate by MS **[21]**.

Solid samples, which include liver and muscle, require a more intensive sample pretreatment stage. The analyte(s) must be exposed to extracting solvents to optimise the conditions for extraction. This is accomplished, in part, by mechanical dispersion using a mincer and/or a homogeniser. The most popular approach for tissue break-up is the homogenisation of samples in water, acid or an aqueous buffer. Following centrifugation, the supernatant may be treated in various ways, made acidic or alkaline and extracted directly **[14, 22]** or pretreated further e.g. removal of fats using solvent extraction **[4]**. For the phenol-type β-agonists, a deconjugation step is required; useful studies have been carried out which show that the main salbutamol metabolite in calf tissue samples, including liver, is the sulphate conjugate **[23]**.

The use of ultrasonication to extract β-agonists from a tissue/aqueous acid medium has also been reported **[24, 25]**. An extraction time of 15 min was sufficient and the pH of the supernatant was adjusted to optimise further clean-up steps. In order to digest proteins, enzymatic digestion at 55°C has been described **[26, 27]**. Lyophilization (freeze drying) of rat tissue samples

has been carried out prior to extraction **[28]**. For eye tissues sample pretreatment is analogous to that of liver and muscle samples **[4]**.

1.3 SAMPLE CLEAN-UP/EXTRACTION OF β-AGONISTS

The main goal of the sample pretreatment step(s) is to produce a primary extract which can be easily cleaned-up using an appropriate extraction procedure. Various types of sample clean-up procedures are available: conventional liquid-liquid partitioning, solid phase extraction methodology, matrix solid phase dispersion and immunoaffinity chromatography. In some cases these procedures may be used in combination to obtain highly purified extracts.

1.3.1 Liquid-liquid extraction

Originally, liquid-liquid extraction was the standard method for isolating drug compounds from biological samples. Unfortunately, this technique is relatively time-consuming especially for the extraction of polar compounds or a combination of compounds with various chemical properties. The main approach to β-agonist extraction has been to adjust the pH of the sample above the pK_a of the compound (usually > 9.0) to achieve deprotonation, followed

by partitioning with an organic solvent. Most of the earlier methods for clenbuterol analysis adopt this type of clean-up strategy. Diquet *et al.* devised an extraction method for clenbuterol from mouse plasma, the compound being extracted at high pH into chloroform **[29]**. Although the recovery of drug was low (45%), the use of an internal standard, yohimbine, allowed reproducible measurement of the levels present. Higher recovery, from urine, of 80% was reported by Brunn, using dichloromethane as the extraction solvent **[30]**. Diethyl ether was the chosen solvent for the extraction of clenbuterol from bovine urine **[12]** and rat brain tissue **[8]**. The latter publication focusses on the lipophilic characteristics of clenbuterol; the drug penetrates through the blood-brain barrier in rats at pre- or post-natal development. For the more hydrophilic compound, salbutamol, ethyl ether was the extraction medium chosen **[31]**. In our laboratory, extraction of salbutamol with diethyl ether was found not to be possible, due presumably, to its hydrophilic character.

For sample extracts which are determined by mass spectrometry, the clean-up step must be particularly intensive. Blanchflower and co-workers performed multiple diethyl ether extractions in combination with acid back extractions to achieve a purified extract suitable for GC-MS analysis of clenbuterol **[32]**. Girault *et al.* measured clenbuterol in a wide range of bovine biological samples; pretreated samples were extracted at high pH using ethyl acetate, back extracted into dilute acid, before washing (ethyl acetate: hexane, 2:1) and final re-extraction into ethyl acetate **[14, 33]**. Forster *et al.*

used a similar procedure but with tertiary butyl methyl ether (TBME) as the main extraction solvent **[34]**. Fuerst *et al.* improved sample clean-up by washing plasma samples with dichloromethane prior to extraction of clenbuterol with TBME **[35]**. Other authors have reported extraction procedures for clenbuterol **[4, 11]** and the more hydrophilic compound, fenoterol **[18]**, based on the use of this solvent. For the latter extraction, adjustment of pH to exactly 9.5 allowed for the isolation of fenoterol from its conjugates.

In some cases, more than one organic solvent is required to extract the compound effectively. Horiba *et al.* studied the occurrence of mabuterol and its metabolites in urine samples **[36]**. Extraction, after sample evaporation and salting out steps, was carried out at pH 10 using ethyl acetate: acetone (3:1). Acidic and neutral metabolites were selectively removed after pH manipulation and further solvent extraction. A specific double extraction procedure for salbutamol in plasma samples was reported by Loo *et al.* **[37]**. Selective extraction of salbutamol from its main sulphate metabolite was achieved using methyl acetate. The solvent was evaporated, washed with a tetrahydrofuran/TBME mixture and extracted into a basic solution for analysis. Increased recoveries have been obtained by using a more polar extraction solvent mixture. This is achieved by the addition of a small proportion (10-30%) of a more polar solvent such as butanol. Recoveries of > 90% were obtained for aniline-type β-agonists using a 9:1 mixture of diethyl ether: 2-

butanol **[6]** or TBME: n-butanol **[38]**. For the extraction of a wider range of β-agonists, representing compounds from both groups, t-butanol: ethyl acetate (3:7) was the chosen medium **[27]**. An alternative approach is the combination of ion pair/liquid-liquid extraction methodology for compounds which are charged in biological matrices. Di(2-ethylhexyl)phosphate (DEHP) has been used as an ion-pair reagent for salbutamol extraction into chloroform from plasma samples **[39]**. The compound was then back extracted into dilute acid prior to HPLC analysis. This procedure was also adopted by Wu *et al.*, but with the inclusion of an internal standard, metaproterenol **[40]**. Tan and Soldin carried out preliminary C_{18} SPE clean-up prior to ion pair extraction using both DEHP and 1-heptane sulphonic acid for salbutamol and an internal standard, fenoterol **[41]**. The extraction solvent was ethyl acetate and a salbutamol recovery of 80% was achieved. A more rapid alternative, also based on DEHP/ ethyl acetate extraction was described by Miller and Greenblatt **[42]**. The most recently-reported work in this area, by Sagar *et al.*, described the use of the ion pair reagent, sodium dodecyl sulphate, in combination with ethyl acetate for the extraction of salbutamol from human plasma (recovery 90%) **[43]**.

1.3.2 Solid phase extraction methodology

In recent years, solid phase extraction (SPE) and immunoaffinity chromatography (IAC) have superceded traditional solvent extraction procedures for the isolation of drugs from biological matrices. These types of techniques, which perform sample clean-up and in some cases, analyte preconcentration, are particularly advantageous as they can be easily automated, require low solvent usage, and are generally less time consuming and less labour intensive. From the point of view of β-agonists, solid phase extraction cartridges have a variety of special properties which allow better extraction of the more hydrophilic compounds, such as salbutamol and terbutaline. The two main SPE approaches reported for β-agonists have been either off-line extraction (adsorption, reversed phase, ion-exchange and mixed phase) or on-line extraction (reversed phase and ion exchange).

1.3.2.1 Off-line extraction

1.3.2.1.1 On-column liquid-liquid partitioning

Adsorption columns, containing hydrophilic packing material (diatomaceous earth) have been used to adsorb and distribute urine and tissue supernatants over a large surface area, after which the β-agonists of interest

may be eluted from the column with a water-immiscible solvent. The columns have a large capacity and hence are suitable for large sample volumes (up to 20 ml). Before application, the samples are pH-adjusted to 10 or greater to render the compound uncharged and allow ease of extraction into the organic phase. An early use of this type of column was described by Eddins *et al.* for application to clenbuterol in urine samples **[44]**. Alkalinised urine was applied to a "Clinelut" column and allowed to equilibrate for 10 min before elution of clenbuterol with three column volumes of hexane. This method was modified slightly by Degroodt *et al.* to include animal tissues extracts **[45]**. Brambilla *et al.* also adopted this approach for application to the aniline-type compounds in urine **[46]** and vitreous humour **[47]**. In this case the extraction solvent was dichloromethane. Other elution solvents used were t-butyl methyl ether for clenbuterol **[17]** and a mixture of toluene: dichloromethane (3:1) for clenbuterol and cimaterol **[10]**. This adsorption mechanism has not been extended to include the phenolic β-agonists as they are not eluted from the column using non-polar or semi-polar solvents. Leyssens *et al.* used an alumina neutral SPE column for improved clean-up of liver homogenates: the aniline β-agonists were easily extracted (e.g. mabuterol recovery 85%) but for the phenolic compounds (e.g. salbutamol and terbutaline) the recovery was poor ($< 40\%$) **[27]**.

1.3.2.1.2 Reversed-Phase SPE

β-agonists are particularly suited to reversed-phase SPE due, in part, to their relatively non-polar aliphatic moiety which can interact on hydrophobic (octadecyl (C_{18}) and decyl (C_8)) stationary phases. Optimum retention is achieved by adjusting the sample pH to $>$ 10, as for liquid extraction. Much of the scientific literature deals specifically with C_{18} SPE, with authors adopting very similar approaches for sample application, washing and elution procedures. In most cases methanol or a solvent of similar polarity was used to activate the column packing material prior to equilibrating with water or an alkaline buffer. After sample application, water or water/methanol (usually with a high water content) washed off matrix interferences before elution of the β-agonist(s) with methanol. The literature is replete with examples of C_{18} SPE used in this way to extract clenbuterol **[48, 49]**, salbutamol **[24, 50]**, terbutaline **[51]**, fenoterol **[52]**, cimaterol **[9]** and bambuterol **[53]**.

1.3.2.1.3 Ion-exchange SPE

Drug retention through ion-exchange mechanisms has been reported for the more polar, ionisable β-agonists like salbutamol and terbutaline. These compounds have been retained on either XAD-2 cation exchange columns or

unmodified silica columns. For the former type of column, procedures for the extraction of salbutamol **[54]** or salbutamol conjugates **[21]** have been described. Salbutamol is charged over the entire pH range allowing a retention mechanism with the negatively-charged resin. The drug was removed from the column with methanol. Terbutaline was separated from plasma on a AGX-2 resin, eluted in alkaline buffer and re-extracted into butanol **[55]**. Unmodified silica, also acts as an ion-exchanger with basic analytes. Retention occurs at pH values between 5.5 and 8.0, where the surface hydroxyl groups on the silica become increasingly ionised and attract the positively-charged amine on the β-agonist molecule. SPE procedures, based on silica retention, have been decribed for application to salbutamol **[56]** and salbutamol and terbutaline **[57]** in plasma samples.

1.3.2.1.4 Mixed phase interactions

The SPE columns already described are suitable for individual β-agonists or groups of β-agonists possessing similar chemical properties. However, difficulties can arise when a multicomponent extraction procedure is required. Optimisation of the residue extraction procedure for clenbuterol-like compounds can result in a reduced recovery for salbutamol-like substances **[13]**. To improve retention of the more polar compounds, some authors have tried "mixed phase" columns which have both lipophilic and ion-exchange properties, depending on the pH and the elution solvent. In practice, the

sample is added to the column at a neutral pH and the drug is retained by hydrophobic interaction. Then the column is acidified to protonate the drug and promote its ion-exchange retention on the SO_3^- group on the resin. Methanol serves as a broad spectrum wash solvent and the compounds of interest are eluted in methanol containing a small percentage of concentrated ammonia.

Dumasia *et al.* developed a mixed phase SPE procedure for selected β-agonists from horse urine **[19]**. In this case 1.0 M acetic acid was used to promote ion-exchange conditions, and ethyl acetate containing 2% ammonium hydroxide was the elution solvent. Gabiola adopted a similar procedure to extract salbutamol, terbutaline and clenbuterol, including conjugates, from bovine urine **[58]**. Leyssens *et al.* extended the procedure to cover seven β-agonists, containing compounds from both major classes **[27]**. The only difference was the elution solvent, i.e. dichloromethane: isopropanol (8: 2) containing 2% ammonia. Recoveries from urine were adequate (e.g. clenbuterol 51%, mabuterol 82%) but liver samples required two additional clean-up steps (liquid extraction and alumina SPE), resulting in a procedure only suitable for the clenbuterol-type compounds. Montrade *et al.* improved the recovery of the salbutamol-like compounds after optimisation of the percentage ammonia in the elution solvent. A 3% (v/v) concentration in ethyl acetate resulted in good recovery of thirteen β-agonists in urine samples **[13]**.

1.3.2.2 On-line solid phase extraction

On-line SPE is a clean-up technique which is used in conjunction with chromatographic procedures, principally HPLC. The analyte is retained on a preconcentration column positioned between the injector and the analytical column and, after a washing step, desorbed and re-routed to the analytical column for separation and detection.

On-line methods have been reported for β-agonist extraction based on either reversed-phase or ion-exchange principles. With respect to the former category, Sagar *et al.* described an on-line reversed-phase preconcentration procedure for terbutaline **[59]** and terbutaline and salbutamol **[60]** from plasma samples. Plasma was injected directly onto a small C_{18} column (10 cm x 1.5 mm) and, after washing with water, the β-agonists were back-flushed with mobile phase to the analytical column. Similar procedures for terbutaline retention were described by Edholm *et al.* **[61]** and Berquist and Edholm **[62]**. Tamisier-Karolak *et al.* used a combination of off-line and on-line reversed-phase SPE to produce highly purified extracts, containing both salbutamol and fenoterol, for HPLC analysis **[63]**. On-line ion-exchange SPE was described by Oosterhuis and van Boxtel **[64]**; salbutamol and bemathan were retained on a cation exchange (Partisil SCX) column followed by desorption and subsequent separation on a reversed phase column.

1.3.3 Immunoaffinity chromatography (IAC)

IAC is a SPE-type technique which relies on antigen: antibody interactions to preconcentrate the analyte(s) of interest. The technique has only recently been applied to the extraction of β-agonists, but already it has found widescale acceptance due to its high specificity and sample clean-up efficiency. A review by van Ginkel covers many of the characteristics of the IAC procedure **[65]**. To produce antibodies suitable for IAC columns, an animal (e.g. rabbit) is immunised with the test analyte conjugated to a protein (e.g. human serum albumin). Serum is harvested from the treated animals and immunoglobulins with specific cross-reactivity to the analyte (and similar compounds) is purified by ammonium sulphate precipitation. The purified immunoglobulin is bound to a sepharose gel and incorporated into a column. IAC may be applied either off-line as an extraction procedure or on-line as an extraction/preconcentration procedure.

The first selective on-line extraction/concentration procedure for a β-agonist (clenbuterol) based on IAC was carried out by Haasnoot *et al.* **[66, 67]**. Urine or tissue supernatants were applied to the column by valve switching and, after washing with water, clenbuterol was desorbed and flushed to a C_{18} analytical column with 0.01 M acetic acid. This system, could not be developed for multicomponent analysis because of the low cross-reactivity shown by the antibody to other β-agonists. Schilt *et al.* overcame this

problem by off-line concentration of the eluate from the the IAC column and used GC-MS to detect the low cross-reacting compounds in the low ppb range **[68]**. Other IAC methods performing in a similar way have been described for salbutamol extraction from urine **[69]** and tissue **[28]**.

van Ginkel *et al.* **[25]** explored the possibility of a less specific antibody which could be directed against a broader range of β-agonists. A mixture of antibodies, cross-reacting with both the N-t-butyl and the N-iso-propyl groups of β-agonists, was produced. This resulted in a more robust multi-β-agonist extraction/preconcentration procedure, and, coupled with GC-MS determination, gave good recovery of four compounds. With further advances in clean-up capability expected, it is likely that IAC procedures in conjunction with HPLC and MS, will soon become the standard analytical technique for β-agonist analysis.

1.4 SEPARATION OF β-AGONISTS

After the extraction/clean-up step, chromatographic separation is usually required for selective detection of β-agonist(s). Reversed-phase HPLC has been the most popular separation technique for β-agonists, due to the hydrophobic interaction of the molecules with C_{18} or C_8 stationary phases. Separation methods based on these principles has been reported for clenbuterol **[8]**, salbutamol **[42]** and a mixture of β-agonists **[9]**. For compounds which

are charged (salbutamol, terbutaline), ion-pair chromatography has been used. Typical ion-pair reagents are heptane sulphonic acid **[41]** and sodium dodecyl sulphate **[10]**. Alternatively, charged compounds can interact on a cation exchange column **[57, 64]**. Because the β-agonist molecule contains a chiral centre, enantiomers have been separated by chiral HPLC **[70, 71]**. In this case the stationary phase is made up of β-cyclodextrins or glycoproteins bound to silica. Normal phase HPLC has been less popular. One reported normal phase method for the separation of salbutamol involved a silica column and a mobile phase of 0.25% acetate buffer in methanol **[56]**.

Separation of β-agonists by gas chromatography (GC) is not ideal as these relatively polar compounds must first be derivatized. However, the main advantage of using GC for separation purposes is the choice of very sensitive detectors, including mass spectrometers. The separation may be carried out using the classical packed column **[72]** or, for improved efficiency and better applicability to multiresidue analysis, use of a capillary column is the preferred option **[19, 27, 58]**.

High performance thin layer chromatography (HPTLC) may be used, but generally this technique has been applied only as a qualitative technique. Extracts are spotted onto silica plates and compounds of interest are separated as bands by the mobile phase. Henion *et al.* reported the separation of clenbuterol from its urine matrix on a HPTLC plate **[73]**. The compound was converted to an azo dye for visual confirmation prior to removal of the

developed spot for tandem mass spectrometric analysis. Similar procedures were carried out for other aniline-type β-agonists in a variety of matrices **[10, 30, 46]**. Methods have been reported for the separation of salbutamol followed by conversion to its indoaniline dye and detection by absorption microdensitometry **[24, 54]**.

Capillary zone electrophoresis (CZE) and related methods have also appeared in the literature, but only for measurement of relatively high concentrations of β-agonists **[74-76]**. Whether or not they can be applied to biological sample extracts at the ppb level remains to be seen.

1.5 DETECTION OF β-AGONISTS

After a suitable extraction/clean-up procedure has been carried out, the β-agonists may be detected by spectrometric, electrochemical, immunological or mass spectrometric techniques, the choice of which depends on the required sensitivity. Published methods for β-agonists in biological samples have focussed on either measurement of drug levels after therapeutic dosing or after illegal (growth enhancing) use. In general, the HPLC-based detector options have been suitable for measuring β-agonists in the range 1 to 15 ng/ml, the plasma concentrations which occur following therapeutic doses **[56]**. Residue testing, however, requires detection systems which can detect β-agonists at sub ng/g level (0.5 ng/g is the maximum residue limit (MRL) set in the UK for

clenbuterol in edible tissues) **[77]**. For this purpose the more sensitive immunoassay or mass spectrometric methods are used and, in the case of the latter technique, additional information concerning the structure of the β-agonist molecule(s) may be obtained which allows for confirmation of the presence of specific residues.

1.5.1 Spectrometric Detection

Measurement of β-agonists by HPLC with UV spectrometric detection has resulted in low detection limits due to the presence of strong chromophores. Methods have been reported for clenbuterol at wavelengths between 222-245 nm **[8, 44, 66]**, and a mixture of β-agonists (clenbuterol, cimaterol, salbutamol) at a compromise wavelength of 260 nm **[9]**. In the case of the method reported by Botterblom *et al.* **[8]**, the method was limited by the detector sensitivity (limit of detection 33 ng/ml), a problem which may be overcome by using a post-column derivatization procedure. In this procedure, reagents (consisting of sodium nitrite and nitric acid to cause diazotization, ammonium amidosulphonate to remove excess nitric acid and N-(1-naphtyl)ethylenediamine which binds to the diazonium salt to form a light-absorbing product) are introduced into the mobile phase, post-column, to derivatise the β-agonists into diazo dyes. The absorbance for the products (derivatized clenbuterol; λ_{max} = 493 nm and derivatized cimaterol λ_{max} =

537 nm) was higher and resulted in limits of detection for the compounds in liquid and solid samples of 0.1 ng/l and 0.2 ng/g, respectively **[10, 78]**. A combination of UV and electrochemical detection has recently been shown to result in unequivocal confirmation of residue-positive samples **[17]**.

An alternative detector option, fluoresence detection, confers the additional advantage of selectivity to the detection of β-agonists. This type of detection is particularly suitable for the resorcinol and catechol β-agonists due to the intrinsic fluoresence of the aromatic phenolic structure. Many methods have been reported for salbutamol detection, with typical excitation and emission wavelengths at 225 nm and 310 nm, respectively **[7, 39, 42]**. These wavelengths are suitable for the detection of other related compounds like metaproterenol **[20, 40]** and terbutaline **[20]**. The sensitivity of the detection may vary, but detection limits of 1 ppb and lower have been reported **[39, 56]**.

Quantification of analyte bands for HPTLC-based methods is accomplished by visual observation or densitometric measurements. For the latter detection mode, light of suitable wavelength is directed onto the TLC plates and the amount of light transmitted or reflected by the band is measured. Clenbuterol may be detected as a coloured band after reaction with Ehrlichs reagent **[45]**. Salbutamol was converted to its indoaniline derivative and quantified by absorption microdensitometry at 650 nm **[24, 54]**.

1.5.2 Electrochemical Detection

Electrochemical detection is suited to β-agonists due to the presence of the oxidisable amino/hydroxyl groups on the aromatic part of the molecule and the amino group on the aliphatic moiety. Interesting and informative electrochemical studies have been carried out which demonstrate the behaviour of salbutamol **[80]** and clenbuterol and mabuterol **[6]** at a glassy carbon electrode (GCE). The studies show that β-agonists, as a class, are oxidised irreversibly at carbon electrodes at high positive potentials.

The bulk of the literature reports HPLC linked with electrochemical detector methods for detection of therapeutic levels of β-agonists in urine and plasma. Hauck and Brugger described the detection of clenbuterol (at +1.2 V vs Ag/AgCl reference) above 10 ng/ml using a GCE **[48]**. A more sensitive electrochemical detection method, also employing a GCE (set at +1.15 V vs the reference electrode), reported clenbuterol determination as low as 1 ng/ml **[29]**. Qureshi and Eriksson developed a method based on either GCE or carbon paste electrode (CPE) detection of aniline type compounds at +0.75 V **[6]**. Limits of detection of 0.5 ng/ml (clenbuterol) and 2.0 ng/ml (mabuterol) were achieved and an electrode pretreatment step (oxidation of electrode at high positive potentials) was described. A GCE detector was used to measure salbutamol and fenoterol **[41]** and salbutamol enantiomers **[70]**. Other forms of the carbon electrode used in this field were

the carbon paste rotating electrode for salbutamol **[64]** and the vitreous carbon electrode for terbutaline **[81]**. Sagar *et al.* developed methods based on detection of the phenolic β-agonists (salbutamol, terbutaline) using a micro carbon fibre electrode **[43, 59-60]**. Advantages of this type of approach include low charging current and increased mass transport. The electrode potential used was +1.3 V and electrode pretreatment procedures were described. A novel two-GCE detector approach for salbutamol and fenoterol was reported by Tamisier-Karolak *et al.* **[63]**. The operating potentials of the two electrodes were set at +0.5 V (the potential where no oxidation occurred) and +0.9 V (optimum oxidation) and the difference in signals was recorded. A limit of detection of 0.5 ng/ml was reported.

1.5.3 Immunoassay Procedures

Immunoassays, including radioimmunoassay (RIA) and enzyme immunoassay (EIA), are highly favoured procedures due to their low detection capability, allowing the measurement of residues at 0.5 ng/g and lower. The EIA option has proved more popular in recent years but both techniques can handle large sample numbers and may require less sample clean-up than for other techniques. The procedures leading to the production of antibodies (described in section 1.3.3), are relevant also for immunoassay techniques.

Commercial kits for both techniques are available for single or multianalyte determination.

1.5.3.1 Radioimmunoassay

The first RIA procedure for a β-agonist compound was reported by Kopitar and Zimmer for the pharmacological studies on clenbuterol in animal tissue **[82]**. Delahaut *et al.* described the development of a more specific RIA method for the determination of clenbuterol after its administration to animals as a growth enhancing agent **[11]**. The antiserum had 100% cross-reactivity to clenbuterol and lower cross-reactivity to other β-agonists ($< 10\%$), and was applicable to extracts from plasma, urine and faeces containing less than 0.5 ng/g (ml) level. A kit has been produced for measurement of salbutamol using an anti-salbutamol antiserum **[69]**. A method by Loo *et al.* **[37]** described the use of a specific RIA for the detection of free forms of salbutamol after the conjugates were removed from plasma by liquid-liquid extraction. Adam *et al.* reported an interesting RIA procedure for the determination of salbutamol using a monoclonal antibody **[83]**. The antibody was synthesized in mice against the O-(3-carboxypropionyl) derivative of salbutamol linked to bovine serum albumin. Moreover, the antibody showed a high cross-reactivity (75%) to clenbuterol. Rominger *et al.* developed a specific RIA procedure for fenoterol which consisted of an antibody cross-

reacting with the different sterioisomers of fenoterol and radiolabelled [^{125}I]-fenoterol **[18]**. The procedure measured fenoterol at the low pg/ml level in biological fluid extracts.

Radioreceptor assays have been developed which are similar to RIA but which use the binding of β-agonists by receptors, instead of antibodies, as the analytical principle **[12]**. The method was based on competition between a radioactive tracer (^{3}H-dihydroalprenolol) and aniline-type β-agonists for binding to receptors (plasma membranes). Although the limit of detection reported, 2.4 ppb, was relatively high, this approach merits further investigation.

1.5.3.2 Enzyme-immunoassay

EIA has progressed in the last decade as a sensitive and reliable determination procedure in residue analysis. The first EIA for clenbuterol was developed by Yamamoto and co-workers **[84]**, a selective double antibody and heterogenous immunoassay based on competition for binding between clenbuterol and its β-D-galactosidase-labelled analogue to a clenbuterol specific antibody, followed by selective binding of the antibody-bound enzyme hapten with a second, immobilised antibody. The activity of the enzyme was determined fluorometrically after addition of substrate. Many of the principles developed in this procedure have been incorporated into EIA procedures

presently being used. The EIA procedure developed by Degand *et al.* was based on the competition between clenbuterol and diazo-clenbuterol-horseradish peroxidase (HPO) for the antiserum **[22]**. The procedure had the capability of determining clenbuterol at levels below 0.5 ppb. An EIA procedure, containing an antiserum specific for clenbuterol and also utilising a HPO-based enzyme conjugate, was developed to monitor urine and tissues from clenbuterol-medicated farm animals **[85]**. The antiserum had cross-reactivities to salbutamol and cimaterol of 26.3% and 1.3%, respectively. EIA kits containing a salbutamol-based enzyme conjugates, either salbutamol-4-carboxymethylether-HPO **[4]** or salbutamol hemisuccinate-HPO **[86-88]** were used to determine clenbuterol in urine and tissue extracts. Angeletti *et al.* **[89]** and Paleologo-Oriundi **[90]** reported EIA assays which could determine four β-agonists (clenbuterol, mabuterol, salbutamol and terbutaline) in biological samples (urine, serum), using anti-β-agonist and anti-clenbuterol antisera, respectively. The latter assay was advantageous as no sample clean-up step was required.

An EIA kit with a hydroxyclenbuterol-alkaline phosphatase enzyme conjugate and an anti-clenbuterol antiserum (produced as described by Yamamoto and Iwata **[84]**) was used to measure clenbuterol **[91]** and salbutamol **[26]** in urine and tissues, respectively. An EIA procedure which contained an anti-salbutamol antiserum was developed and described by Degand *et al.* **[92]**. The antiserum showed principal cross-reactivities relative

to salbutamol (100%) of 115% (clenbuterol), 65% (mabuterol), 31% (terbutaline) and 13% (cimaterol), and the procedure required mimimal sample clean-up step and could be used to determine conjugated residues qualitatively without a prior hydrolysis step. A novel and rapid approach, developed by Ploum *et al.* **[93]**, was the use of test-strip enzyme immunoassays for the direct detection of clenbuterol in urine. The antiserum was prepared as described previously by Yamamoto and Iwata **[84]** and the clenbuterol conjugate was clenbuterol horseradish peroxidase. Indication of clenbuterol presence, down to 5 ng/ml, was achieved visually, by the appearance of a blue colour on the strip.

1.5.4 Mass Spectrometric Detection

Mass spectrometric (MS) detection, above all other techniques, provides unequivocal identification of drugs and metabolites. With respect to β-agonists, the EC has stated that for confirmatory analysis of veterinary drug residues, identification of the analyte must be based on at least four characteristic fragment ions. To satisfy this criterion it may be necessary to combine the data from two MS ionisation techniques, for example electron impact (EI) and chemical ionisation (CI) mass spectrometry. In the EI mode, the (derivatised) β-agonist molecule is volatilised and bombarded by energetic electrons, to produce a protonated molecular ion (M^+). In the CI mode, a

reactant gas (e.g. ammonia) is bombarded with electrons to become ionised and collides with the β-agonist molecule thereby ionising it. The spectrum is then scanned for molecular ion and fragment ions, after which the MS may be set to monitor the most abundant ions (selected ion monitoring; SIM). The peak area measured is proportional to the concentration of analyte.

A prerequisite of β-agonist analysis by GC-MS is the derivatisation of the polar groups (hydroxyls, amino) on the molecule. The silyl derivatives are most commonly used; a typical silylation procedure was described by Fuerst *et al.* **[35]** whereby sample extracts containing clenbuterol were treated with hexamethyldisilazane (HMDS) in acetonitrile at 30°C. Garcia-Regueiro *et al* extracted salbutamol, using the method of Fuerst *et al.* **[35]**, and clenbuterol from urine by adsorption SPE to produce extracts for GC-MS. The compounds were derivatised with bis(trimethylsilyl)trifluoracetamide and separated on a capillary column (100 μm). Detection limits of 1.5 ng/ml in urine were reported **[94]**. Girault and Fourtillan found that trimethylsilyl (TMS) derivatives were problematic; the derivatisation step resulted in low sensitivity and poor reproducibility and the O-TMS derivative was prone to hydrolysis. They proposed the use of a dipentafluoropropionyl derivative **[14]**. The clenbuterol perfluoracyl derivative was scanned in the CI mode and the ion at m/z 368 was monitored resulting in detection limits as low as 10 pg/g in tissue samples. Several authors have reported the use of two derivatives and two detection modes for a more reliable confirmation. Montrade and co-workers

described a procedure applicable to thirteen β-agonists in urine samples **[13]**. The extracted analytes were derivatised to either their TMS or cyclic-2-dimethylsilamorpholine (DMS) derivative. van Rhijn *et al.* carried out similar studies on clenbuterol **[95]**. This paper described the application of TMS and DMS derivatives for the identification of clenbuterol from urine extracts. The derivatives were ionised in both EI and CI modes, and the combination gave high-intensity diagnostic ions; m/z 86 for both derivatives in the EI mode and m/z 349 and 351 (TMS derivative) and 391 and 393 (DMS derivative) in the CI mode. Dumasia *et al.* **[19]** carried out a multicomponent analysis procedure based also on the use of the TMS and DMS derivatives of β-agonists using the EI mode. The spectra of the TMS derivative showed a base peak at m/z 86 (tertiary butyl amino compounds) or 72 (isopropylamine compounds). For the cyclic DMS derivatives, the molecular ion peak was dominant at m/z 346, as well as two fragment ions at $(M-15)^+$ and $(M-43 \text{ or } 57)^+$ for the loss of the isopropyl or t-butyl groups, respectively. Boronic acid derivatives have also been used to improve the abundance of high mass ions. Blanchflower *et al.* scanned a methylboronic acid derivative of clenbuterol in the EI mode **[32]**. The resulting spectra showed three prominant peaks (m/z 243, 285 and 300), and using SIM, levels of clenbuterol in urine extracts was determined. In the procedure of Polettini *et al.* **[38]**, a comparative study of different derivatising reagents for clenbuterol was carried out. The TMS derivative showed a major response at m/z = 86, but limited abundance for

all other ions. The trifluoroacetic anhydride (TFAA) derivative led to the formation of three or more different products, resulting in spectra difficult to interpret. The boronic acid derivatives (phenylboronic acid and 1-butaneboronic acid) showed the most abundant ions in the high mass range (at m/z 243, 327, 342 and at 243, 347, 362, respectively). The 1-butaneboronic acid derivative was chosen as optimum for the further development of a GC-MS method for clenbuterol (limit of detection, 0.5 ppb).

For additional information and improved confirmation of β-agonists by scanning daughter ions, GC-tandem MS (GC-MS-MS) has been used **[27, 73]**. A recent development to provide complementary results from GC-MS analysis is cryotrapping GC-fourier transform-infra-red spectrometry **[96]**. Sample extracts are derivatised as for GC-MS and the spectra obtained are compared to standards for positive identification.

Coupling of liquid chromatography with mass spectrometry (LC-MS or LC-MS-MS) has been more difficult due to the need to remove the liquid mobile phase prior to entering the MS. However, the advantage of this type of determination is that its more suited to polar compounds and hence derivatisation procedures are not necessary. Thermospray LC-MS was described by Blanchflower and Kennedy **[97]** for the identification of clenbuterol; the m/z 277 fragment ion was monitored (by SIM) in urine extracts. Thermospray LC-MS was also suitable for measuring the sulphate ester of salbutamol which shows a prominant peak at the m/z 318 ion **[21]**.

Thermospray LC-MS-MS was used to monitor seven β-agonists **[98]**; the compounds all exhibited the loss of 74 mass units from the molecular ion or from prominant fragment ions, thought to be due to the loss of water and methylpropene. A recent development, electrospray ionisation MS, has been used to monitor various β-agonists in aqueous-based standards **[15]** and for clenbuterol in urine extracts **[99]**.

1.6 CONCLUSIONS

β-agonists are relatively polar compounds which can be extracted from biological matrices using the more conventional solvent extraction procedures or by some form of column chromatography (SPE, IAC). Liver or eye are the preferred sample types to monitor illegal usage, but for monitoring levels following therapeutic use plasma or urine may be analysed. For tissue samples procedures are required to homogenise the sample, followed by digestion and extraction of the compound(s).

As clenbuterol has been the most effective repartitioning agent, the bulk of the literature has reported determination procedures for its analysis in various biological samples. However, with the introduction as growth enhancing agents of other compounds in this class, such as salbutamol and cimaterol, and the possibility of new chemically-designed β-agonists, multi-residue methods are now required. The most effective means of extracting β-agonists of both groups (i.e. aniline-type and the more hydrophilic phenolic-type) is achieved through the use of IAC or "mixed phase" SPE cartridges. The main criterion for the selection of a detection system for illegal β-agonist use is sensitivity. The two most used techniques have been immunoassay and GC-MS. The main advantages of the former technique have been high sensitivity and specificity for the test analyte. The vast majority of the published immunoassay procedures detect the analytes below 1 ppb.

Procedures based on GC-MS detection reach the sensitivity of immunoassay but usually require larger sample sizes (e.g. 5-10 g liver). This technique can provide unequivocal confirmation of the β-agonist(s). The procedure adopted by Montrade *et al.* **[13]**, for instance, satisfies the EC criteria and allows for identification of thirteen β-agonists in urine extracts at the low ppb level. LC-MS has great potential to become a confirmation technique in the field of β-agonists due to its applicability to polar analytes. However, as yet, it has not challenged GC-MS in terms of sensitivity.

The most active phase of β-agonist research for human therapeutic use seems to be past, as selective and effective compounds are now readily available for asthma or other bronchial diseases. β-agonists are still being administered illicitly as feed additives in animal production and hence development of analytical procedures for their detection must continue. The reported cases of intoxication in humans following consumption of clenbuterol-incurred liver serves as a strong reminder of such abuse **[100, 101]**. The development of suitable methodology with the required sensitivity and specificity will help to ensure the safety of edible tissues and protect the health of the consumer.

1.7 REFERENCES

1. P.J. Buttery and J.M. Dawson, in J.P. Hanrahan (ed.), Beta-agonists and Their Effects on Animal Growth and Carcass Quality, Elsevier, London, 1987, p.20.
2. R.F. Witkamp and A.S.J.P.A.M. van Miert, in Proceedings of the FLAIR Workshop, Thessaloniki, Greece, (eds: H.A. Kuiper and L.A.P. Hoogenboom), Oct 30-31, 1992, p.75.
3. H.H.D. Meyer and L. Rinke, in Proceedings of the 51st Easter School in Agricultural Science: The Control of Fat and Lean Deposition, University of Nottingham, Sutton Bomington, April 1991, p.61.
4. H.H.D. Meyer and L.M. Rinke, *J. Anim. Sci.*, **69**, 1991, 4538.
5. I. Dursch, H.H.D. Meyer and S. Jager, *Anal. Chim. Acta*, **275**, 1993, 189.
6. G. Ali Qureshi and A. Eriksson, *J. Chromatogr.*, **441**, 1988, 197.
7. N. Kurosawa, S. Morishima, E. Owada and K. Ito, *J. Chromatogr. (Biomed. Applic.)*, **305**, 1984, 485.
8. M.H.A. Botterblom, M.G.P. Feenstra and E.B.H.W. Erdtsieck-Ernste, *J. Chromatogr. (Biomed Applic.)*, **613**, 1993, 121.
9. H.H.D. Meyer, L. Rinke and I. Dursch, *J. Chromatogr. (Biomed. Applic.)*, **564**, 1991, 551.

10 D. Courtheyn, C. Desaever and R. Verhe, *J. Chromatogr. (Biomed. Applic.)*, **564**, 1991, 537.

11. Ph. Delahaut, M. Dubois, I. Pri-bar, O. Buchman, G. Degand and F. Ectors, *Food Addit. Contam.*, **8**, 1991, 43.

12. V. Helbo, M. Vandenbroeck and G. Maghuin-Rogister, in Proceedings EuroResidue 2 Conference, Veldhoven, The Netherlands, May 3-5, 1993, 362.

13. M.-P Montrade, B. Le Bizec, F. Monteau, B. Siliart and F. Andre, *Anal. Chim. Acta*, **275**, 1993, 253.

14. J. Girault and J.B. Fourtillan, *J. Chromatogr.*, **518**, 1990, 41.

15. L. Debrauwer and G. Bories, *Rapid Comm. Mass Spectrom.*, **6**, 1992, 382.

16. R.J.H. Pickett and M.J. Sauer, *Anal. Chim. Acta*, **275**, 1993, 269.

17. H. Hooijerink, R. Schilt, W. Haasnoot and D. Courtheijn, *J. Pharm. Biomed. Anal.*, **9**, 1991, 485.

18. K.L. Rominger, A. Mentrup and M. Stiasni, *Arzneim.-Forsch/Drug Res.*, **40**, 1990, 887.

19. M.C. Dumasia and E. Houghton, *J. Chromatogr. (Biomed. Applic.)*, **564**, 1991, 503.

20. K. Selinger, H.M. Hill, D. Matheou and L. Dehelean, *J. Chromatogr. (Biomed. Applic.)*, **493**, 1989, 230.

21. G.D. Bowers, D.M. Higton, G.R. Manchee, J. Oxford and D.A. Saynor, *J. Chromatogr.*, **554**, 1991, 175.

22. G. Degand, A. Bernes-Duyckaerts and G. Maghuin-Rogister, *J. Agric. Food Chem.*, **40**, 1992, 70.

23. M.-P. Montrade, S. Riverain, B. Le Bizec and F. Andre, in Proceedings of the FLAIR Workshop, Thessaloniki, Greece (eds; H.A. Kuiper and L.A.P. Hoogenboom), Oct 30-31, 1992, p.143

24. J. De Groof, J.-M. Degroodt, B. W. de Bukanski and H. Beernaert, *Z. Lebensm. Forsch.*, **193**, 1991, 126.

25. L.A. van Ginkel, R.W. Stephany and H.J. Van Rossum, *J. Assoc. Off. Anal. Chem.*, **75**, 1992, 554,

26. L. Howells, M. Sauer, R. Sayer and D. Clark, *Anal Chim. Acta*, **275**, 1993, 275.

27. L. Leyssens, C. Driessen, A. Jacobs, J. Czech and J. Raus, *J. Chromatogr. (Biomed.Applic.),* **564**, 1991, 515.

28. F. Ramos, M.C. Castilho, M.I.N. da Silveira, J.A.M. Prates and J.A.R. Dias Correia, *Anal. Chim. Acta*, **275**, 1993, 279.

29. B. Diquet, L. Doare and P. Simon, *J. Chromatogr.*, **336**, 1984, 415.

30. H. Brunn, *Fleischwirtsch.*, **68**, 1988, 1476.

31. M. Weisberger, J.E. Patrick and M.L. Powell, *Biomed. Mass Spectrom.*, **10**, 1983, 556.

32. W.J. Blanchflower, S.A. Hewitt, A.Cannavan, C.T. Elliot and D. G. Kennedy, *Biol. Mass Spectrom.*, **22**, 1993, 326.

33. J. Girault, J. Gobin and J.B. Fourtillan, *Biomed. Environ. Mass Spectrom.*, **19**, 1990, 80.

34. H.J. Forster, K.L. Rominger, E. Ecker, H. Peil and A. Wittrock, *Biomed. Environ. Mass Spectrom.*, **17**, 1988, 417.

35. P. Fuerst, C. Fuerst and W. Groebel, *Dtsch. Lebensm. Rundsch,* **85**, 1989, 35.

36. M. Horiba, T. Murai, K. Nomura, T. Yuge, K. Sanai and E. Osada, *Arzneim.-Forsch/Drug Res.*, **34**, 1984, 1668.

37. J.C.K. Loo, N. Beaulieu, N. Jordan, R. Brien and I.J. M^{c} Gilveray, *Res. Comm. Chem. Path. Pharm.*, **55**, 1987, 283.

38. A. Polettini, M.C. Ricossa, A. Groppi and M. Montagna, *J. Chromatogr. (Biomed. Applic.*), **564**, 1991, 529.

39. M.J. Hutchings, J.D. Paull and D.J. Morgan, *J. Chromatogr. (Biomed. Applic.)*, **277**, 1983, 423.

40. Y.Q. Wu, R. Shi, R.L. Williams and E.T. Lin, *J. Liq. Chromatogr.*, **14**, 1991, 253.

41. Y.K. Tan and S.J. Soldin, *J. Chromatogr. (Biomed. Applic.)*, **311**, 1984, 311.

42. L.G. Miller and D.J. Greenblatt, *J. Chromatogr.*, **381**, 1986, 205.

43. K.A. Sagar, C. Hua, M.T. Kelly and M.R. Smyth, *Electroanalysis.*, **4**, 1992, 481.

44. C. Eddins, J. Hamann and K. Johnson, *J. Chromatogr. Sci.*, **23**, 1985, 308.

45. J.-M. Degroodt, B.W. de Bukanski, H. Beernaert and D. Courtheyn, *Z. Lebensm. Unters. Forsch.*, **189**, 1989, 128.

46. G.F. Brambilla, S. Castelli, A. Riberzani, M. Montana and F. Manca, *Indust. Aliment.*, **29**, 1990, 674.

47. G.F. Brambilla, U. Agrimi and E. Pierdominici, *Ital. J. Food Sci.*, **3**, 1991, 303.

48. M. Hauck and E. Brugger, *Deutsche Lebensm.-Rundschau.*, **85**, 1989, 178.

49. C. Presa, M. Ramos and T. Reuvers, *Alimentaria*, **27**, 1990, 85.

50. T. Emm, L.J. Lesco, J. Leslie and M.B. Perkal, *J. Chromatogr. (Biomed. Applic.)*, **427**, 1988, 188.

51. H.J. Leis, H. Gleispach, V. Nitsche and E. Malle, *Biomed. Environ. Mass Spectrom.*, **19**, 1990, 382.

52. J.G. Leferink, J. Dankers and R.A.A. Maes, *J. Chromatogr. Biomed. Applic.)*, **229**, 1982, 217.

53. C. Lindberg, S. Jonsson, J. Paulson, and A. Tunek, *Biomed. Environ. Mass Spectrom.*, **19**, 1990, 218.

54. P.V. Colthup, F.A.A. Dallas, D.A. Saynor, P.F. Carey, L.F. Skidmore and L.E. Martin, *J. Chromatogr.*, **345**, 1985, 111.

55. S.E. Jacobsson, S. Jonsson, C. Lindberg and L.A. Svensson, *Biomed. Mass Spectrom.*, **7**, 1980, 265.

56. R.E. Bland, R.J.N. Tanner, W.H. Chern, J.R. Lang and J.R. Powell, *J. Pharm. Biomed. Anal.*, **8**, 1990, 591.

57. P.T. M^{c} Carthy, S. Atwal, A.P. Sykes, J.G. Ayres, *Biomed Chromatogr.*, **7**, 1993, 25.

58. C. Gabiola, in Proceedings of EuroResidue 2 Conference, Veldhoven, The Netherlands, May 3-5, 1993, 289.

59. K. Sagar, M.T. Kelly and M.R. Smyth, *J. Chromatogr. (Biomed Applic.)*, **115**, 1992, 109.

60. K. Sagar, M.T. Kelly and M.R. Smyth, *Biomed. Chromatogr.*, **7**, 1993, 29.

61. L.E. Edholm, B.M. Kennedy and S. Berquist, *Chromatographia*, **16**, 1982, 341.

62. S. Berquist and L.E. Edholm, *J. Liq. Chromatogr.*, **6**, 1983, 559.

63. T. Tamisier-Karolak, B. Delhotal-Landes, P. Jolliet-Riant, J. Milliez, D. Jannet, J. Barre and B. Flouvat, *Thera. Drug Monit.*, **14**, 1992, 243.

64. B. Oosterhuis and C.J. Van Boxtel, *J. Chromatogr. (Biomed. Applic.)*, **232**, 1982, 327.

65. L.A. van Ginkel, *J. Chromatogr (Biomed. Applic.)*, **564**, 1991, 363.

66. W. Haasnoot, M.E. Ploum, R.J.A. Paulussen, R. Schilt and F.A. Huf, *J. Chromatogr.*, **519**, 1990, 323.

67. W. Haasnoot, A.R.M. Hamers, R. Schilt and C.A. Kan, in M.R.A. Morgan, C.J. Smith and P.A. Williams (eds.), *Food Safety and Quality Assurance-Applications of Immunoassay Systems*, Elsevier, London, 1992, 185.

68. R. Schilt, W. Haasnoot, M.A. Jonker, H. Hooijerink and R.J.A. Paulussen, in N. Haagsma, A. Ruiter and P.B. Czedik-Eysenberg (eds.), Proceedings of the EuroResidue Conference, Noordwijkerhout, The Netherlands, May 21-23, 1990, 320.

69. Ph. Delahaut, M. Dubois, Y. Colemonts, A. Boenke and G. Maghuin-Rogister, in Proceedings of the EuroResidue 2 Conference, Veldhoven, The Netherlands, May 3-5, 1993, 257.

70. Y.K. Tan, S.J. Soldin, *J. Chromatogr. (Biomed. Applic.)*, **66**, 1987, 187.

71. A. Walhagen, L.E. Edholm, B.M. Kennedy and L.C. Xiao, *Chirality*, **1**, 1989, 20.

72. C. Lindberg, S. Jonsson, J. Paulson and A. Tunek, *Biomed. Environ. Mass Spectrom.*, **19**, 1990, 218.

73. J. Henion, G.A. Maylin and B.A. Thompson, *J. Chromatogr.*, **271**, 1983, 107.

74. W. Schmollack, M. Tkaczyk and J. Koerbl, *Zentralbl. Pharm. Pharmakother. Laboratoriumsdiagn.*, **126**, 1987, 1.

75. M.T. Ackermans, J.L. Beckers, F.M. Everaerts and I.G.J.A. Seelen, *J. Chromatogr.*, **590**, 1992, 341.

76. S. Fanali, *J. Chromatogr.*, **545**, 1991, 437.

77. B. Boenisch and J.F. Quirke, in Procceedings of the FLAIR Workshop, Thessaloniki, Greece, (eds: H.A. Kuiper and L.A.P. Hoogenboom), Oct 30-31, 1992, p. 102.

78. J.-M. Degroodt, B.W. de Bukanski, J. de Groof and H. Beernaert, *Z. Lebensm. Unters. Forsch.*, **192**, 1991, 430.

79. P.T. Mc Carthy, S. Atwal, A.P. Sykes, J.G. Ayres, *Biomed. Chromatogr.*, **7**, 1993, 25.

80. K.A. Sagar, M.R. Smyth and R. Munden, *J. Pharm. Biomed. Anal.*, **11**, 1993, 533.

81. B.M. Kennedy, A. Blomgren, L.E. Edholm and C. Roos, *Chromatographia*, **24**, 1987, 895.

82. Z. Kopitar, A. Zimmer, *Arzeimittel-Forschung*, **26**, 1976, 1450.

83. A. Adam, H. Ong, D. Sontag and A. Rapaille, *J. Immunoassay*, **11**, 1990, 329.

84. I. Yamamoto and K. Iwata, *J. Immunoassay*, **3**, 1982, 155.

85. C.T. Elliot, W.J. McCaughey and H.D. Shortt, *Food Addit. Contam.*, **10**, 1993, 231.

86. W. Haasnoot, A.R.M. Hamers, G.D. van Bruchem, R. Schilt and L.M.H. Frijns, in M.R.A. Morgan, C.J. Smith and P.A. Williams (eds.), *Food Safety And Quality Assurance-Applications of Immunoassay Systems,* Elsevier, London, 1992, p. 237.

87. W. Haasnoot, S.M. Ezkerro and H. Keukens, in Proceedings of the EuroResidue 2 Conference, Veldhoven, The Netherlands, May 3-5, 1993, 347.

88. S.D. Bucknall, A.L. MacKensie, M.J. Sauer, D.J. Everest, R. Newman and R. Jackman, *Anal. Chim. Acta*, **275**, 1993, 227.

89. R. Angeletti, M. Paleologo Oriundi, R. Piro and R. Bagnati, *Anal. Chim. Acta*, **275**, 1993, 215.

90. M. Paleologo-Oriundi, G. Giacomini, F. Ballaben, F. Berti, F. Benedetti, R. Bagnati and E. Bastiani, *Food Agric. Immunol.*, **4**, 1992, 73.

91. M.J. Sauer, R.J.H. Pickett and A.L. MacKensie, *Anal. Chim. Acta*, **275**, 1993, 195.

92. G. Degand, A. Bernes-Duyckaerts, Ph. Delahaut and G. Maghuin-Rogister, *Anal. Chim. Acta*, **275**, 1993, 241.

93. M.E. Ploum, W. Haasnoot, R.J.A. Paulussen, G.D. van Bruchem, A.R.M. Hamers, R. Schilt and F.A. Huf, *J. Chromatogr. (Biomed. Applic.)*, **102**, 1991, 413.

94. J.A. Garcia-Regueiro, B. Perez and G. Casademont, *J. Chromatogr.*, **655**, 1993, 73.

95. J.A. van Rhijn, W.A. Traag and H.H. Heskamp, *J. Chromatogr. (Biomed. Applic.)*, **619**, 1993, 243.

96. T. Visser, M.J. Vredenbregt, A.P.J.M. de Jong, L.A. van Ginkel, H.J. van Rossum and R.W. Stephany, *Anal. Chim. Acta*, **275**, 1993, 205.

97. W.J. Blanchflower and D.G. Kennedy, *Biomed. Environ. Mass Spectrom.*, **18**, 1989, 935.

98. J.A. van Rhijn, W.A. Traag, H.H. Heskamp and T. Zuidema, in Proceedings of the EuroResidue 2 Conference, Veldhoven, The Netherlands, May 3-5, 1993, 572.

99. L. Debrauwer and G. Bories, *Anal. Chim. Acta*, **275**, 1993, 231.

100. J.F Martinez-Navarro, *Lancet* , **336**, 1990, 1311.

101. C. Pulce, D. Lamaison, G. Keck, G. Bostvironnois, J. Nicholas and J. Descortes, *Vet. Human Toxicol.*, **33**, 1991, 480.

CHAPTER 2

VOLTAMMETRIC STUDY OF SELECTED β-AGONISTS AT UNMODIFIED AND NAFION-MODIFIED ELECTRODES WITH APPLICATION TO THE ANALYSIS OF REAL SAMPLES

2.1 INTRODUCTION

As discussed in chapter 1 (section 1.5.2), the electrochemical approach to the analysis of β-agonists is highly advantageous due to the presence of oxidisable substituents (amino, hydroxyl) on the aromatic ring. The primary electrode reaction is the oxidation of the hydroxyl moietie(s), the mechanism involving a one electron/one proton process. In the case of the catechol β-agonists (e.g. isoprenaline, rimiterol), the highly reactive o-quinone is formed. Aniline-type β-agonists, containing an oxidisable amino group on the aromatic ring, are also readily oxidised by a suitable electrode. The secondary amino group on the aliphatic part of the molecule also becomes electroactive when deprotonated; for the electrooxidation of methadone at a carbon paste electrode (CPE), the amino group becomes electroactive at pH values > 5 **[1]**. Carbon-based electrodes have been the most suitable for oxidation of these moieties and many authors have used them to investigate the electrochemical behaviour of selected β-agonists. Clenbuterol and mabuterol, two stucturally-similar β-agonists, were oxidised irreversibly at high positive potentials (+0.75 V vs the saturated calomel electrode (SCE)) at both glassy carbon electrodes (GCEs) and CPEs **[2]**. The large positive potentials required were due to the electrophilic substituents (Cl, CF_3) on the aromatic ring. The irreversible oxidation of the compounds, i.e. absence of reductive waves on the reverse scan, is a consequence of a chemical follow-up reaction

of the oxidation products. This was supported by the appearance of small reduction/oxidation waves in subsequent scans at considerably lower positive or negative potentials at the carbon electrodes. The compounds and their products adsorbed on the electrode surface causing inhibition of the current response. This was remedied by addition of 30-40% acetonitrile or by using an electrochemical cleaning step i.e. holding the potential at -1.5 V for 5 min. From this study the authors then developed a sensitive HPLC-ED method for the separation and detection of the two compounds in plasma extracts at the low ppb level. Hooijerink *et al.* developed a similar HPLC-ED method for the detection of clenbuterol only, using a GCE set at a potential of +1.25 V vs the Ag/AgCl reference electrode **[3]**. The detector signal was found to deteriorate due to the adsorption of oxidation products and/or matrix components present in the urine extracts. This led to the development of an electrochemical cleaning procedure; oxidation of the electrode at high positive potentials. The oxidative voltammetric behaviour of salbutamol at the GCE was also studied using cyclic voltammetry (CV) and differential pulse voltammetry (DPV) **[4]**. A pH study (half wave potential ($E_{P/2}$) vs pH) using CV revealed that the oxidation of the compound was strongly influenced by pH. The phenolic hydroxyl group oxidation gave rise to a single peak in the pH range studied. The peak shifted towards negative potentials as the pH increased in such a way that two different lines with different slopes were observed. The two lines intersected at pH 9.0 which is the expected pK_a value

of salbutamol **[5]**. Coulometric analysis of the compound showed that two electrons were involved in the oxidation process. The compound was subsequently determined in tablet formulations by DPV (at optimum pH of 5.0) giving rise to a peak at +0.75 V vs the SCE reference electrode. Tan *et al.* carried out a hydrodynamic study (i.e. profiles of current vs potential) for salbutamol and internal standard, fenoterol at the GCE **[6]**. An applied potential of +0.60 V was required to initiate an electrochemical response for salbutamol whereas for fenoterol a response was initiated at considerably lower potentials. Furthermore, the detection limit for fenoterol was found to be lower due to a larger signal being generated by the oxidation of the three hydroxyl groups. Sagar *et al.* developed HPLC-ED methods for phenolic β-agonists (salbutamol, terbutaline) using a micro carbon fibre electrode **[7-9]**. Hydrodynamic studies for salbutamol **[7, 9]** showed that the optimum response occurred at very high positive potentials (+1.3 V vs the Ag/Ag_3PO_4 reference electrode). The carbon fibre electrode, when compared to the macro-GCE, was more advantageous both in terms of lower detection limits (1 ng/ml compared to 3 ng/ml) and a less troublesome electrochemical cleaning procedure. For the purposes of understanding the processes occurring at the electrode surfaces, the use of a CPE is particularly advantageous. The benefits gained from using CPEs, i.e. ease and speed of fabrication and low background currents, have been previously demonstrated for methadone electrooxidation **[1]**. Moreover this present work shows that CPE's may be

more suitable than GCE's for electrochemical studies involving β-agonists due to their greater discriminating power for secondary processes. The electrochemical response of the CPE was investigated for three selected β-agonists possessing catechol (salbutamol) or resorcinol (metaproterenol, fenoterol) ring structures.

To understand the electrochemical process occurring on carbon paste electrodes, both pH and scan rate studies were carried out. The regeneration of the carbon paste activity is essential for reproducible analyte response. Loss of electrode activity has been attributed to adsorption of reaction products, of the analyte itself or other electroactive organic surfactants. Use of high anodic potential activation procedures as suggested by Adams and co-workers **[10]**, and effectively demonstrated by Tuñon and co-workers **[1]**, results in a very reproducible response with relative standard deviations of less than 1%. For this work, electrochemical pretreatment steps were optimised for the three compounds studied.

Chemically-modified electrodes have received a great deal of attention in recent years. By concentrating the analyte in the modified layer of the electrode, voltammetric determinations can be greatly improved with respect to sensitivity and selectivity. Modification of the electrode with electrocatalytic moieties or specially functionalized polymers provides conventional electrochemical electrodes with a significant functional enhancement for analytical applications. One such polymer, i.e. Nafion,

possesses almost ideal properties for preparation of chemically modified electrodes. Nafion is chemically inert, non-electroactive, hydrophilic, thermally stable and insoluble in water **[11]**. Studies by Martin's group **[11]** and **[12]**, have shown that the polymer's sulphonated (SO_3^-) moiety shows a remarkably high affinity for hydrophobic cations. This has led to the development of various carbon-based electrodes modified with the Nafion polymer.

The Nafion-modified glassy carbon electrode (NMGCE) has been used in flow analysis for the determination of cationic drugs and neurotransmittors **[13, 14]**. Results show that Nafion favours cations considerably over neutral species while exhibiting a shielding effect for anions. Further modifications of the electrode has resulted in the combination of Nafion with other materials such as mercury thin films **[15]**, platinum particles **[16]**, methylene blue dye **[17]** and solvents (for example, tributylphosphate) **[18]**. A Nafion-modified graphite electrode incorporating viologens has been developed for catalytic purposes **[19]**. A recent development is the use of a polyaniline-Nafion thin film on a platinum electrode. Polyaniline contains oxidisable groups which form positive charge sites on electrooxidation. Current flows if anionic species "move into" the polymer and counterbalance the positive charge. Nafion, containing negatively-charged SO_3^- groups, can take on this role, and together with the polyaniline, attract positively-charged groups like alkali and alkaline earth metal ions **[20]**. Nafion-modified carbon fibre

ultramicroelectrodes have been applied to the analysis of neurotransmitters and metal ions **[21, 22]**. A recent article by Litong *et al.* reported a chronopotentiometric stripping analysis method for adenosine with a NMGCE **[23]**. Gao *et al.* reported the use of a Nafion-1,10-phenanthroline-modified CPE for the determination of Fe(II) **[24]**. The purpose of the Nafion was two-fold in this application: firstly to immobilise the complexing agent on the electrode surface, and secondly to act as an ion-exchanger for the positively-charged iron complex. The design of the electrode is quite different to the Nafion-modified CPE used for β-agonist accumulation. In the paper of Gao *et al.*, the Nafion was incorporated into the graphite-nujol paste mixture. For the fabrication of the electrode described in this present study, an aliquot of Nafion solution was simply added to the surface of a prepared CPE. A thin film, formed after drying, was sufficient to act as an ion-exchanger for various β-agonists at optimum pH and Nafion film conditions.

2.2 EXPERIMENTAL

2.2.1 Reagents and materials

A Britton-Robinson (BR) buffer solution was prepared containing 11.48 ml acetic acid (99.7%), 13.5 ml phosphoric acid (85%) and 12.44 g boric acid per litre. The pH (range varying from 2 to 12) was adjusted using 2 M sodium hydroxide. Carbon paste was prepared containing 5 g carbon (Spectro pure grade, Fluka Chemical Co.) and 1.8 ml Nujol oil. Lauryl sulphate, Triton-X, fenoterol hydrobromide, metaproterenol hemisulphate, salbutamol and isoproterenol were purchased from Sigma (St.Louis, MO). L(-)-epinephrine was purchased from Janssen Chemica (Beere, Belgium). Terbutaline was a gift from The National Food Centre, Ireland. The pharmaceutical preparation (Berotec) was prepared by Boehringer Ingelheim, (Barcelona). All standard solutions were prepared using deionised water (obtained by passing distilled water through a Milli-Q water purification system) and stored in the dark at 5°C. Chemicals obtained from Aldrich Chemicals were cholic acid (Milwaukee, USA), Amberlite-XAD-2 (Steinheim, Germany) and Nafion (Gillingham, UK). Dilutions of the Nafion stock solution were prepared using water: isopropanol (1:1). Asolectin soyabean (phospholipids) was received from Fluka Biochemika (Switzerland), and silicon OV17 was purchased from Merck (Darmstadt, Germany). The

extraction solvent mixture consisted of ethyl acetate (Farmitalia Earlo Erba, Milan) and amyl alcohol (Panreac, Barcelona) (9:1).

2.2.2 Apparatus

The experiments were carried out in an all-glass cell designed for a three-electrode potentiostatic circuit. Cyclic voltammetric experiments were carried out using a VA Scanner E612 and VA detector E611 (both Metrohm) coupled to a Graphtec WX4421 recorder. Differential pulse voltammetric experiments were carried out using a Model 663-VA stand (Metrohm) equipped with a rotating carbon paste disc electrode (18 mm^2) and coupled to a Model 626 Polarecord (Metrohm). The reference electrode was a Ag/AgCl electrode, whereas the counter electrode was a platinum electrode.

2.2.3 Methods

2.2.3.1 Cyclic voltammetry at carbon paste electrodes

Studies on the effect of pH, using BR buffer pH 2-12, were carried out at concentrations of $1x10^{-4}$ M (salbutamol) and $1x10^{-5}$ M (fenoterol and metaproterenol). Scan rate studies, electrochemical activation procedures and calibration curves were carried out in the optimum pH. Selected modifying

compounds were evaluated for their capacity to promote an adsorption-type process for salbutamol and fenoterol. The modifier was either added to the electrolyte solution (cholic acid, lauryl sulphate, Triton X), incorporated into the carbon paste (silicon OV17, phospholipids, Amberlite XAD-2) or applied in the form of a film on the electrode surface (Nafion).

2.2.3.2 Differential pulse voltammetry at Nafion-modified electrodes

The Nafion-modified electrode was prepared by pipetting 0.01 ml of an appropriate concentration of Nafion solution onto the surface of a previously prepared CPE. The resulting film was air-dried using a domestic hair-dryer. All stripping analyses were carried out in 20 ml BR buffer, pH 2.0. In operation, the CPE was immersed in the buffer solution and rotated at a constant speed (accumulation times typically 10-60 s, depending on the compound concentration) with electrolysis at 0 V. After a 5 s rest period, the compound was removed by stripping anodically from 0 V to +1.2 V and the peak current measured.

2.2.3.3 Analysis of a pharmaceutical preparation

The pharmaceutical preparation was diluted 1: 500 with deionised water and a 0.05 ml aliquot was injected into the cell. 0.05 ml aliquots of fenoterol solution ($2x10^{-5}$ M) were added and a 30 s accumulation time was employed.

2.2.3.4 Extraction methodology

Urine and serum samples obtained from healthy individuals, were stored frozen until assay. After thawing, 5 ml aliquots of urine were fortified with an appropriate fenoterol concentration and 2.0 ml of 0.2 M sodium hydroxide solution was added. After vortexing, 20 ml of solvent mixture was added and the flask shaken by hand for 7 min. The layers were then allowed to separate for 15 min. 18 ml of the upper organic layer was removed using a glass pipette, evaporated to dryness under a stream of nitrogen at 70°C, and redissolved in 2 ml BR buffer, pH 3.0. Differential pulse voltammetry at a Nafion-modified electrode was undertaken as described in section 2.2.3.2 using an accumulation time of 45 s.

For serum, a similar procedure was adopted, but using only 1.0 ml aliquots of sample and appropriately-reduced volumes of extraction solvent (10 ml) and sodium hydroxide (1 ml) solutions. In this case, for improved

sensitivity, the extract was injected into a reduced volume of BR buffer (10 ml).

2.3 RESULTS AND DISCUSSION

2.3.1 Electrochemical behaviour at bare carbon paste electrodes

The cyclic voltammetric behaviour of salbutamol, fenoterol and metaproterenol at bare carbon paste electrodes in BR buffer, pH 8.0, is shown in Figure 2.1. The electrochemical behaviour of the three compounds at these electrodes over the pH range 2-12 in all cases yields one main irreversible oxidation process, which shifts towards more negative potentials as the pH increases. The presence of a very distinct secondary process was also observed for salbutamol. In the case of fenoterol and metaproterenol, this secondary process was also observed but was less distinct and appeared only in the pH range 6-10. For salbutamol this process appears in the pH range 6-12 and becomes more pronounced as the pH increases.

Figure 2.2 shows the relationship between half-peak potential and pH for the two processes of salbutamol at bare CPEs. Linearity for the first process was observed in the pH range 1-11, giving a negative slope of 50.7

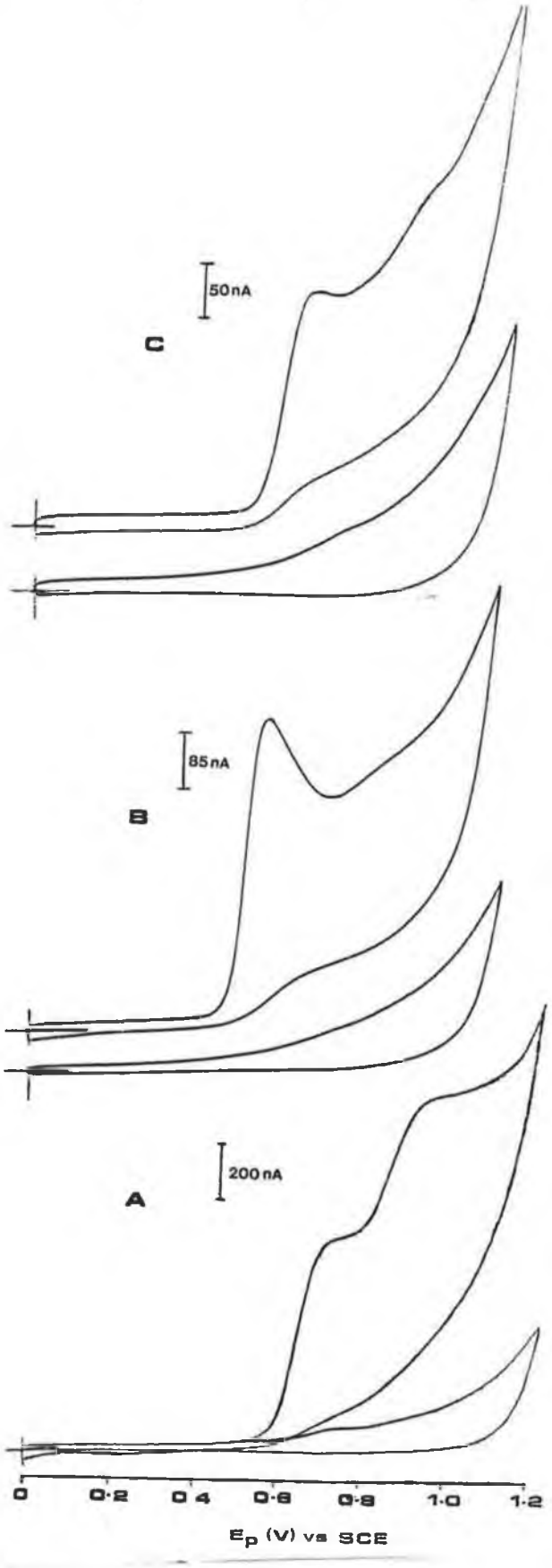


Figure 2.1 Cyclic voltammograms of (a) salbutamol (concentration $1x10^{-4}$ M), (b) fenoterol and (c) metaproterenol (concentration $1x10^{-5}$ M) at carbon paste electrodes (pH=8.0, Scan rate 70 mVs^{-1}, background scan shown below each compound.).

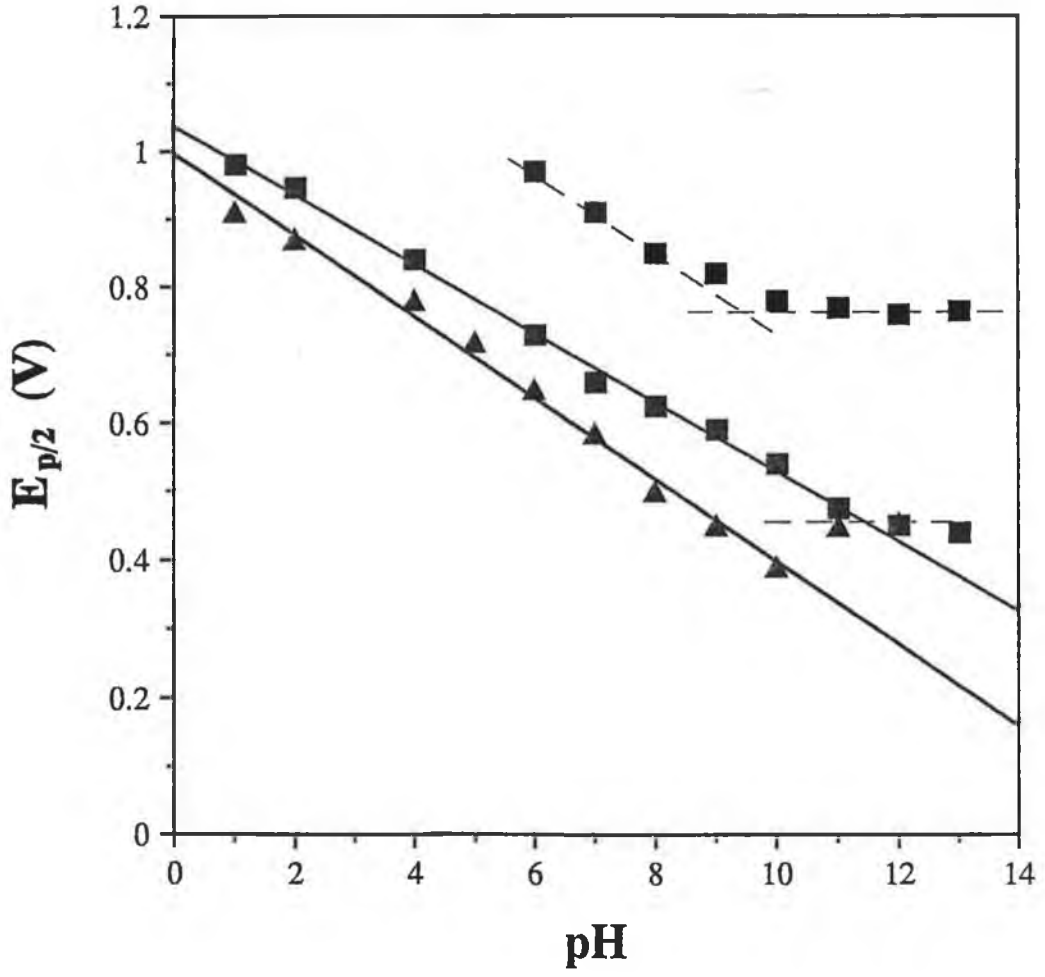


Figure 2.2 The relationship between half-peak potential and pH in cyclic voltammetry for $1x10^{-4}$ M salbutamol (■) and $1x10^{-5}$ M fenoterol (▲) at bare carbon paste electrodes (Scan rate=70 mVs^{-1}. — indicates main process, --- indicates secondary process.)

mV per pH unit. For the second process linearity was observed in the pH range 6-8, giving a negative slope of 60.0 mV per pH unit. The main process is most likely due to oxidation of the phenolic hydroxyl group. This type of process can be observed in both acid and basic media. Amino groups can be oxidised only in the non-protonated form and at higher potentials than the hydroxyl aromatic group, i.e. amino groups are not electroactive in acidic media. For example, no process for the amino group in methadone was observed at pH < 5)**[1]**. Hence the first process can only be assigned to the oxidation of the hydroxyl group and the second process, which cannot be observed in acidic media, is due to the amino group oxidation. A break in the $E_{p/2}$ vs pH plot for the main process appears between 9.5-10.5, which is in the region close to the value reported by other workers **[4, 5]**. This break, however, is not as distinct as that exhibited at a glassy carbon electrode **[4]**, and may be due to some interaction of the compound with the pasting material. The pK_a value, determined from the intersection of the two lines of the secondary process was, however, more distinct indicating a pK_a value of 9.3. It is interesting to note that this secondary process was not observed at the glassy carbon electrode, indicating the greater discriminating power of the CPE for electrode processes occurring at similar potentials.

Figure 2.2 also represents the relationship of half-peak potential and pH for fenoterol. Linearity for the main process of fenoterol was observed in the pH range 1-10, giving a slope -59.2 mV per pH unit. The lower potential

required to oxidise fenoterol compared to salbutamol is due to the nature of the hydroxyl substituents in the molecule. Coulometric studies aimed at finding the number of electrons involved in the processes proved fruitless owing to adsorption effects. Both fenoterol and metaproterenol are likely to exhibit several electron transfer reactions due to the hydroxyl groups in the structure. In the case of metaproterenol (results not shown), linearity was observed in the pH range 2-6, giving a slope of -60 mV per pH unit. A change in the slope occurs after pH 6, and at pH 10 the process is independent of pH. The presence of small reduction waves, which move to more negative potentials as the pH increased, was seen for salbutamol and fenoterol and may be due to products formed after oxidation. Such behaviour was reported also for the oxidation of other related compounds, i.e. clenbuterol and mabuterol, at CPE's [2]. The relationship of peak current with pH for salbutamol showed a decrease in current for the first process as the pH increases, after which the second process appears and subsequently the current for this process increases with the pH. A similar behaviour was observed for fenoterol. For metaproterenol, the peak current decreased with increasing pH. The optimum pH for further study, based on obtaining a good signal coupled with adequate separation from the background discharge, was in the range 4-5 for all the compounds studied.

Scan rate studies were then carried out to assess whether the processes at carbon paste electrodes were under diffusion or adsorption control. Table

2.1 gives a summary of results, indicating the concentrations tested, the relationship of peak current with scan rate/square root of scan rate, and whether the process was diffusion or adsorption controlled. From the results one can see that for all three compounds, at high concentrations, a diffusion-controlled process occurs. However, at low concentrations, close to the limit of detection of the technique, an adsorption process was observed for both salbutamol and metaproterenol while for fenoterol a diffusion process was found at low concentrations.

Electrode pretreatment procedures based on activation at high anodic potentials were then developed for the three compounds. For salbutamol analysis, the electrode was kept in a quiescent solution for 30 s under electrolysis at +1.55 V. The electrolysis was then switched off and the solution stirred for 15 s. For fenoterol and metaproterenol the activation potential used was lower, i.e. +1.3 V, and the activation and stirring times were 20 s and 30 s, respectively. Using such activation procedures, relative standard deviations of $< 0.5\%$ (n = 5) were achieved. Using the activation procedure, calibration ranges were established for the three compounds. For salbutamol, a linear response was obtained in BR buffer, pH 4.0, in the range 2.5×10^{-6} to 4.75×10^{-5} M according to the following equation:

$$i_p\ [nA] = 1.37 \times 10^{+7}\ .C\ [M\ ] - 1.46 \qquad (n=10,\ r=0.9994)$$

COMPOUND	pH	CONCENTRATION (M)	RANGE (mVs^{-1})	EQUATION	PROCESS CONTROL
Salbutamol	4.0	$2x10^{-6}$	2-90	i_p /nA= 0.83 v+ 8.99 r=0.999 n=11	Adsorption
		$1x10^{-5}$	2-100	i_p /nA= 19.60 $v^{1/2}$+ 9.29 r=0.999 n=11	Diffusion
Fenoterol	5.0	$3.3x10^{-7}$	2-40	i_p /nA= 4.58 $v^{1/2}$- 2.29 r=0.998 n=6	Diffusion
		$1x10^{-5}$	2-90	i_p /nA= 74.23 $v^{1/2}$- 36.61 r=0.998 n=9	Diffusion
Metaproterenol	5.0	$5x10^{-7}$	2-100	i_p /nA= 0.23 v- 36.61 r=0.998 n=8	Adsorption
		$5x10^{-6}$	2-100	i_p /nA= 15.16 $v^{1/2}$+ 8.36 r=0.998 n=8	Diffusion

Table 2.1 Characterisation of the rate-controlling step for the selected phenolic β-agonists using cyclic voltammetry at bare carbon paste electrodes .

For fenoterol a linear response was established in acetate buffer pH 5.0, in the range $5.0x10^{-8}$ to $1.6x10^{-6}$ M according to the equation:

$$i_p\ [nA] = 6.01x10^{+7}\ .C\ [M\ ] + 2.28 \qquad (n=9,\ r=0.9994)$$

Linearity for metaproterenol was achieved also in acetate buffer, pH 5.0, in the range $4.0x10^{-7}$ to $4.25x10^{-5}$ M giving the following equation:

$$i_p\ [nA] = 1.71x10^{+7}\ .C\ [M\ ] + 8.00 \qquad (n=10,\ r=0.9991)$$

2.3.2 Electrochemical studies at a Nafion-modified carbon paste electrode (NMCPE)

None of the modifiers tested, with the exception of lauryl sulphate and Nafion, showed higher faradaic currents than those achieved at a bare CPE. In some cases (in particular with cholic acid, Amberlite XAD-2 and Triton X), very high capacitative currents were recorded. Lauryl sulphate gave almost a three-fold increase in peak current for fenoterol (using $2x10^{-4}$ M lauryl sulphate), but an accumulation process did not occur with time. Results obtained from using a Nafion-modified electrode for both salbutamol and fenoterol showed large increases in peak current as the accumulation time increased. Differential pulse voltammetry was performed to obtain accumulation curves for the compounds and hence to ascertain their linear range. Moreover, cyclic voltammetry with the modified electrode permitted determination of the rate-controlling step within the Nafion layer itself. The remainder of this work concentrated on studying Nafion-modified electrodes in more detail.

2.3.2.1 Influence of pH

A pH study, using BR buffer of pH 2-8, was used to evaluate the accumulation behaviour of the three compounds at Nafion-modified CPE

surfaces (compound concentration $2.5x10^{-7}$ M, 0.5% Nafion film and accumulation time 30 s). Figure 2.3 shows that the best accumulation of all three compounds occurred at pH 2.0, and decreased rapidly as the pH is increased. Of the three compounds studied, salbutamol showed considerably lower peak currents under the same conditions. In conclusion, it appears that for optimum analytical conditions, a pH value of 2.0 would be the most suitable. However, the effect of the background current, which is more pronounced at lower pH values, must also be considered. pH values between 2.0 and 3.0 are therefore the best compromise for analyte selectivity.

2.3.2.2 Influence of pulse height, scan rate and deposition potential

The pulse height was varied over the range 10 to 100 mV in optimum buffer conditions and a 0.5% Nafion-modified electrode. The analyte peak current increased significantly with pulse height up to 50 mV, after which further increase in pulse height resulted only in a small increase in peak current. Hence a pulse height of 50 mV was used for all further work.

The influence of scan rate on the peak current was studied in the range 2-25 mVs^{-1}. The optimum scan rate was either 5 or 10 mVs^{-1}, both of which gave similar peak currents. However, a scan rate of 10 mVs^{-1} was chosen for all further work due to the shorter analysis times achieved. The response of the stripping peak at various initial accumulation potentials was also carried

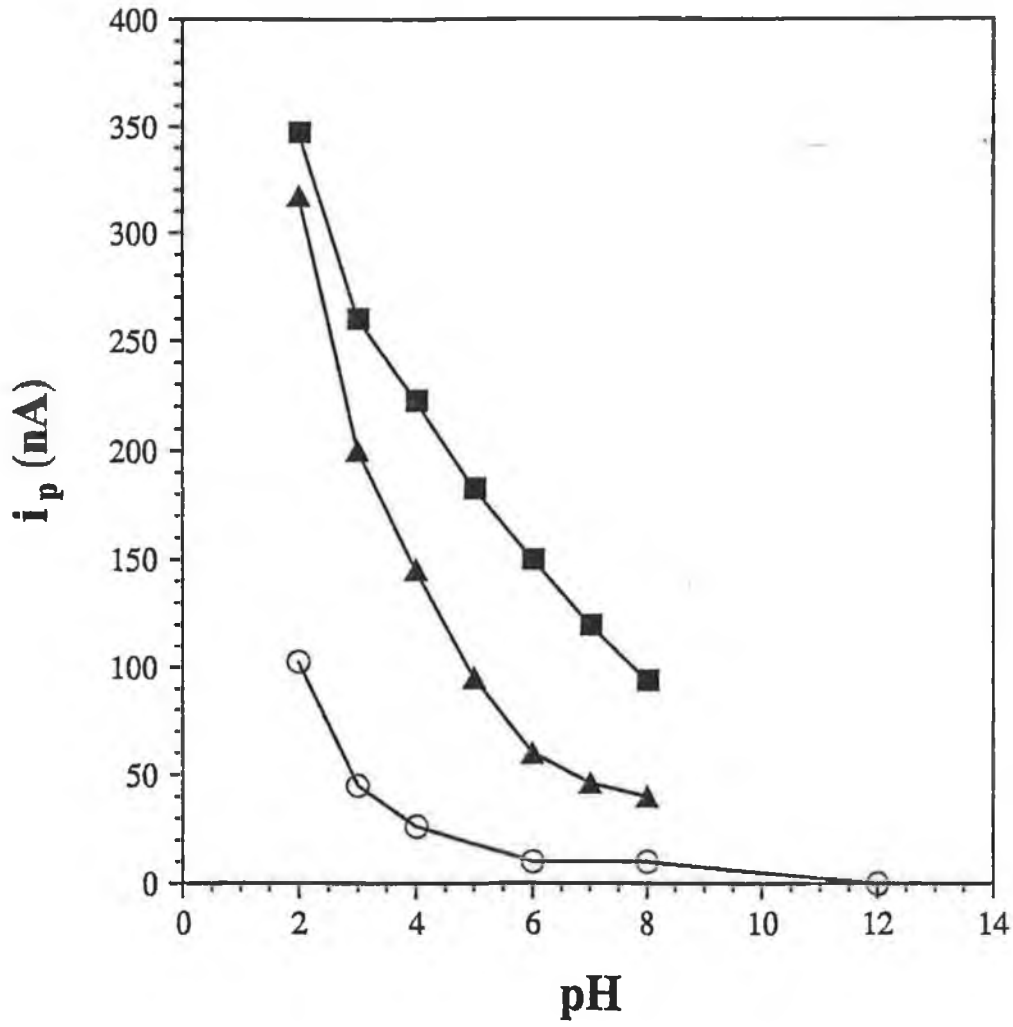


Figure 2.3 Effect of pH on the accumulation of 2.5×10^{-7} M concentrations of fenoterol (■), metaproterenol (▲) and salbutamol (o) at Nafion modified CPE's (0.5%) using a 30 s preconcentration time using differential pulse voltammetry (DPV)(ΔE=50mV υ=10mV s^{-1}).

out in the range -0.2 V to +0.7 V. No real difference in peak current was recorded in the range -0.2 V to +0.1 V, but from +0.2 V onwards a significant decrease in the response was observed. An electrolysis potential of 0 V was chosen as the optimum accumulation potential.

2.3.2.3 Accumulation studies

Accumulation studies on an electrode modified with a 0.5% Nafion solution were carried out in BR buffer, pH 2.0. Figures 2.4, 2.5 and 2.6 show typical accumulation curves of fenoterol, metaproterenol and salbutamol, respectively, at various concentration levels. The fenoterol and metaproterenol accumulation curves at Nafion-modified electrodes are very similar in nature. Fenoterol exhibited a good linear response up to 120 s at 2.5×10^{-8} M, after which the slope levelled off. At the 1.0×10^{-7} M level the linear response was observed up to 45 s, and at higher levels of 2.5×10^{-7} and 5.0×10^{-7} M, saturation of the electrode response occurred after 30 s. At low levels of metaproterenol of 2.5×10^{-8} M, a linear signal was observed up to 60 s. A 45 s preconcentration time may be used for concentration levels of 1.0×10^{-7} and 2.5×10^{-7} M, respectively. For a concentration of 5.0×10^{-7} M a linear response may be achieved up to 30 s, as with fenoterol. Salbutamol exhibited a much better accumulation onto the Nafion membrane as shown in Figure 2.6. For the entire concentration range 2.5×10^{-8} to 5.0×10^{-7} M, linearity was obtained

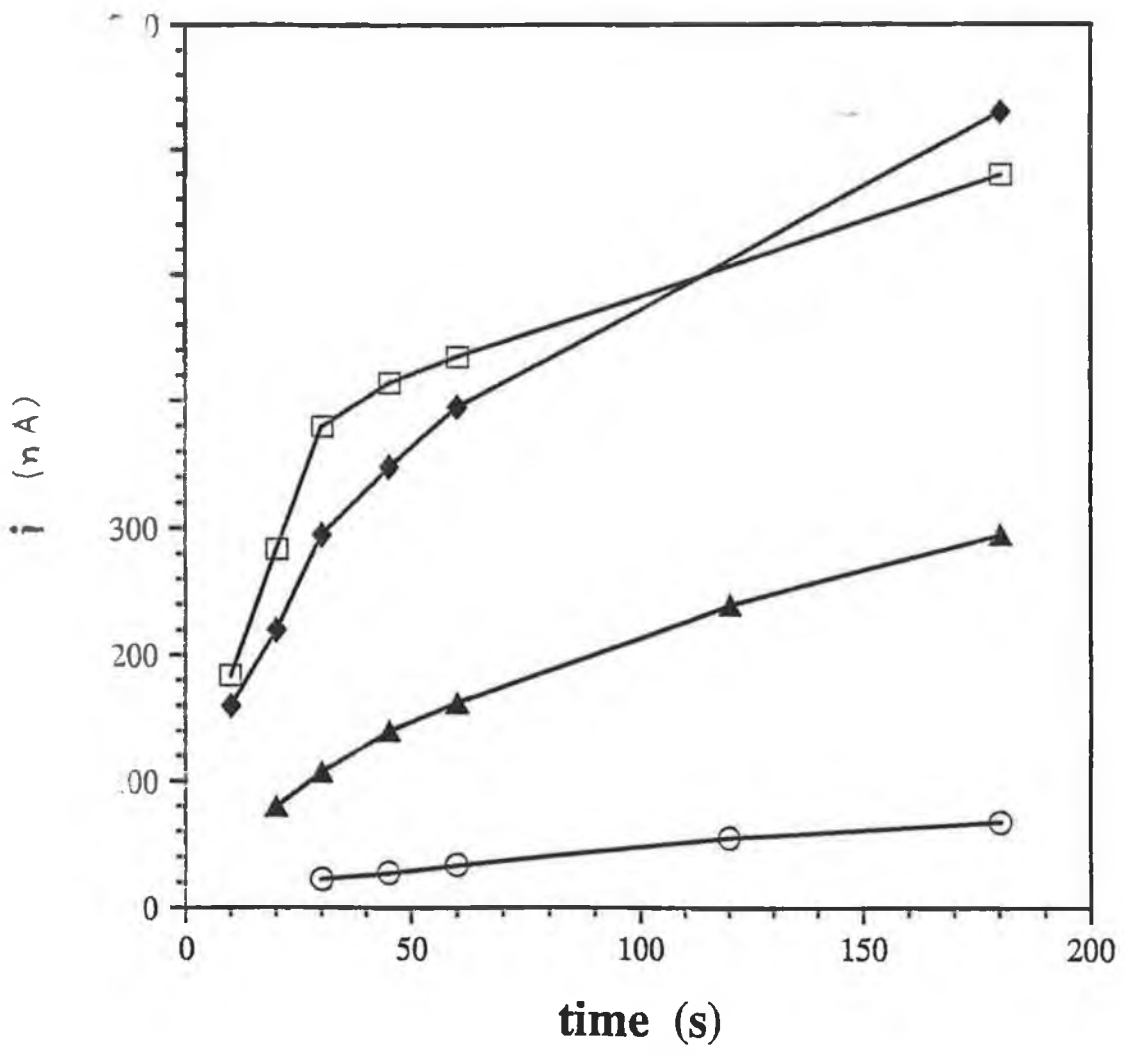


Figure 2.4 Accumulation curves of fenoterol using differential pulse voltammetry (DPV) with a 0.5% Nafion modified CPE in BR buffer, pH 2.0; 2.5×10^{-8} M (o), 1.0×10^{-7} M (▲), 2.5×10^{-7}M(◆) and 5.0×10^{-7}M (□) (experimental conditions as in figure 2.3)

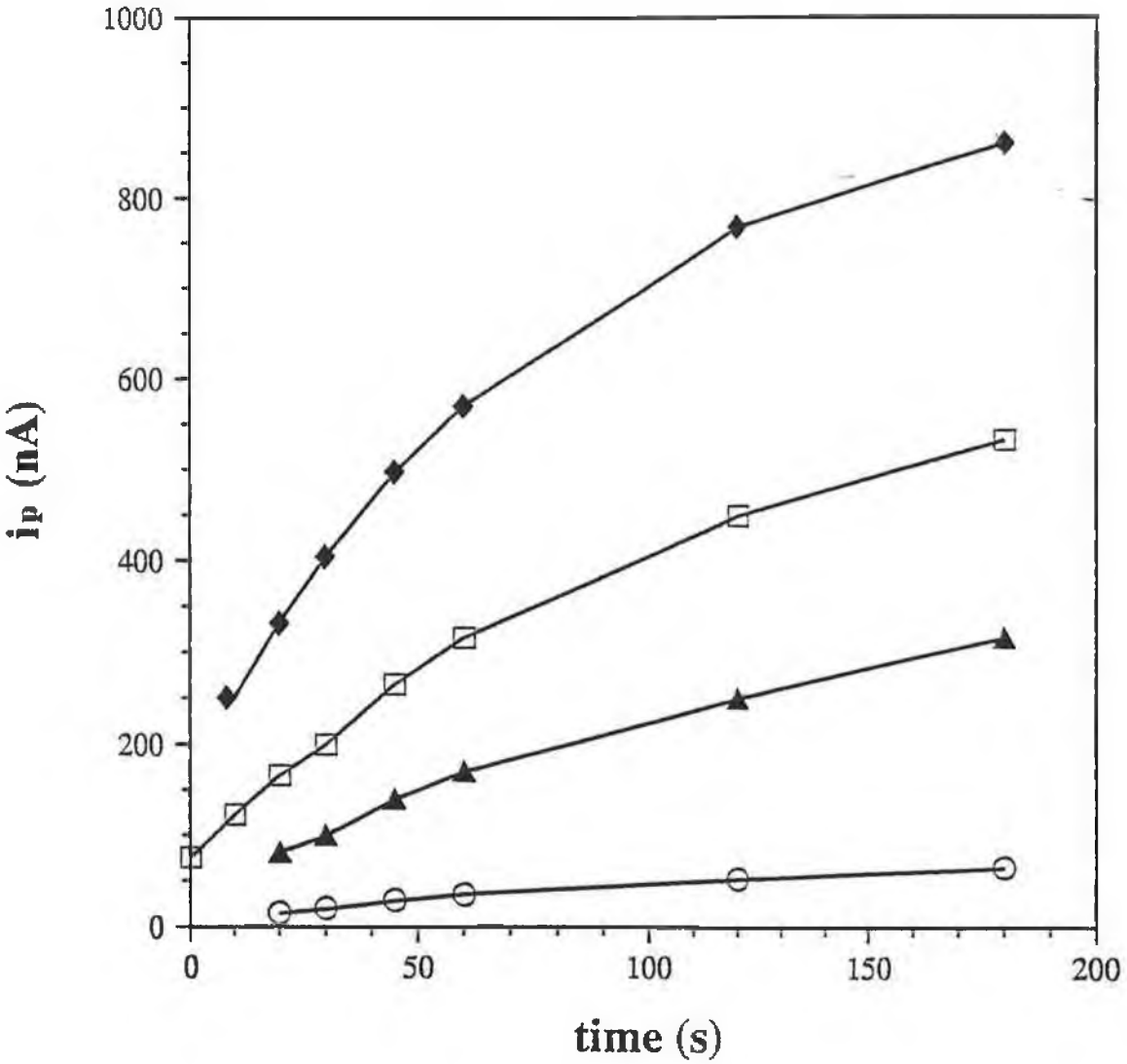


Figure 2.5 Accumulation curves of metaproterenol in DPV with a 0.5 % Nafion modified CPE at pH 2.0; 2.5×10^{-8} M (o), 1.0×10^{-7} M (▲), 2.5×10^{-7} M (□) and 5.0×10^{-7} M (◆) (experimental conditions as in figure 2.3)

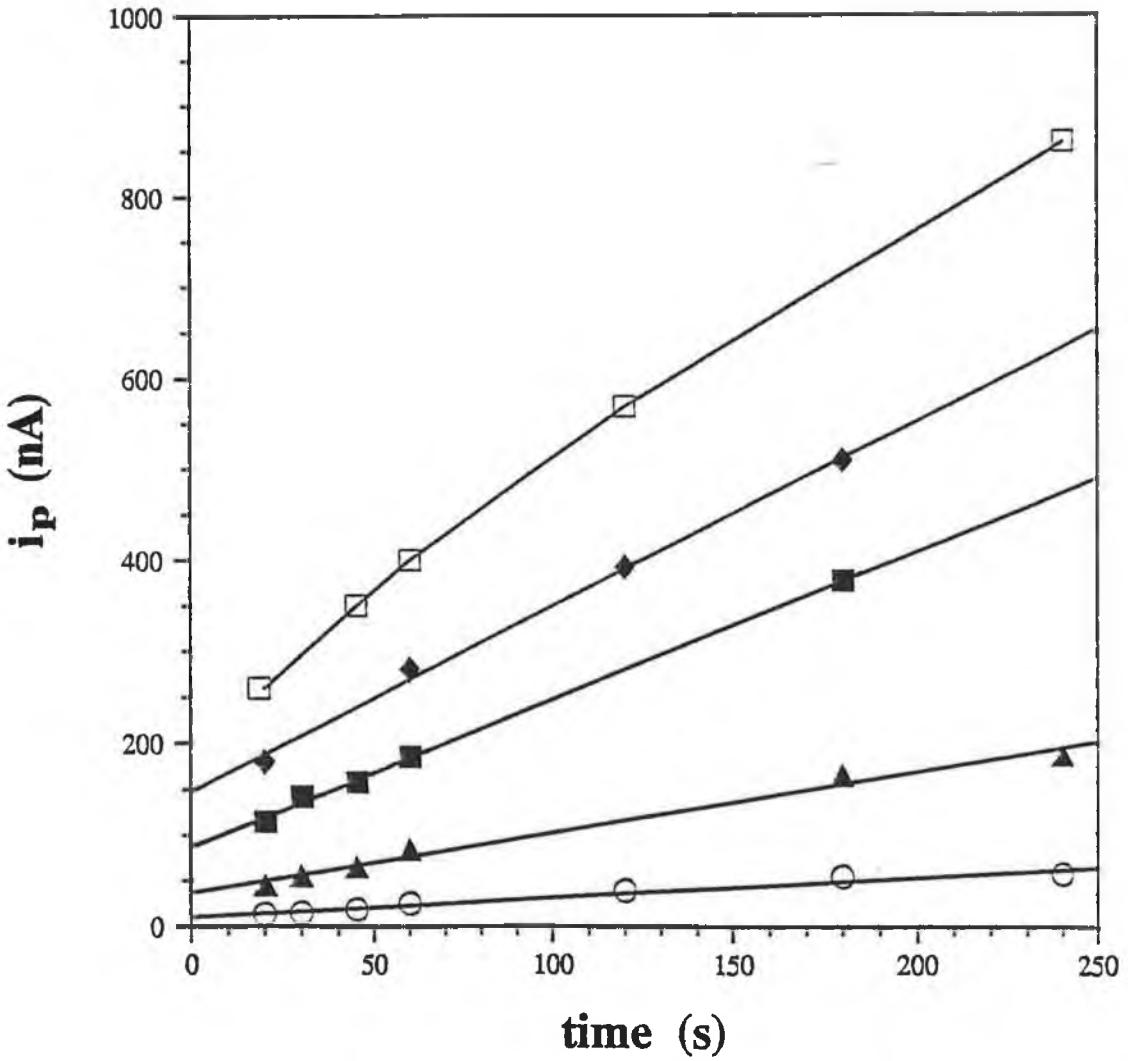


Figure 2.6 Accumulation curves of salbutamol in DPV with a 0.5% Nafion modified CPE at pH 2.0: 2.5×10^{-8} M (o) 1.0×10^{-7} M (▲), 2.5×10^{-7} M (■), 5.0×10^{-7} M (◆) and 1.0×10^{-7} M (□) (experimental conditions as in Figure 2.3)

up to 180 s. Higher accumulation times of 240 s at low compound concentrations resulted in a change of slope of the line. At high levels of $1.0x10^{-6}$ M a linear response was observed up to 60 s. This means that, due to a structural difference in the salbutamol molecule to that of fenoterol and metaproterenol, longer accumulation times and higher concentrations may be used. However, for the same concentration level and accumulation time the peak currents obtained for fenoterol and metaproterenol are almost twice that of salbutamol.

2.3.2.4 Cyclic voltammetry

To understand the rate-controlling step of the process at the Nafion membrane, scan rate dependency studies were carried out. For both films studied (using 0.5% and 2.0% Nafion solutions), the dependence of the peak current (i_p) on the scan rate (v) was found to be non-linear. When the peak current was plotted versus the square root of the scan rate, a linear response was recorded for all three compounds, indicative of a diffusion-controlled process. The results are summarised in Table 2.2. The main conclusion to be drawn from these results is that the rate-controlling process is the diffusion of the compounds from one part of the Nafion layer to another.

COMPOUND	NAFION (%)	CONCENTRATION (M)	RANGE (mVs^{-1})	EQUATION	PROCESS CONTROL
Salbutamol	0.5	$1x10^{-6}$	5-70	i_p /nA= 34.90 $v^{1/2}$ -19.20 r=0.997 n=8	Diffusion
	2.0	$1x10^{-6}$	5-50	i_p /nA= 51.84 $v^{1/2}$ -2.14 r=0.995 n=6	Diffusion
Fenoterol	0.5	$5x10^{-7}$	5-80	i_p /nA= 52.60 $v^{1/2}$ -41.01 r=0.999 n=9	Diffusion
	2.0	$5x10^{-7}$	5-70	i_p /nA= 86.60 $v^{1/2}$ -65.09 r=0.998 n=8	Diffusion
Metaproterenol	0.5	$5x10^{-7}$	5-90	i_p /nA= 43.29 $v^{1/2}$ -33.95 r=0.999 n=8	Diffusion
	2.0	$5x10^{-7}$	5-80	i_p /nA= 55.54 $v^{1/2}$ -26.69 r=0.998 n=7	Diffusion

Table 2.2 Characterisation of the rate-controlling step for the selected phenolic β-agonists using cyclic voltammetry at Nafion-modified carbon paste electrodes using pH = 2.0 and a 30 s preconcentration time.

2.3.2.5 Medium Exchange

Medium exchange experiments were then carried out, using fenoterol as a model for the other compounds, on unmodified and modified carbon paste electrodes. At a modified electrode a medium exchange experiment resulted in a peak (compound concentration; 2.5×10^{-7} M, t_{acc}; 30s) which was 76% in height with respect to that of the original (n = 5, CV = 2.8%). Incorporating medium exchange and a 30 s stirring step, a peak 57.9% in height of the original was obtained (n = 5, CV = 3.1%). No peak was recorded after medium exchange using an unmodified surface. These experiments demonstrate clearly that a strong attraction exists between this class of compound and the Nafion polymer.

2.3.2.6 Response of some endogenous neurotransmitters at the NMCPE

Epinephrine and isoproterenol, two naturally-occurring catecholamines with hydroxyl groups at positions 3 and 4 on the aromatic ring were also evaluated for their electrooxidation at the NMCPE. In a BR buffer pH 2.0, both compounds gave rise to a peak at +0.62 V (using the optimum conditions already described), but no accumulation occurred with time. Figure 2.7 shows a voltammogram of a mixture of epinephrine (+0.62 V) and fenoterol (+0.92 V) at a common concentration of 2.5×10^{-7} M, showing the

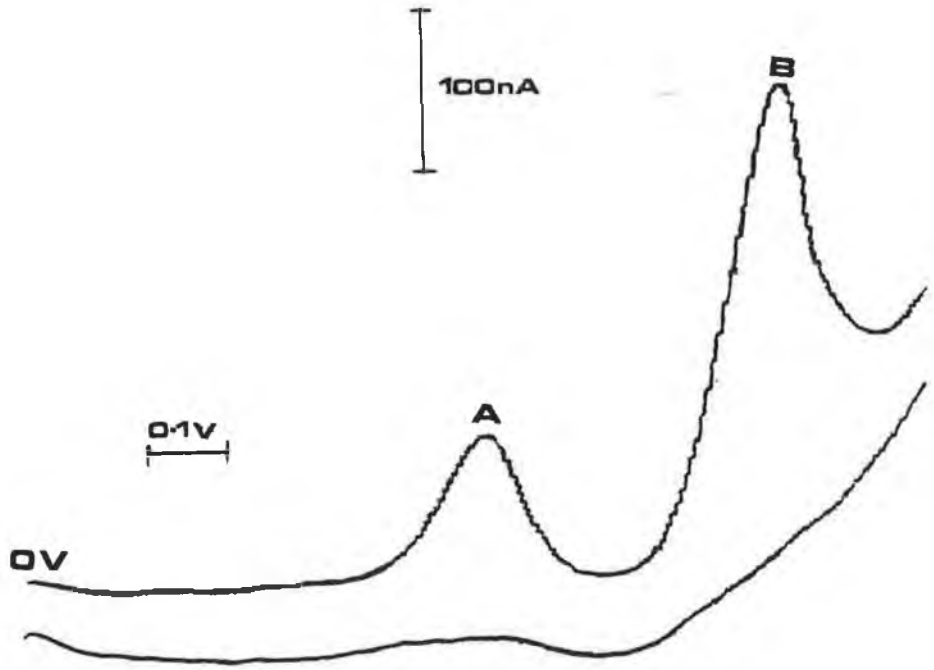


Figure 2.7 Differential pulse voltammograms of 2.5×10^{-7} M concentrations of (A) epinephrine and (B) fenoterol using a 0.5% Nafion electrode in BR buffer, pH 2.0. (pulse height 50 mV, scan rate 10 mV s^{-1})

good selectivity of the voltammetric method.

2.3.3 Application of the NMCPE to fenoterol determination in real samples

2.3.3.1. Effect of pH of supporting electrolyte

The results obtained in section 2.3.2.1, indicate that a solution of low pH favours high accumulation of the phenolic-type β-agonists. Although BR buffer of pH 2.0 gave rise to higher peak current the effect of background current became more pronounced at such low pH values. A BR buffer of pH 3.0 combined good analyte response with satisfactory separation from the background current and hence was used for all further work.

2.3.3.2 Optimisation of Nafion concentration

The effect of increasing the concentration of Nafion at the CPE surface was investigated. Figure 2.8 shows the accumulation profile of $5x10^{-7}$ M fenoterol on unmodified and modified surfaces. No preconcentration of fenoterol occurred with the unmodified electrode. Using Nafion films, however, a preconcentration process was observed resulting in large increases in current up until 0.5% Nafion. Further increases in Nafion concentration

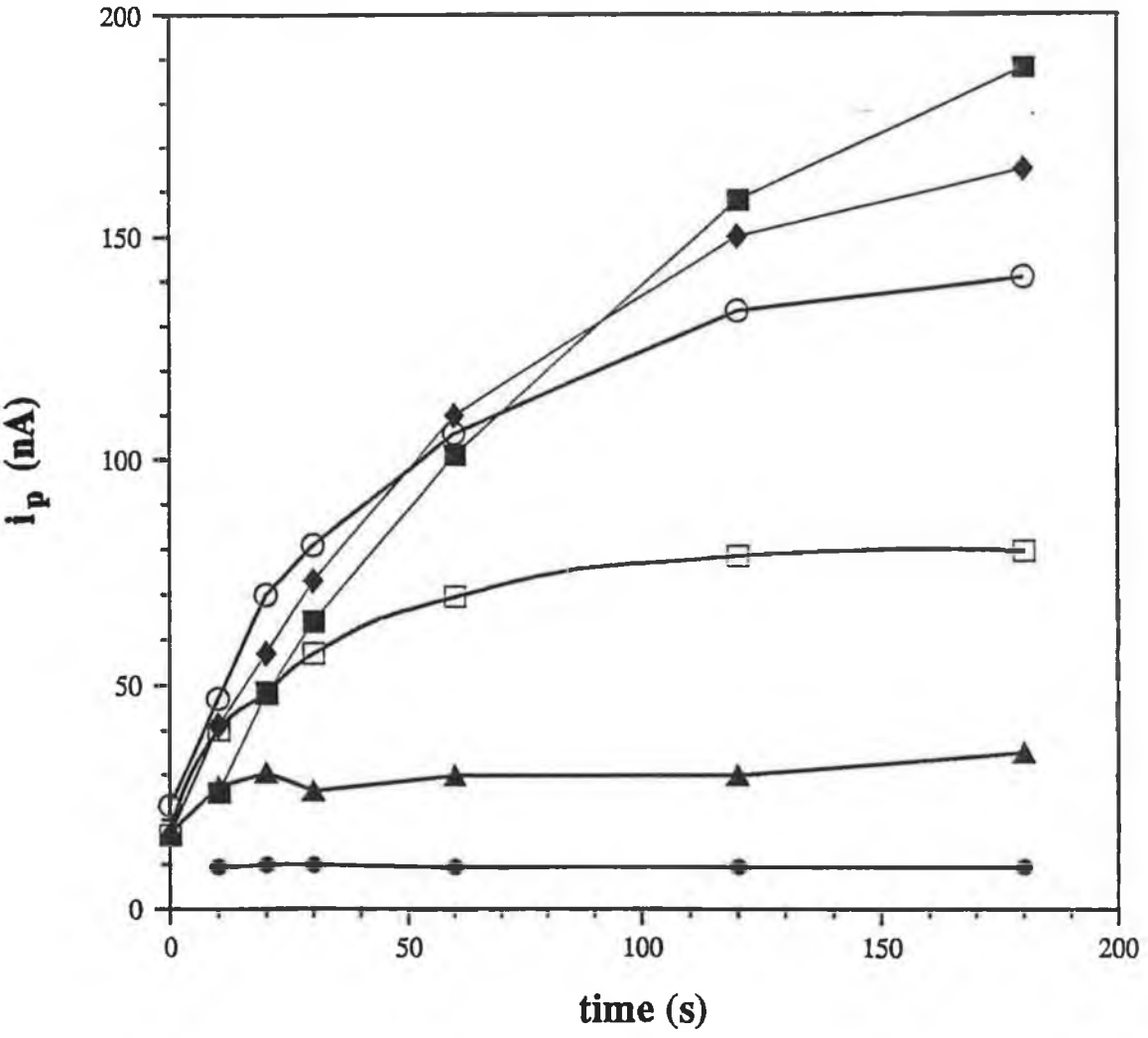


Figure 2.8 Comparison of the DPV responses of fenoterol at a bare carbon paste electrode and at various Nafion-modified CPE's for 5.0×10^{-7} M fenoterol in pH 3.0 BR buffer; Unmodified CPE (●); 0.1% Nafion electrode (▲); 0.2% Nafion Electrode (□); 0.5% Nafion electrode (o); 0.8% Nafion electrode (◆); 2.0% Nafion electrode (■). experimental conditions as for figure 2.7

(up to 2.0%) resulted in insignificant increases in peak current, suggesting that 0.5% may be the optimum concentration of Nafion required. Moreover, a lower current was obtained for higher % Nafion films at low accumulation times, most likely due to resistance of the diffusion of the compound by a thicker layer. From this study, two Nafion concentrations (0.5% and 2.0%) were selected; accumulation studies, calibration curves and reproducibility studies were carried out to investigate whether there is any advantage in using thicker films to enhance the accumulation process.

2.3.3.3 Fenoterol accumulation studies

Accumulation curves, on electrodes modified with 0.5% and 2.0% Nafion, were carried out at four concentration levels of fenoterol. Figure 2.9 (0.5% Nafion) shows the preconcentration of the compound with time. This Figure demonstrates that at low concentrations of fenoterol ($2.5x10^{-8}$ and $5.0x10^{-8}$ M) there was a linear response up to 90 s after which the slope changes (r = 0.998, n = 5 and r =0.999, n =5, respectively). For higher concentrations, i.e. $1.0x10^{-7}$ and $5.0x10^{-7}$ M, linearity was observed up to 60 s and 30 s, respectively (r = 0.999, n = 5 and r = 0.999, n = 3, respectively). For all the concentrations studied there were non-zero intercepts, indicating accumulation of fenoterol during scanning. Accumulation studies with 2.0% Nafion films (results not shown) revealed longer linearity

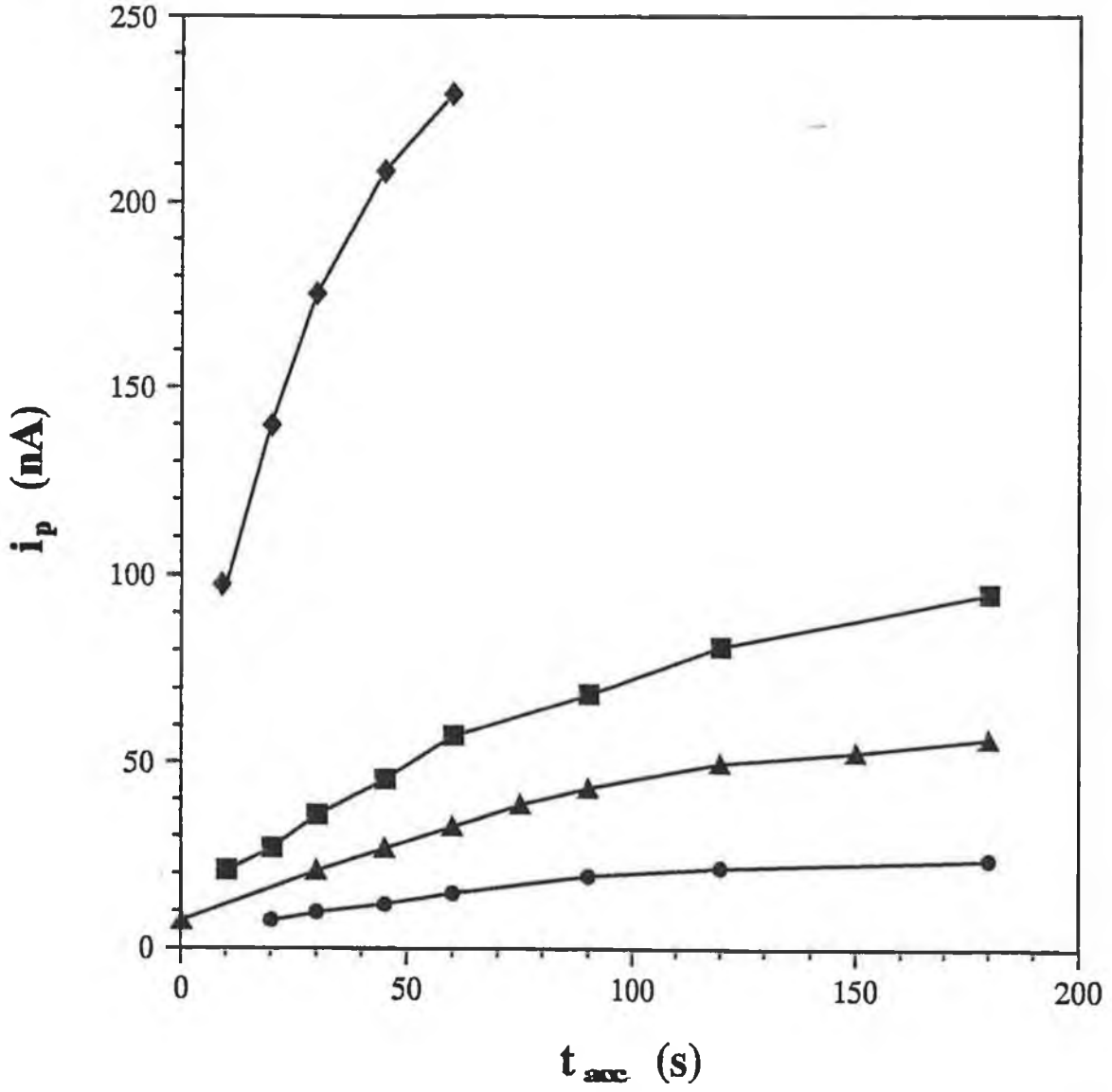


Figure 2.9 Accumulation curves of fenoterol obtained with a 0.5% Nafion electrode using DPV at pH 3.0 and using the same experimental conditions as for figure 2.7: 2.5×10^{-8} M (●), 5.0×10^{-8} M (▲); 1.0×10^{-7} M (■) and 5.0×10^{-7} M (◆).

(180 s) at low concentrations of $2.5x10^{-8}$ M. For concentrations above this level the linear profile was the same as for 0.5 % Nafion. Overall the currents obtained using the higher Nafion concentration were greater but only by a relatively small amount. Non-zero intercepts were obtained as for 0.5% Nafion.

2.3.3.4 Calibration curves

For both Nafion concentrations there was good linearity for all concentrations studied, up to and including 30 s. Using this preconcentration time and a 0.5% Nafion film a calibration curve covering one order of magnitude, $5.0x10^{-8}$ to $5.0x10^{-7}$ M, was observed. The following calibration equation was obtained:

$i/nA = 4.08x10^{+8}$ C/M $+6.61$ $(n = 9, r = 0.9990)$

Using the same preconcentration time and a 2.0% Nafion film, a linear calibration curve covering one order of magnitude, $2.5x10^{-8}$ to $6.0x10^{-7}$ M, was observed. The following equation was obtained:

$i/nA = 3.45x10^{+8}$ C/M $+3.33$ $(n = 9, r = 0.9994)$

It was possible to measure concentrations of the drug compound below $2.5x10^{-8}$ M by applying longer accumulation times, but co-adsorption of interferences was observed. The limit of detection of fenoterol using a 45 s preconcentration time (calculated using a signal-to-noise ratio of 3) was $9x10^{-9}$ M.

2.3.3.5 Reproducibility

The reproducibility of the Nafion-modified electrode was evaluated at two concentration levels of fenoterol ($1.0x10^{-7}$ M and $5.0x10^{-7}$ M); acceptable coefficients of variation, for ten consecutive runs, of 2.21 and 5.25% (0.5% Nafion film) and 3.52% and 4.95% (2.0% Nafion film) respectively, were obtained. The higher variation observed at higher concentrations of compound may be due to modification of the electrode surface by a higher proportion of adsorption products. A fresh NMCPE was fabricated for each study. The electrode may be prepared reproducibly in a reasonably short period of time (typically 20 min).

2.3.3.6 Choice of Nafion concentration for real samples

Apart from the slightly higher faradaic current obtained with a 2.0% Nafion film, no real advantages were derived from its use over the 0.5% film.

Results from cyclic voltammetry (section 2.3.2.4) show that higher capacitance currents are present for thicker films. Moreover, other authors have reported that thicker films retain a more powerful barrier to the diffusion of the analyte **[13, 24]**. Hence, for these reasons, a 0.5% Nafion film was chosen for application to real samples.

2.3.4 Analysis of fenoterol in real samples

The level of fenoterol determined directly in a commercially available pharmaceutical product was 4.78 mg/ml fenoterol hydrobromide (n = 4, CV = 3.81%). This compares reasonably well with the stated level of 5.0 mg/ml. At the 95% confidence level, the value of t (experimental) was less than that of t (theoretical) showing that the method has no systematic error.

For biological matrices, fenoterol was added in the following manner; urine samples were fortified with fenoterol to achieve final concentrations of 2.5×10^{-7} M and 5.0×10^{-7} M, and serum samples were fortified with fenoterol to achieve final concentrations of 1.0×10^{-6} M and 5.0×10^{-6} M. Figure 2.10 shows typical voltammograms of fenoterol in a serum extract and subsequent standard additions. The results of these analyses are summarized in Table 2.3. Good recovery was achieved from both sample types.

A paper by Rominger *et al.* indicates that urine samples containing incurred residues of fenoterol (the availability of which were outside the scope

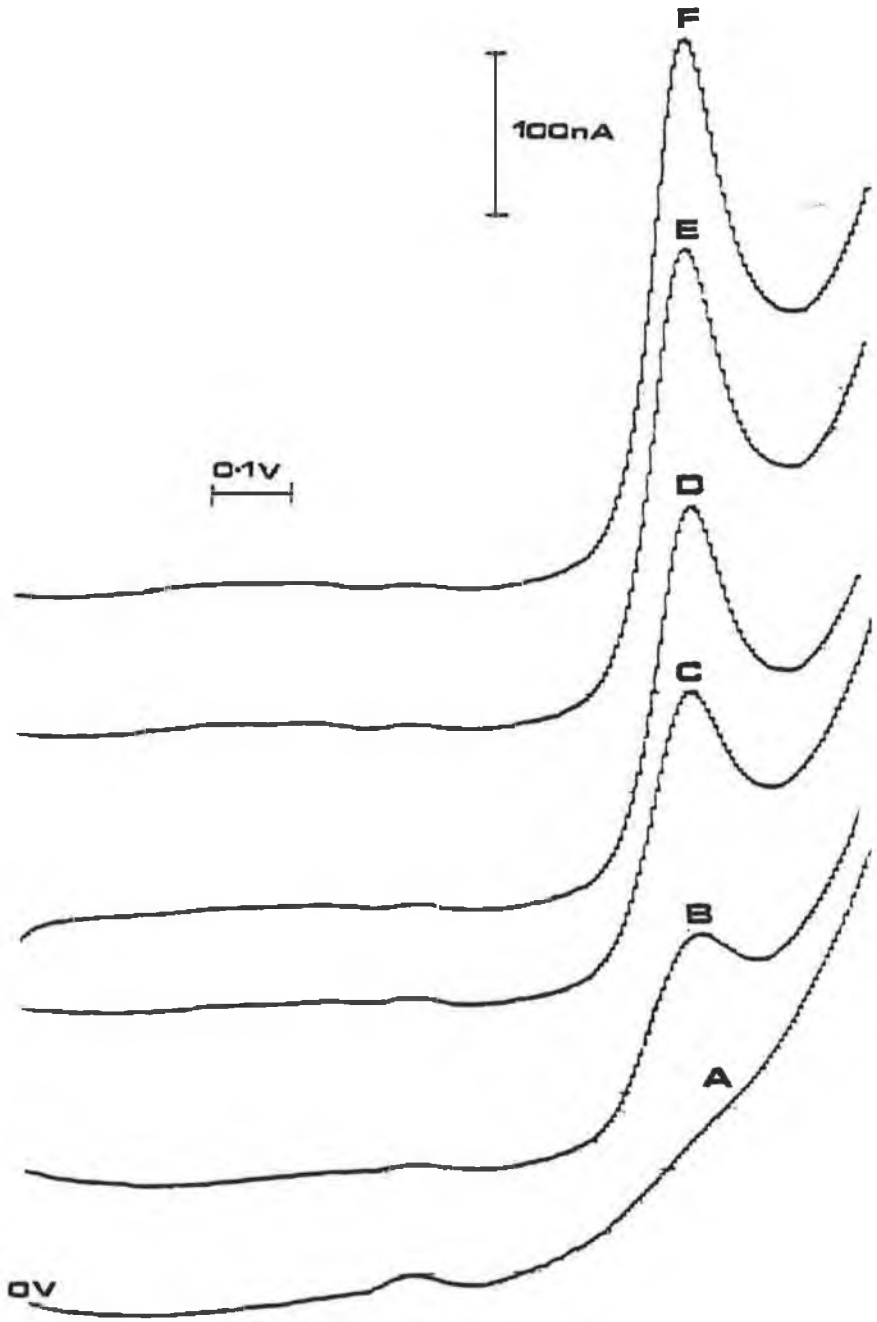


Figure 2.10 Differential pulse voltammograms of fenoterol in BR buffer, pH 3.0 using a 0.5% Nafion electrode and the same experimental conditions as figure 2.7: (A) blank serum extract, (B) extract containing $5x10^{-6}$ M fenoterol and (C)-(F) additions of 6 µl of $1.0x10^{-4}$ M fenoterol (cell volume 20 ml)

Sample	Fenoterol added (M)	n	level determined (M)	CV (%)	Average % recovery
Urine	$2.5x10^{-7}$	3	$2.30x10^{-7}$	11.3	91.8
	$5.0x10^{-7}$	3	$4.62x10^{-7}$	2.95	92.4
Serum	$1.0x10^{-6}$	3	$8.72x10^{-7}$	3.05	87.2
	$5.0x10^{-6}$	3	$3.92x10^{-6}$	6.41	78.3

Table 2.3 Results obtained for fenoterol-fortified urine and serum samples after using the extraction procedure and differential pulse voltammetry (conditions: 0.5% Nafion electrode, pH 3.0, pulse height 50 mV, scan rate 10 mV s^{-1}, t_{acc} 45 s).

of this project) are almost completely conjugated **[25]**. To ensure determination of total fenoterol (i.e. free and conjugated), a deconjugation procedure was recommended. A publication by van Ginkel *et al.* used suc d'helix pomatia juice (enzyme solution) for the hydrolysis of glucuronide and sulphate conjugates in urine samples **[26]**; samples were incubated with buffer, pH 5.2 overnight (18 h) at 37°C. Such a procedure may be carried out in fenoterol-incurred urine prior to extraction and the free form of the compound analysed using the technique developed in this study.

2.4 CONCLUSIONS

The electrochemical behaviour of three β_2-agonists on unmodified and modified carbon paste electrodes has been reported. The compounds are oxidised at high positive potentials on CPE's (at pH 4.0, salbutamol +0.84 V, fenoterol +0.78 V, metaproterenol +0.89 V), giving rise to two processes. The rate-controlling step is mainly diffusion controlled, although adsorption processes are present for salbutamol and metaproterenol in low concentrations, close to the limit of detection of the technique.

Application of a thin film of Nafion on the electrode surface allows the preconcentration of the three compounds and increases the sensitivity of the technique over bare carbon paste electrodes. An ion-exchange mechanism between the protonated (positively charged) compounds and the negatively charged Nafion membrane is observed in the pH range studied. Enhanced signal at low pH values may be explained by a partition equilibrium (Donnan equilibrium) which occurs when the Nafion membrane, saturated with protons, remains neutral ($SO_3^- H^+$) and the analyte forms an ion-pair with a background electrolyte anion. Cyclic voltammetry results show that the oxidation process on Nafion films is diffusion-controlled. Such a phenomenon was also reported by Hoyer *et. al.* in relation to metal ions on Nafion-modified glassy carbon electrodes **[15]**.

Epinephrine and isoproterenol, two compounds in this class posessing an aromatic ring structure with two hydroxyl groups, were also evaluated for their ability to adhere to Nafion films. However, results from these experiments, using the same conditions described above, showed that both compounds did not accumulate with time. A possible explanation for the non-accumulation for these compounds may be that only substances with an aromatic ring which has three carbon atoms between the hydroxyl groups can bind to the Nafion film (i.e. the SO_3H group). The compounds studied have this structural characteristic, whereas epinephrine and isoproterenol, possessing only two carbon atoms between the hydroxyl groups, do not. To further check on this theory, another β-agonist, terbutaline, which has this three carbon atom difference, was evaluated for its performance on the Nafion film. This compound also accumulated with a response similar to that of fenoterol. It is postulated therefore, that the Nafion electrode will accumulate those compounds with the hydroxyl groups placed three carbon atoms apart on the aromatic ring.

The higher affinity of salbutamol to Nafion films is an interesting finding. While this compound has three carbon atoms between the hydroxyl groups, it differs structurally from fenoterol and metaproterenol in the positioning of one hydroxyl group on the aromatic ring and the other hydroxyl group as a methanolic side-chain. It was postulated that this difference caused higher accumulation of compound without saturating the electrode.

The NMCPE has been used successfully to determine fenoterol in complex matrices. Optimum conditions were achieved with a BR buffer, pH 3.0 and a 0.5% Nafion film. Application of the electrode to a pharmaceutical preparations was possible after a suitable dilution of the sample was made. The solvent extraction procedure, developed for biological samples, proved to be selective for fenoterol with good recoveries obtained at the levels tested. This extraction procedure may be suitable for other resorcinol-type β-agonists.

2.5 REFERENCES

1. J.R. Barrierra Rodriguez, A. Costa Garcia and P. Tunon Blanco, Electrochim. Acta, **34** , 1989, 957.
2. G.A. Qureshi and A. Eriksson, *J. Chromatogr.*, **441**, 1988, 205.
3. H. Hoojerink, R. Schilt, W. Haasnoot and D. Courtheijn, *J. Pharm. Biomed. Anal.*, **9**, 1991, 485.
4. K.A. Sagar, M.R. Smyth and R. Munden, *J. Pharm. Biomed Anal.*, **11**, 1993, 533.
5. B.G. Katzung, *Basic and Clinical Pharmacology*, Prentice Hall International Ltd, London, 1989, p.3.
6. Y.K. Tan and S.J. Soldin, *J. Chromatogr. (Biomed Applic.)*, **311**, 1984, 311.
7. K.A. Sagar, C. Hua, M.T. Kelly and M.R. Smyth, *Electroanalysis*, **4**, 1992, 481.
8. K.A. Sagar, M.T. Kelly and M.R. Smyth, *J. Chromatogr. (Biomed. Applic.)*, **115**, 1992, 109.
9. K.A. Sagar, M.T. Kelly and M.R. Smyth, *Biomed. Chromatogr., 7*, 1993, 29.
10. M.E. Rice, Z. Galus and R.N. Adams, *J. Electroanal. Chem*, **143**, 1983, 89.
11. M.N. Szentirmay and C.R. Martin, *Anal. Chem.*, **56**, 1984, 1898.

12. R.B. Moore, J.E. Wilkerson and C.R. Martin, *Anal. Chem.*, **56**, 1984, 2572.

13. J. Zhou and E. Wang, *Anal. Chim. Acta*, **249**, 1991, 489.

14. J. Wang, P. Tuzhi and T. Golden, *Anal. Chim. Acta*, **194**, 1987, 129.

15. B. Hoyer, T.M. Florence and G.E. Batley, *Anal Chem.*, **59**, 1987, 1608.

16. K. Itaya, H. Takahashi and I. Uchida, *J. Electroanal. Chem.*, **208**, 1986, 373.

17. Z. Lu and S. Dong, *J. Chem. Soc. Faraday Trans.*, **84**, 1988, 2979.

18. P. Audebert, B. Divisia-Blohorn, P. Aldebert and F. Michalak, *J. Electroanal. Chem.*, **322**, 1992, 301.

19. K. Shigehara, E. Tsuchida and F.C. Anson, *J. Electroanal. Chem.*, **175**, 1984, 291.

20. J.-Y. Sung and H.-J. Huang, *Anal. Chim. Acta*, **246**, 1991, 275.

21. P. Capella, B. Ghasemzadeh, K. Mitchell and R.N. Adams, *Electroanalysis.*, **2**, 1990, 175.

22. H. Huiliang, D. Jagner and L. Renman, *Anal. Chim. Acta*, **207**, 1988, 17.

23. J. Litong, L.Shenghui, B. Zhuping and F. Yuzhi, *Electroanalysis.*, **5**, 1993, 611.

24. Z. Gao, P. Li, G. Wang and Z. Zhao, *Anal. Chim. Acta.*, **241**, 1990, 137.

25. K.L. Rominger, A. Mentrup and M. Stiasni, *Arzneim.-Forsch Drug Res.*, **40**, 1990, 887.

26. L.A. van Ginkel, R.W. Stephany and H.J. Van Rossum, *J. Assoc. Off. Anal. Chem.*, **75**, 1992, 554.

CHAPTER 3

DEVELOPMENT AND VALIDATION OF CLENBUTEROL EXTRACTION FROM LIVER USING MATRIX SOLID PHASE DISPERSION (MSPD) WITH DETERMINATION BY IMMUNOASSAY

3.1 INTRODUCTION

The β-adrenergic agonist, clenbuterol, has been widely (but illegally) used in beef production due to its growth enhancing capability when administered to animals at multiple concentrations of the therapeutic dose. Work by Meyer and Rinke has shown that liver tissue contains detectable residues for 2 weeks after withdrawal of clenbuterol from the animals diet **[1]**. Rapid and efficient extraction techniques are essential, therefore, to control illegal usage of clenbuterol and to ensure the safety of meat.

Current methodology for the extraction of clenbuterol residues in tissues has been extensively reviewed in Chapter 1. To produce a sample extract sufficiently purified for a chromatographic or immunological determination, the analyst must perform extensive sample homogenisation, solvent extractions and column chromatography. In addition to the obvious disadvantage of the length of time required to perform these analyses, the methods require the excessive use of hazardous organic solvents. Extractions based on solid phase extraction (SPE) overcome the problems associated with large solvent usage. For this technique a homogenised tissue sample is added to a suitable SPE column and the compounds of interest are isolated from the other sample components based on interactions with the column polymer phase.

This approach, while it leads to a reduction in the use of organic solvents, does not eliminate labour-intensive steps such as sample homogenisation, centrifugation and enzyme digestion.

Alternatives to the above approach have been investigated by Barker, Long and co-workers at the Louisiana State University, USA. Their initial research focussed on finding suitable tissue-adsorbing solid supports which could retain the bulk of the sample matrix but expose the test analyte for subsequent elution. They first evaluated the performance of diatomaceous earth as a tissue dispersant; the sample was mixed with diatomaceous earth to obtain a semi-dry column packing material from which drug compounds, e.g. benzimidazole anthelmintics **[2]**, were eluted. Further developments, based on the same concept, were carried out using octadecylsilane (C_{18}) material as the tissue dispersant. The C_{18} material acts as both a solid support (the silica base) and as a tissue solubiliser (the non-polar C_{18} moieties). Samples which are mixed or blended with this material would be expected to disperse in such a manner that the more neutral or lipophilic compounds would solubilise in the polymer phase. Thus, triglycerides and the less polar ends of phospholipids, steroids and other tissue components, would be expected to insert into the phase that is bonded to the surface and pores of the silica particles (i.e. the C_{18} phase). More polar components would associate through hydrophilic interactions with themselves and with the more polar ends of the compounds already inserted in the polymer.

Scanning and transmission electron microscopy studies of the uncoated and tissue-coated C_{18} material surface showed that the mechanical blending action disrupts the organelle structure within the tissue. This allows the more hydrophilic regions of proteins and moderate to polar drugs to extend outwards away from the non-polar C_{18}/lipid region. Water also associates with these hydrophilic ends. In practice, tissue sample (0.5 g) and C_{18} material (2.0 g) are blended, using a mortar and pestle, for between 30 and 40 seconds. The resulting mixture is packed into a plastic column to a volume of 4.5 ml and wash/elution solvents are added to separate the analyte(s) from sample components. Figure 3.1. illustrates the structure of a prepared column.

Barker and co-workers have published a wide range of extractions based on MSPD methodology. In many cases an assay was developed for the isolation of a single compound (e.g. ivermectin **[3]**, chloramphenicol **[4]**). In others, the methods were developed to isolate a class of compounds (benzimidazoles **[5]**, halogenated pesticides **[6]**) or several compound classes (organophosphates and beta-lactams **[5]**) from a sample. The versatility of the procedure to isolate a wide range of unrelated compounds is best demonstrated in reference **[5]**. Residues (pesticides, anthelmintics and antibiotics in order of increasing polarity) were fractionated using an non-polar solvent (hexane), a semi-polar solvent (ethyl acetate) or a solvent of high polarity (methanol). Extracts were determined by GC-nitrogen phosphorus detection or HPLC

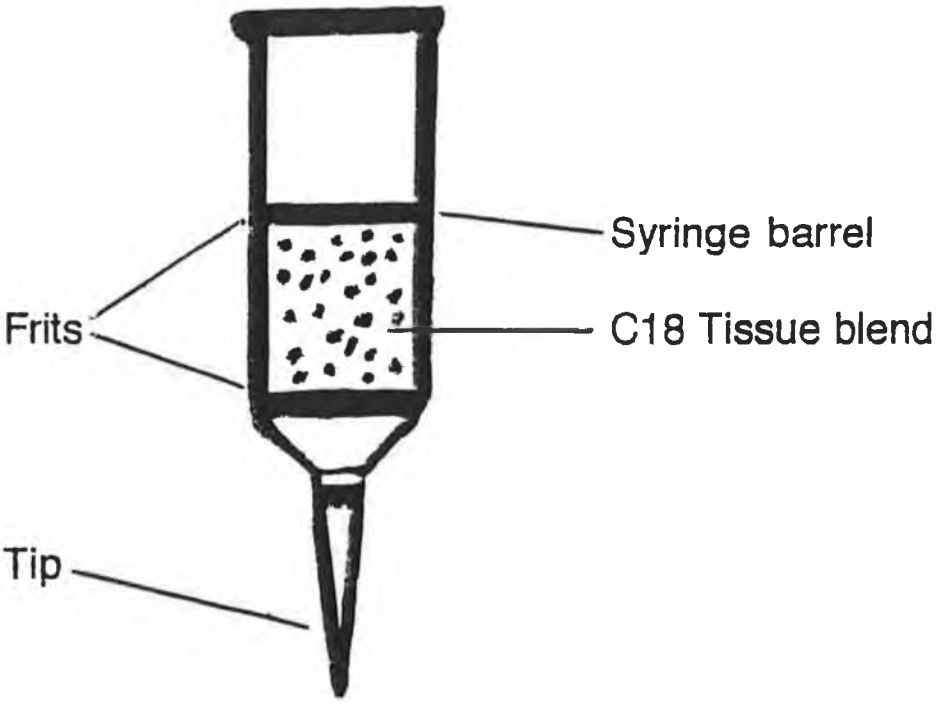


Figure 3.1 A prepared MSPD column.

-photo diode array detection. A similar procedure was used to extract eight sulphonamides from pork tissue **[7]**. In this case hexane was used as a wash solvent and sulphonamides were eluted with dichloromethane. Small modifications in the MSPD procedure were reported by Long *et al.* **[8]**, who proposed the addition of activated florisil to the MSPD column to adsorb fats and water, and Schenk *et al.* **[3]**, who introduced a post-MSPD alumina SPE step for improved sample clean-up. The tissue disruption and distribution and the subsequent elution profile from an MSPD column may be influenced by blending the sample in the presence of acids, bases, salts, chelators or other modifiers. For example, tetracyclines are obtained in higher recovery by incorporating oxalic acid and other chelating agents (such as EDTA) into the MSPD column **[9, 10]**. Thus, the polarity of the target compound may be altered by protonation or deprotonation so as to cause a drug or metabolite to be retained longer or eluted earlier. MSPD methodology is not reserved solely for tissue samples; some publications report MSPD extraction of antibiotics **[4]**, benzimidazoles **[11]** and the herbicide, chlorosulfuron, **[12]**, from milk samples. Work at the National Food Centre (Dublin) has extended the application of MSPD for sulphonamide analysis through its linkage with a TLC determination step to provide a kit method for use in industry **[13, 14]**. Other applications developed by the NFC and associated laboratories have been for ivermectin in fish **[15]** and gestagens in tissue **[16]**. A general summary of the applications of MSPD for a range of analytes, based on their

polarity, is shown in Figure 3.2. This study demonstrates the extension of the MSPD method to the extraction of β-agonists from liver samples. Residues extracted by MSPD are normally determined by chromatographic-based techniques like HPLC and GC. This is due, mainly, to the multianalyte detection capability of these techniques, and also because the detection limits required are relatively high (10-100 ppb). To monitor clenbuterol, in the context of illegal usage, such detection limits are unsuitable (the MRL for clenbuterol set by the UK is 0.5 ppb). Immunoassays are commonly used to determine residues of banned substances (e.g. steroids). Coupled with an efficient extraction procedure, immunoassay techniques have the capability to measure residues as low as 10 ppt **[17]**. The two immunoassay procedures most used in the field of drug residue analysis have been radioimmunoassay (RIA) and enzyme immunoassay (EIA).

Radioimmunoassays for the quantitative detection of small molecular analytes such as veterinary drugs generally employ tritiated antigens and specific antibodies. A fixed amount of radiolabelled antigen is added to the sample extract and following competitive binding by an antibody, the bound complex is separated (demonstrated in Figure 3.3). The level of radioactivity is determined in the bound complex. To quantify the level of unlabelled antigen (the analyte) in the sample extract, a calibration curve is prepared using a range of standards which compete with the radiolabelled antigen for binding to the antibody. Fewer radiolabelled molecules are bound to the

MATRIX SOLID PHASE DISPERSION

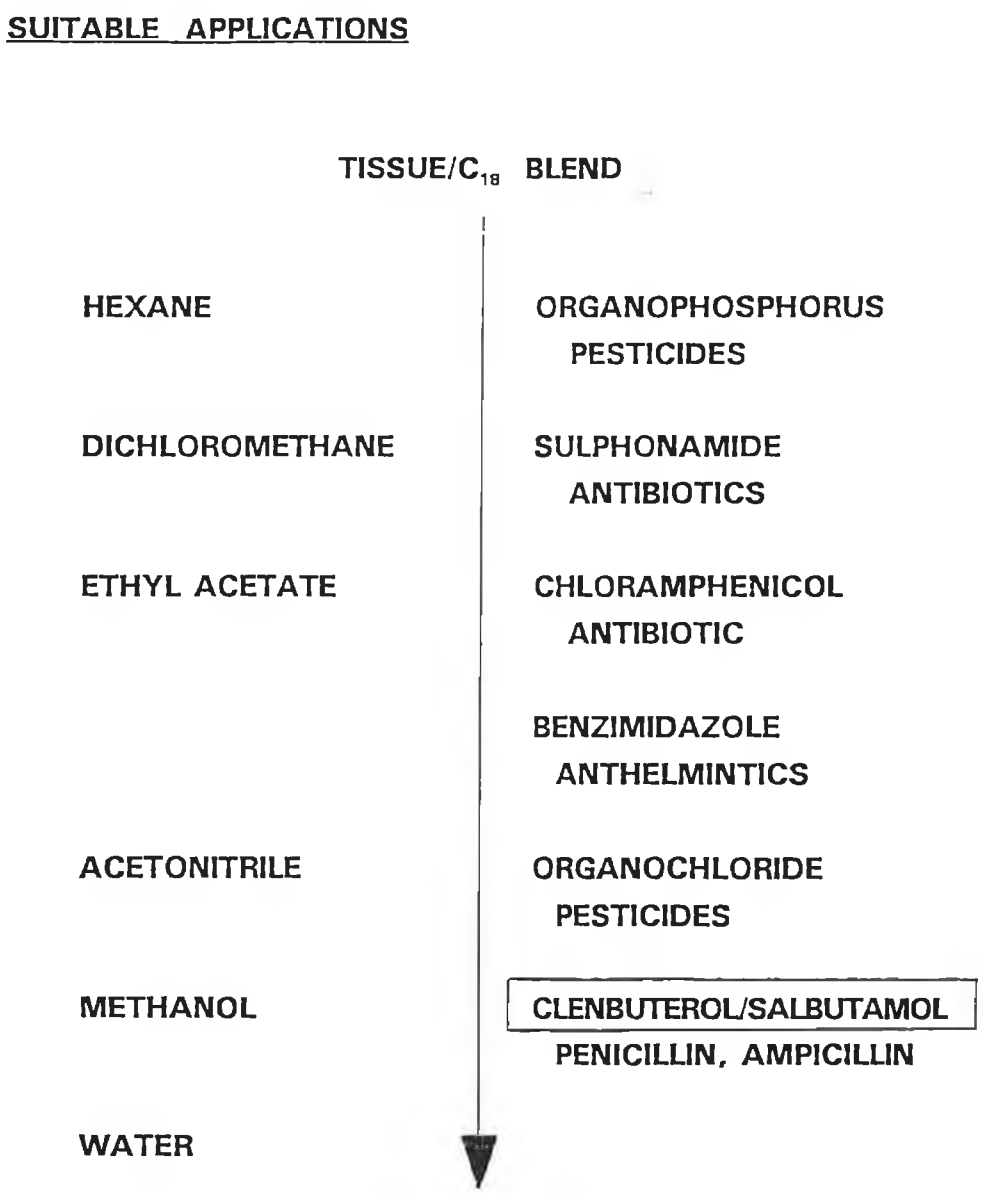


Figure 3.2 The extraction of an analyte from an MSPD column depends on the selection of an organic solvent of similar polarity.

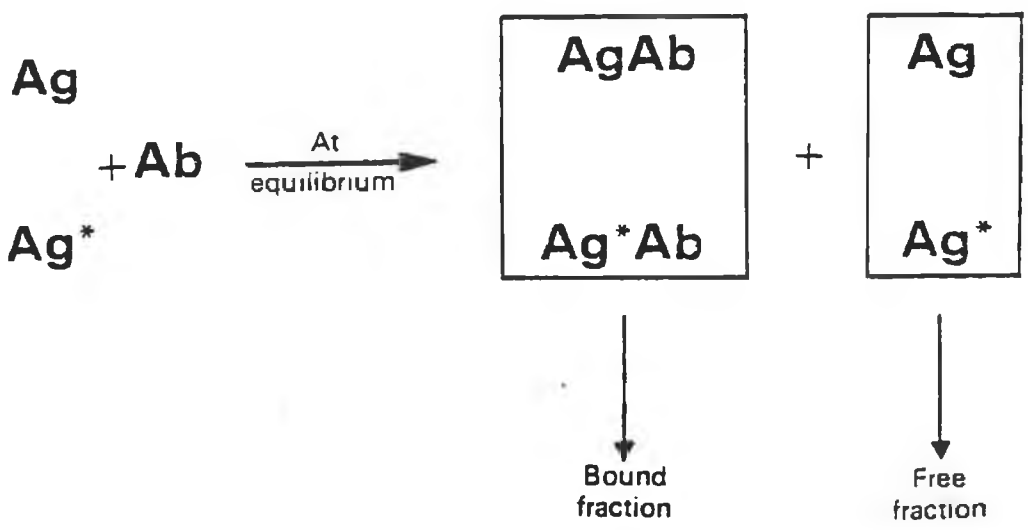


Figure 3.3 Principle of radioimmunoassay: Ag = unlabelled antigen; Ag^* = radiolabelled antigen; Ab = antibody; AgAb = antigen-antibody complex.

antibody as the concentration of the unlabelled antigen increases and consequently, a lower signal (i.e. radioactivity) is counted. The resulting standard curve between antibody-bound radiolabelled antigen (%) against concentration of unlabelled antigen is sigmoid in nature. The procedures leading to the production of antiserum have been discussed in Chapter 1 (section 1.3.3). The antiserum is then tested for titre (a measure of the concentration of cross-reacting antibodies in the serum) and specificity (the measure of the exclusiveness of cross-reactivity to the analyte(s) of interest). In addition to polyclonal antiserum (i.e. antibodies derived from several clones of lymphocytes) as described in Chapter 1, monoclonal antiserum (i.e. antibodies derived from a single clone of lymphocyte) may be prepared and used in the RIA procedure.

The labelling of antigens is generally accomplished by the replacement of carbon, hydrogen or iodine with ^{14}C, ^{3}H or ^{125}I, respectively. For RIA procedures for the determination of small molecules, however, labelling with tritium [^{3}H] is most common as Iodine [^{125}I] is a large molecule which can interfere with antibody binding. It is essential for efficient RIA that the radiolabelled and unlabelled antigen have equal affinity for the antibody. One particular RIA procedure which utilises the [^{125}I]-analyte tracer is that described by Rominger *et al.* for the determination of fenoterol **[17]**. For radioimmunoassay, the antisera is diluted to a concentration where it binds with 50% of the selected concentration of radiolabelled antigen. This situation

represents the optimum for competition when the standards or sample extracts are introduced into the RIA procedure.

The first RIA procedure, prepared for determination of clenbuterol residues (in the context of illegal usage) was developed by Delahaut *et al.* **[18]**. As in other immunoassay procedures (both RIA and EIA), the immunogen was prepared by the method of Yamamoto and Iwata **[19]**. The antiserum was specific for clenbuterol with lower cross-reactivity to salbutamol, terbutaline and cimaterol (< 10% relative to clenbuterol). Loo and co-workers developed a RIA procedure, using a radiolabelled [^{3}H]-salbutamol antigen, specific only for salbutamol (but not salbutamol conjugates) in plasma extracts **[20]**. Adam *et al.* also reported a RIA procedure for salbutamol, but using a monoclonal antibody **[21]**. Interestingly, the antibody showed a high cross-reactivity to clenbuterol (75%). Rominger *et al.* used an RIA procedure which had an antibody cross-reacting with the different sterioisomers of fenoterol; this compound was detected in biological fluid extracts at the sub ppb level **[17]**.

Enzyme-linked immunosorbent assay (ELISA) provides an alternative to the RIA procedure and has become the more favoured technique in the field of residue analysis. There are advantages in using ELISA over RIA; the availability of cheap and commonly available equipment and the absence of radiolabelled compounds and scintillation solvents. Figure 3.4 shows different types of ELISA's applied in residue analysis. The choice of enzyme and its

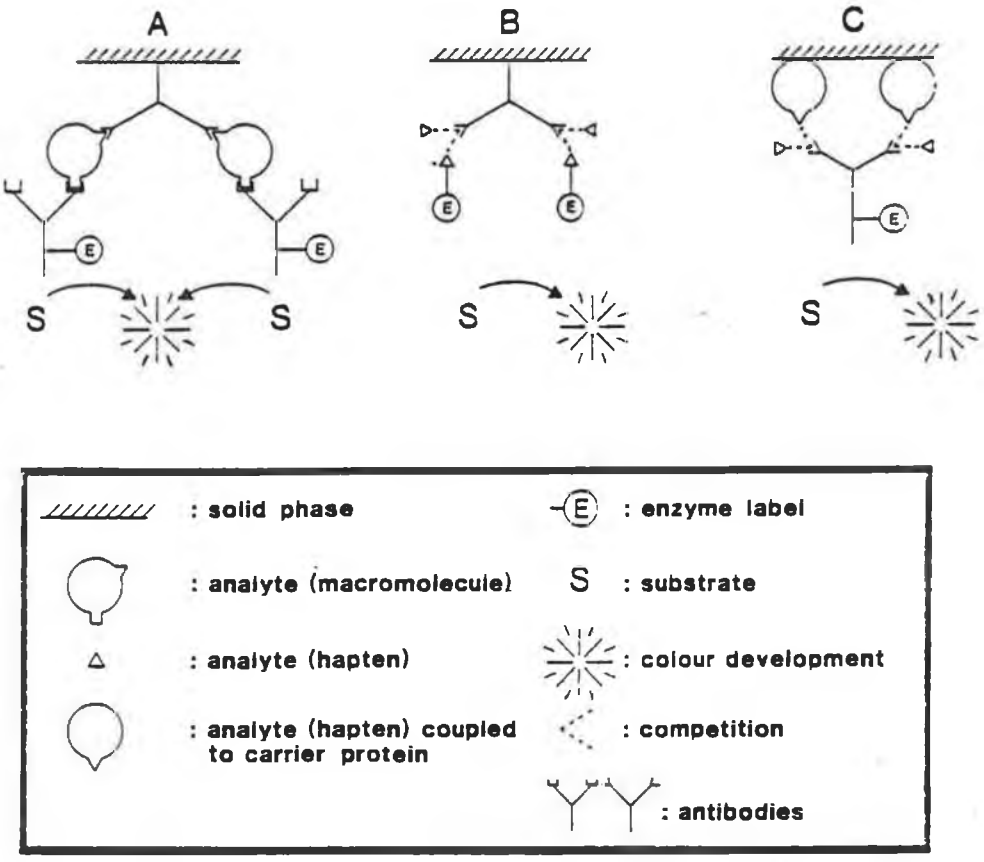


Figure 3.4 Schematic presentation of different ELISA systems applied in food/residue analysis: A: sandwich ELISA, B: competetive ELISA using enzyme-labelled analyte, C: competetive enzyme-labelled antibody.

specific activity are primary considerations in relation to assay sensitivity as it will determine the strength of the signal at the assay end-point. Typical enzymes used are β-galactosidase, alkaline phosphatase and peroxidase. The enzyme is conjugated to either the antigen or antibody through a lysine amino group of the enzyme or, where glycoprotein enzymes such as peroxidase are used, through carbohydrate groups.

The first EIA for clenbuterol was developed by Yamamoto and Iwata **[19]**, a double-antibody and heterogenous immunoassay based on competition for binding between clenbuterol and its β-D-galactosidase-labelled analogue for a limited amount of antibody, followed by binding of the enzyme-labelled analogue and a second antibody. The activity of the bound enzyme was determined fluorometrically after addition of substrate. Degand *et al.* used an EIA procedure with a clenbuterol-horseradish peroxidase (HPO) enzyme conjugate to measure clenbuterol in bovine tissues at less than 1 ppb **[22]**. EIA kits containing salbutamol-based enzyme conjugates, salbutamol-4-carboxymethylether-HPO **[23]** or salbutamol hemisuccinate-HPO **[24, 25]** were used to determine clenbuterol in urine and tissue samples. Multi-β-agonist EIA kits containing antiserum directed against a number of compounds have also been developed **[26, 27]**. A more rapid approach to β-agonist testing may be achieved using an "on-site" test strip enzyme immunoassay. The antibodies were prepared as previously described **[19]** and the clenbuterol conjugate was clenbuterol horseradish peroxidase. Determination of clenbuterol in urine

samples down to 5 ng/ml, was achieved by the visual appearance of a blue colour on the strip **[28]**. In this following study, a competitive double antibody ELISA was used to determine clenbuterol after MSPD extraction from liver. The ELISA kit contained an anti-immunoglobulin (IgG) antibody, immobilised on the surface of the microtiter wells. When the anti-clenbuterol antibody, the clenbuterol standard (or sample extracts) and the clenbuterol enzyme substrate were added to the wells, two mechanisms occurred; competition between the clenbuterol and enzyme-labelled clenbuterol for binding to the anti-clenbuterol antibody and binding of this antibody by the immobilised anti-IgG antibody. After washing, to remove unbound material, a substrate was added to produce a coloured product which was measured spectrometrically.

For the work carried out in this study, both RIA and EIA (commercially available as kits) were employed to detect clenbuterol in liver extracts produced by MSPD. Both techniques report determination limits of less than 1 ng clenbuterol per gram of liver.

3.2 EXPERIMENTAL

3.2.1 Reagents and Equipment

Hexane ('extra pure' grade) and ethanol (absolute) from Merck (Darmstadt, Germany), dichloromethane and methanol ('Hypersolv' grade) from BDH (Poole, UK) and double-distilled water were used. Other chemicals used were Analar grade from BDH, or equivalent. Clenbuterol hydrochloride from Sigma (St. Louis, MO, USA) was used for fortification of samples.

Immunoassay kits used for determination of clenbuterol were the following: (a) Radioimmunoassay kit, supplied by Laboratoire d'Hormonologie (Marloie, Belgium) was used for determination of clenbuterol. The [^{3}H]-clenbuterol in this kit, with a specific activity of 13 Ci/mmol (481 GBq/mmol), was used for method optimisation. The clenbuterol antiserum in the kit has principal cross-reactivities (relative to 100% for clenbuterol) of 9% (terbutaline), 7.7% (salbutamol, free base), 6.8% (salbutamol, sulphate salt), 2.75% (cimaterol) and 0.11% (pirbuterol). (b) Enzyme-immunoassay kit supplied by R-Biopharm (Germany) with antiserum which had been raised against clenbuterol and had principal cross-reactivities relative to 100% for clenbuterol of 71% (mabuterol), 11% (salbutamol), 6% (terbutaline) and 6% (cimaterol).

For the radioimmunoassay 0.01 M phosphate buffer (pH 7.0), containing 1 g/l gelatin and 0.1 g/l thiomersal, was used. Dextran-coated charcoal was prepared by adding 2 g activated charcoal (Sigma) and 0.25 g dextran T70 (Pharmacia, Uppsala, Sweden) to 500 ml double-distilled water. The scintillation cocktail was Cocktail T (BDH). Preparative grade Bondesil C_{18} (40 μm) packing material, supplied by Analytichem International (Harbor City, CA, USA) was used for the extraction procedure. This material was prepared by placing it in a plastic syringe barrel (50 ml) and washing sequentially with two column volumes each of hexane, dichloromethane and methanol and drying by vacuum aspiration **[6]**. Extraction columns were prepared in prewashed plastic syringe barrels (10 ml) and qualitative filter paper discs (No. 1, Whatman, Maidstone, UK) were used as frits.

3.2.2 Apparatus

A LKB Wallac 1219 Rackbeta liquid scintillation counter was used to count the radioactivity present in extracts from the MSPD procedure. Absorbance measurements on samples determined by EIA were carried out using a Dynatech Minireader with a 410 nm filter.

3.2.3 Methods

3.2.3.1 Samples and Sample Preparation

All samples of bovine liver were stored frozen, until assay. Liver samples from animals certified as not treated with clenbuterol were homogenised for use in fortification studies and for production of liver extract to be used in the standard curve.

3.2.3.2 Fortification

The method was optimised by extraction of samples fortified with 193 Bq [^{3}H]-clenbuterol per 0.5 g tissue. The method was validated by analysis of samples fortified with clenbuterol; levels of 0.5, 1.0 and 2.5 ng clenbuterol in 10 μl ethanol, were added to 0.5 g tissue prior to extraction. Fortification was carried out by pipetting the material on to the surface of the sample and leaving for 10 minutes prior to extraction.

3.2.3.3 Extraction

0.5 g liver was weighed into a glass mortar and 10 μl ethanol or an appropriate clenbuterol solution added. 2 g of the C_{18} packing material was added to the mortar and blended with the tissue by mixing with a pestle for 40 seconds. The mixture was removed from the mortar and transferred to a 10 ml syringe barrel containing two filter paper discs and with a 100 μl pipette tip attached to its outlet. The syringe barrel was tapped to settle the mixture and two filter paper discs were placed on top of the mixture. The mixture was compressed to a volume of 4.5 ml with a syringe plunger (from which the rubber seal and pointed plastic retainer had been removed). The column was washed with 8 ml of hexane and 8 ml of double-distilled water. After each wash solvent had flowed through the column, positive pressure was applied to the top of the syringe barrel with a pipette bulb to remove surplus solvent. 8 ml of methanol was then added to the column, the first 1 ml of eluate (mainly water) was discarded and the remaining eluate collected. This eluate was evaporated to dryness under a stream of nitrogen, at 55°C on a sample concentrator, and redissolved in 0.5 ml of ethanol (for RIA procedure) or 1.0 ml of water (for EIA procedure).

3.2.3.4 Radioimmunoassay procedure

Standard curves (10 to 1000 pg clenbuterol per tube) were prepared both with and without tissue extract. The extract-containing curve was prepared by the addition of 0.1 ml of a pooled extract of control tissue samples to each standard tube. 0.1 ml of sample extracts were assayed, in duplicate. The contents of all tubes were evaporated to dryness under a stream of nitrogen at 40°C and 0.5 ml of phosphate gelatin buffer was added. After vortexing and incubation for 15 minutes at 37°C, 0.1 ml [^{3}H]-clenbuterol and 0.1 ml antiserum were added. The tubes were incubated at 37°C for 15 minutes and overnight at 4°C. 0.5 ml of dextran-coated charcoal suspension was added to separate bound from free clenbuterol and the tubes were centrifuged at 2000 rpm for 10 minutes. The supernatant was decanted into scintillation vials, 10 ml of scintillation cocktail was added and the radioactivity counted.

3.2.3.5. Enzyme immunoassay

The following solutions were added to the wells of the microtiter plate; 0.1 ml of enzyme conjugate, 0.020 ml of standard clenbuterol (0-162 ng/ well) or sample extract and 0.1 ml of antiserum. The microtiter plate was subsequently vortexed and incubated overnight at 5°C. After emptying and

washing the wells, 0.050 ml aliquots of substrate and chromogen were added and the microtiter plate incubated at room temperature for 30 minutes. Finally, 0.1 ml of stop solution (1.0 M sulphuric acid) was added and the absorbance measured using a 410 nm filter.

3.3 RESULTS AND DISCUSSION

3.3.1 Method optimisation

A range of solvents of differing polarities were evaluated for their capacity to elute [^{3}H]-clenbuterol from the MSPD column. 8 ml volumes of each solvent were used as such a volume has been found suitable in previous studies with the MSPD technique **[5, 7]**. The [^{3}H]-clenbuterol was not removed from the column by non-polar solvents, such as hexane, and was only partially removed by solvents of intermediate polarity, such as dichloromethane and diethyl ether. Polar solvents, such as methanol, were found to give almost complete recovery of added [^{3}H]-clenbuterol. The [^{3}H]-clenbuterol was not removed from the column with water. Figure 3.5 shows elution profiles for [^{3}H]-clenbuterol from the columns, obtained by counting 0.5 ml fractions of the methanol eluate. Most of the [^{3}H]-clenbuterol is recovered in the second to fourth ml of the methanol eluate and the first ml,

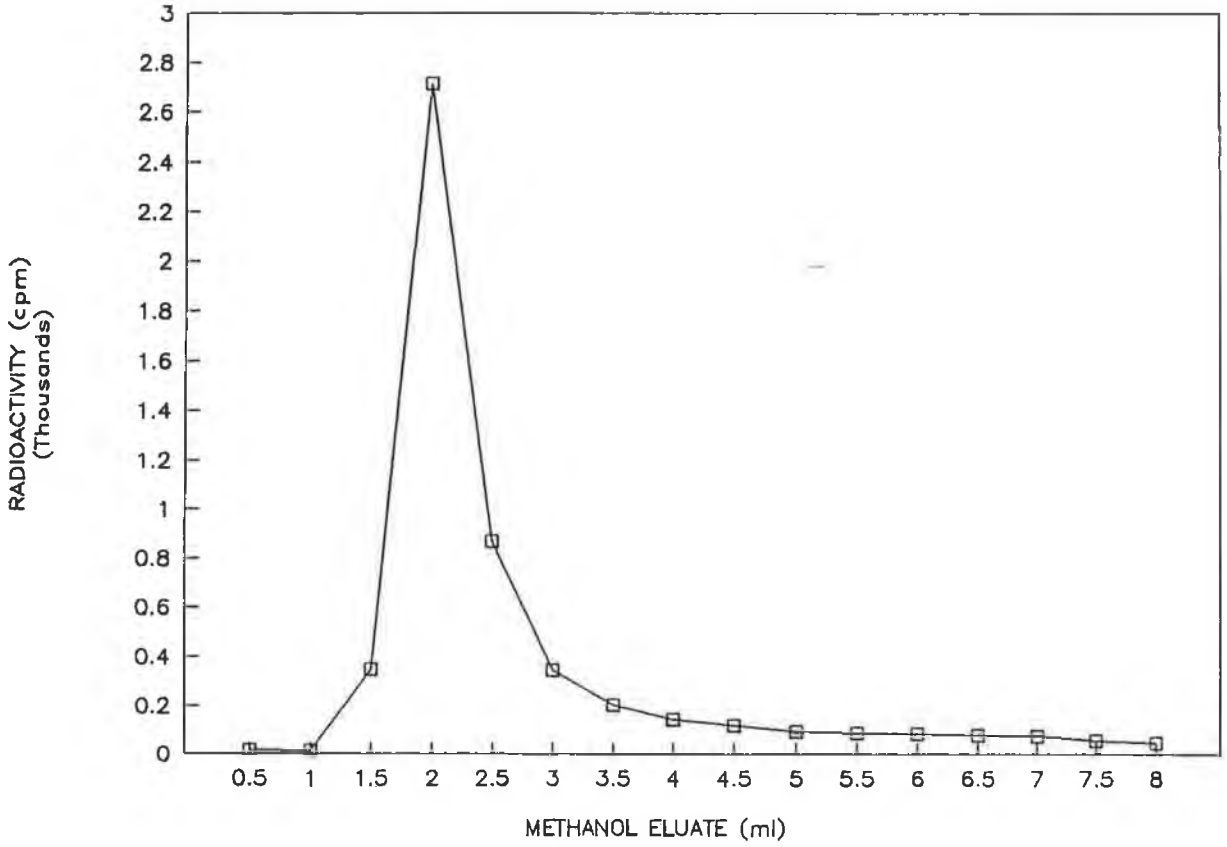


Figure 3.5 Elution of tritiated clenbuterol from the MSPD column.

which is mostly water retained from the aqueous wash step, can be discarded. A suitable clean-up and extraction procedure was the following: the column was washed with 8 ml hexane (to remove non-polar interferences) and 8 ml water (to remove polar interferences) prior to elution with 8 ml methanol. Using these conditions, recovery of [^{3}H]-clenbuterol in the methanol fraction was 101.9% ($\pm$ 6.0%, n = 6).

3.3.2 Method validation by radioimmunoassay

Standard curves for radioimmunoassay were prepared both with and without tissue extract. For the extract-containing curves, sufficient 0.5 g aliquots of a control liver sample were extracted by the MSPD procedure to give a pool of liver extract for the curve. 0.1 g equivalent of control sample extract was added to each standard tube. Figure 3.6 shows the standard curves (calculated from 8 separate assays) prepared with and without tissue extract. Good parallelism between the curves was found, indicating that a curve without tissue extract may be used. The value for the control liver extract in standard curves without extract was 23.6 pg/tube ($\pm$ 3.4 pg, n = 4).

The inter- and intra-assay variations of the method are shown in Tables 3.1 and 3.2, respectively. Results are presented for radioimmunoassay standard curves both with and without tissue extract. Good recovery of

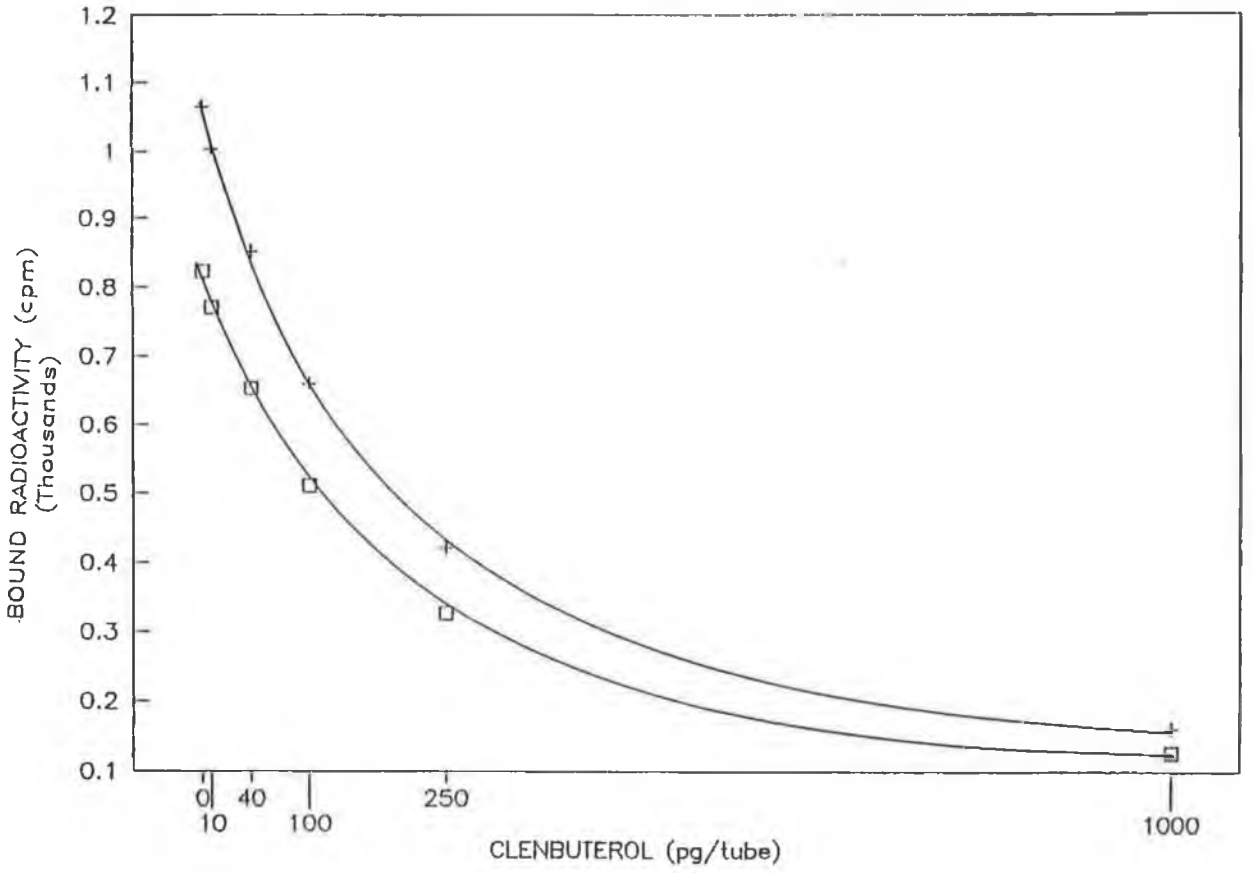


Figure 3.6 Radioimmunoassay standard curves; □ = with extract; + = without extract.

Clenbuterol added (ng/g)	n	Clenbuterol determined (ng/g)			
		(a)		(b)	
		mean ± SD	CV (%)	mean ± SD	CV (%)
1	6	0.98 ± 0.12	12.3	0.93 ± 0.18	19.3
2	6	1.94 ± 0.26	13.2	1.71 ± 0.32	18.8
5	6	4.99 ± 0.84	16.9	3.70 ± 0.60	16.1

Table 3.1 Inter-assay variation for MSPD/RIA determination of clenbuterol in liver; results are calculated on standard curves with (a) and without (b) tissue extract.

Clenbuterol added (ng/g)	n	Clenbuterol determined (ng/g)			
		(a)		(b)	
		mean ± SD	CV (%)	mean ± SD	CV (%)
1	6	1.16 ± 0.07	5.9	0.91 ± 0.05	4.9
5	6	4.69 ± 0.60	12.7	3.88 ± 0.35	8.9

Table 3.2 Intra-assay variation for MSPD/RIA determination of clenbuterol in liver; results are calculated on standard curves with (a) and without (b) tissue extract.

clenbuterol (97%-99%) was achieved for extracts measured against the standard curve containing extract (Table 3.1). For extracts measured against an external standard curve, good recovery of residue was obtained for 1 ng/g (93%) and 2 ng/g (86%), but lower recovery was determined for the 5 ng/g (74%) level. This apparent low recovery is due to inaccuracy of the radioimmunoassay rather than to losses in the extraction procedure; a relatively high non-specific binding (NSB) value occurs in extracts prepared by the MSPD procedure and its negative influence on quantitation increases with concentration of analyte in the extract. The intra-assay results (Table 3.2) show acceptable variation within a single assay for samples fortified at 1 and 5 ng/g.

Overall, high recovery of clenbuterol is obtained from the MSPD column and may be determined in a standard curve prepared with or without tissue extract addition. Use of a standard curve without tissue extract is normally preferred due to the decreased number of tissue samples required to be processed by MSPD and, hence, the reduced time and cost of the assay.

3.3.3 Method validation by enzyme immunoassay

Fortified sample extracts containing 1, 2 and 5 ng/g clenbuterol were determined by EIA and results are shown in Table 3.3. The clenbuterol content in the extracts was measured using a clenbuterol standard curve

without tissue extract and, overall, the estimation of clenbuterol recovery (84%-96%) was higher than that obtained by an equivalent RIA measurement (74%-93% , see Table 3.1).

3.3.4 Sample analyses

Table 3.4 shows the results for analysis of samples containing incurred clenbuterol. The levels determined by the MSPD procedure, using standard curves with and without tissue extract, are compared with results obtained by a solvent extraction method **[29]**. This method consists of protease digestion of the sample (55°C for 2 h), multiple extractions with diethyl ether and determination of the clenbuterol in the extract by radioimmunoassay. Good comparison between the two methods was found. The coefficient of variation (CV) for analysis of residue-positive samples by MSPD/RIA was found to be 10-11% (Table 3.4); This compares well with the solvent extraction method for which a CV value of 17.4% was obtained for a quality control sample (mean = 0.33 ng/g, SD = 0.058, n = 6).

3.3.5 Limit of detection

A tentative limit of detection for the MSPD procedure was determined by RIA analysis of liver samples free of clenbuterol residue. The mean value

Clenbuterol added (ng/g)	n	Clenbuterol determined (ng/g)	
		Mean ± SD	CV (%)
1	4	0.96 ± 0.23	24.0
2	4	1.96 ± 0.20	10.2
5	4	4.20 ± 0.57	13.6

Table 3.3: Inter-assay variation for MSPD/EIA determination of clenbuterol in liver.

Sample	Clenbuterol determined (ng/g)			
	Standard method	MSPD/RIA (n = 6)		MSPD/EIA (n = 4)
		(a) Mean ± SD	(b) Mean ± SD	Mean ± SD
A	3.60	3.61 ± 0.35	3.07 ± 0.34	3.20 ± 0.57
B	1.62	1.84 ± 0.21	1.84 ± 0.19	1.90 ± 0.68

Table 3.4 Clenbuterol levels determined in residue-positive liver samples by MSPD/ RIA and MSPD/EIA, compared with a standard technique; results from MSPD/RIA are calculated on standard curves with (a) and without (b) tissue extract.

for these samples was 0.18 ng/g (± 0.04 ng/g, n = 8), determined in a standard curve without tissue extract; the limit of detection, calculated as the mean plus 3 times the standard deviation, was 0.30 ng/g. Assay of a much larger number of clenbuterol-free samples (for example n = 50 **[30]**) would be required to give a robust limit of detection for the method.

3.4 CONCLUSIONS

The MSPD technique has been developed successfully for application to the analysis of clenbuterol in liver samples. Satisfactory results were obtained when the determination of residue levels was by radioimmunoassay (either with or without tissue extract) and enzyme immunoassay. In the former case, the use of a standard curve without tissue extract is preferred because of the reduced time and cost of the assay. Recovery of clenbuterol from fortified samples was greater than 70% (in most cases around 90%) and results for samples containing incurred residues compared well with a solvent extraction method **[26]**. The developed technique offers a simple, rapid procedure for assay of clenbuterol in tissue samples. The MSPD technique can be applied to assay of residues at levels of 0.5 ppb, or lower, and suitable extracts are produced from the technique for determination by immunoassay.

The MSPD method eliminates many of the problems associated with classical isolation techniques. The method uses small sample sizes (0.5 g), has a minimal number of steps, requires no chemical manipulations (such as pH adjustments) and requires low solvent usage (16 ml). Conversely, a typical solvent extraction method used requires 5.0 g of sample, homogenisation of the sample, protease digestion, a minimum of 30 ml of diethyl ether, pH adjustments and multiple extractions. For a typical analytical run of 10 to 20 samples, the MSPD procedure takes slightly more

than half the operator time required for a solvent extraction procedure.

The inclusion of incurred liver samples in the study provides a better validation of the MSPD method. The bulk of the previously reported MSPD methods do not include such data, due mainly to the time and expense involved in treating animals. The drawback of reporting methods based only on fortified samples is that no account is taken of the form in which the residue may be present in the tissue matrix. Hence there may be a different degree of extraction between fortified and incurred samples. In the described MSPD method for clenbuterol the combination of high analyte recovery and good comparability with an alternative procedure for incurred samples, suggests that both residue types (i.e. fortified and incurred) are extracted .

3.4. REFERENCES

1. H.H.D. Meyer and L. Rinke, in Proceedings of the 51st Easter School in Agricultural Science: *The Control of Fat and Lean Deposition*, University of Nottingham, April 1991, p. 61.
2. S.A. Barker, L. Hsieh and C.R. Short, *Anal. Biochem.*, **155**, 1986, 112.
3. F.J. Schenk, S.A. Barker and A.R. Long, *J. Assoc. Off. Anal. Chem.*, **75**, 1992, 655.
4. A.R. Long, L.C. Hsieh, A.C. Bello, M.S. Malbrough, C.R. Short and S.A. Barker, *J. Agric. Food Chem.*, **38**, 1990, 427.
5. S.A. Barker, A.R. Long and C.R. Short, *J. Chromatogr.*, **475**, 1989, 353.
6. A.R. Long, M.M. Soliman and S.A. Barker, *J. Assoc. Off. Anal. Chem.*, **74**, 1991, 493.
7. A.R. Long, L.C. Hsieh, M.S. Malbrough, C.R. Short and S.A. Barker, *J. Agric. Food Chem.*, **38**, 1990, 423.
8. A.R. Long, M.D. Crouch and S.A. Barker, *J. Assoc. Off. Anal. Chem.*, **74**, 1991, 667.
9. A.R. Long, L.C. Hsieh, M.S. Malbrough, C.R. Short and S.A. Barker, *J. Assoc. Off. Anal. Chem.*, **73**, 1990, 379.
10. A.R. Long, L.C. Hsieh, M.S. Malbrough, C.R. Short and S.A. Barker, *J. Assoc. Off. Anal. Chem.*, **73**, 1990, 864.

11. A.R. Long, L.C. Hsieh, M.S. Malbrough, C.R. Short and S.A. Barker, *J. Assoc. Off. Anal. Chem.*, **75**, 1989, 739.

12. A.R. Long, L. C. Hsieh, M.S. Malbrough, C.R. Short and S.A. Barker, *J. Assoc. Off. Anal. Chem.*, **72**, 1989, 813.

13. P. Shearan, M. O'Keeffe and M.R. Smyth, *Food Addit. Contam.*, **11**, 1994, 7.

14. P. Shearan and M. O' Keeffe, *Analyst* (1994), in press.

15. E. Iosifidou, P. Shearan and M. O'Keeffe, *Analyst* (1994), in press.

16. J. Rosen, K.E. Hellenas, P. Tornquist and P. Shearan, *Analyst* (1994), in press.

17. K.L. Rominger, A. Mentrup and M. Stiasni, *Arzneim.-Forsch/Drug Res.*, **40**, 1990, 887.

18. Ph. Delahaut, M. Dubois, I. Pri-bar, O. Buchman, G. Degand and F. Ectors, *Food Addit. Contam.*, **8**, 1991, 43.

19. I. Yamamoto and K. Iwata, *J. Immunoassay*, **3**, 1982, 155.

20. J.C.K. Loo, N. Beaulieu, N. Jordan, R. Brien and I.J. Mc Gilveray, *Res. Comm. Chem. Path. Pharm.*, **55**, 1987, 283.

21. A. Adam, H. Ong, D. Sondag, A. Rapaille, S. Marleau, M. Bellemare, Ph. Raymond, D. Giroux, J.K. Loo and N. Beaulieu, *J. Immunoassay*, **11**, 1990, 329.

22. G. Degand, A. Bernes-Duyckaerts and G. Maghuin-Rogister, *J. Agric. Food Chem.*, **40**, 1992, 70.

23. H.H.D. Meyer and L.M. Rinke, *J. Anim. Sci.*, **69**, 1991, 4538.

24. W. Haasnoot, A.R.M. Hamers, G.D. van Bruchem, R. Schilt and L.M.H. Frijns, in M.R.A. Morgan, C.J. Smith and P.A. Williams (eds.), *Food Safety and Quality Assurance-Applications of Immunoassay Systems*, Elsevier, London, 1992, 237.

25. W. Haasnoot, S.M. Ezkerro and H. Keukens, in Proceedings of the EuroResidue 2 Conference, The Netherlands, May 3-5, 1993, 347.

26. R. Angeletti, M. Paleologo Oriundi, R. Piro and R. Bagnati, *Anal. Chim Acta,* **275**, 1993, 215.

27. M. Paleologo-Oriundi, G. Giacomini, F. Ballaben, F. Berti, F. Benedetti, R. Bagnati and E. Bastiani, *Food Agric. Immunol.*, **4**, 1992, 73.

28. M.E. Ploum, W. Haasnoot, R.J.A. Paulussen, G.D. van Bruchem, A.R.M. Hamers, R. Schilt and F.A. Huf, *J. Chromatogr. (Biomed. Applic.)*, **564**, 1991, 413.

29. W.J. McCaughey. personnal communication.

30. C.T. Elliot, W.J. McCaughey and H.D. Shortt, *Food Addit. Contam.*, **10**, 1993, 231.

CHAPTER 4

MATRIX SOLID PHASE DISPERSION (MSPD) AS A MULTIRESIDUE EXTRACTION PROCEDURE FOR β-AGONISTS IN BOVINE LIVER WITH DETERMINATION BY IMMUNOASSAY

4.1 INTRODUCTION

The work on MSPD described in the previous chapter demonstrated the suitability of the technique for the extraction of polar compounds. Clenbuterol is not extracted from the MSPD column with non-polar solvents and is only partially removed with solvents of intermediate polarity: the compound elutes fully only in methanol. Other compounds in the β-agonist class, with similar structures and polarity characteristics to clenbuterol, would be expected to elute from the column in methanol; aniline-type compounds like mabuterol and cimaterol differ only in the type of substituents attached to positions 3 and 5 in the aromatic ring and on the substituent attached to the iso-propyl group on the aliphatic part of the molecule (cimaterol only). Cimaterol, in particular, needs to be monitored, as it performs as a very efficient repartitioning agent in a similar way to clenbuterol **[1]**. Salbutamol has also been administered illegally to animals; data from Furst *et al.* indicates widespread use of this substance as a growth enhancing agent in beef production **[2]**. Moreover, because it has a shorter half-life than the aniline-type β-agonists, it would be more difficult to detect. Since the above mentioned compounds, as well as new related compounds, are frequently detected and identified as growth enhancing agents, it appears necessary to move away from monitoring individual compounds and develope, as far as possible, multi-residue analysis methods.

Because β-agonists as a class contain substances of differing chemical properties, variable recoveries from multiresidue methods are usually reported. Optimisation of the residue extraction procedure for clenbuterol-like β-agonists can result in a reduced recovery for salbutamol-like substances **[3-5]**. In the paper by Leyssens *et al.* **[3]**, using a combination of sample clean-up techniques for liver, reasonable recoveries of mabuterol and clenbuterol were achieved (85% and 60% respectively), but the recovery of salbutamol, terbutaline and fenoterol was poor (< 35%). The method reported by van Ginkel *et al.* **[4]**, which uses adsorption column solid-phase extraction (SPE) and immunoaffinity chromatography, gives rise to better salbutamol recovery (40-50%), but overall it was still low compared to the aniline-type compounds (60-70%). A recent paper by Montrade *et al.* which adopted a "mixed phase" SPE clean-up of urine samples gave reasonable extractability of thirteen β-agonists, including some phenolic-type compounds (e.g. 65% for ractopamine and 59% for fenoterol), but again for salbutamol, metaproterenol, and related substances poor recoveries were obtained (< 35%)**[5]**.

Matrix solid phase dispersion (MSPD) provides an alternative extraction methodology for a multi-residue analytical approach to analysis. In this extraction method sample matrix components are dispersed over a large surface area and, simultaneouly, lipid materials and internal membrane lipids associate with the C_{18} material to essentially unfold the structural components. This process allows the more polar compounds (including drug components)

to extend outwards and make them more extractable into organic solvents. The majority of MSPD publications report multiresidue extraction procedures, mostly of veterinary drugs and pesticides. Components of different classes are fractionated from the MSPD column with different solvents depending on their polarity. A paper by Barker and co-workers **[6]** demonstrates this versatility: residues of pesticides, anthelmintics and antibiotics were fractionated from the MSPD column (containing bovine muscle-C_{18} blend) with hexane/benzene, ethyl acetate and methanol, respectively. Recovery of the components, analysed by high performance liquid chromatography, was in most cases greater than 70%. For the extension of MSPD methodology to β-agonist determinations, the water wash step used for clenbuterol extraction (see Chapter 3) was liable to remove hydrophilic compounds, for example salbutamol. This proved to be the case for certain brands of C_{18} material. An alternative option was the linkage of MSPD and SPE, thereby avoiding the necessity for a water wash step. Such an approach was also adopted by Schenk *et al.* for the isolation of ivermectin in muscle tissue samples **[7]**. This chapter examines both options (with and without a water wash step), with the ultimate aim of developing a robust and sensitive procedure with good extraction capability for a variety of β-agonists in liver tissue.

Procedures which aim to determine phenolic-type β-agonists must provide an additional hydrolysis step to deconjugate residues present in biological samples. Compounds with a catechol structure, like rimiterol and

isoprenaline, are metabolised by an O-methylation reaction which occurs at positions 3 and 4 on the aromatic ring (see Figure 4.1). Compounds such as salbutamol, with a phenolic ring structure, are usually metabolised to sulphate (and to a lesser extent, glucuronide) conjugates. The resorcinol β-agonists, like terbutaline and fenoterol, are also conjugated in this way, but in the case of compounds such as clenbuterol and mabuterol sulphate conjugation cannot take place. The effect of such conjugation means that the compounds have very short half-lives (e.g. 0.02 h for rimiterol in man) and hence are eliminated rapidly from the body. For the use of β-agonists as repartitioning agents, drugs with a long half-life are more practical as the active compound persists longer in the treated animal. This may explain why compounds like clenbuterol (with a half-life of 7 h in man) and cimaterol are often chosen as growth enhancing substances in preference to salbutamol, terbutaline etc. However there does not always seem to be a direct relationship between duration of the effect and the half-life of the drug **[8]**.

Conjugated residues pose particular problems in residue analysis: (a) the conjugates may not extract as well as the free compounds due to their increased polarity; and (b) the antiserum used in the immunoassay which is directed against the free compound may have no (or a lower) cross-reactivity to the conjugate. Hydrolysis studies have been carried out with aniline-type β-agonists to confirm that these compounds do not occur as conjugates. For clenbuterol-incurred urine samples, the amount of drug found following

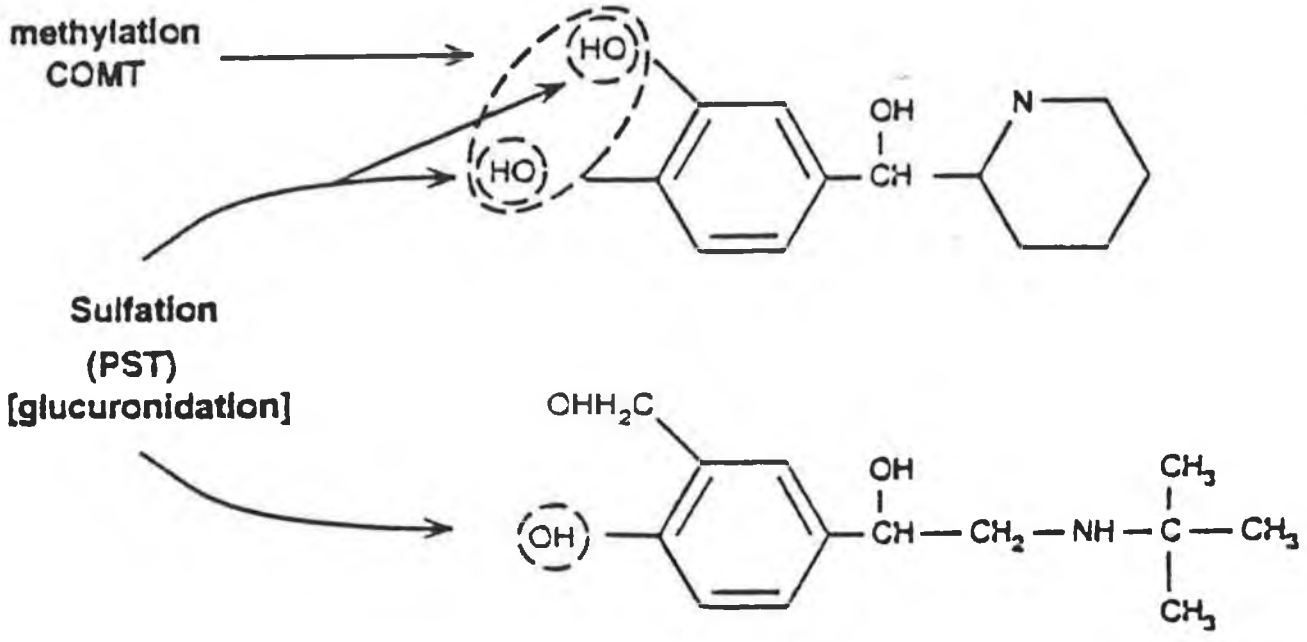


Figure 4.1 Main metabolic pathways for (a) catechol (rimiterol) and (b) resorcinol (salbutamol) β-agonists

hydrolysis was not significantly different to that determined without hydrolysis. Clenbuterol is excreted mainly as the parent compound (24%), mandelic acid metabolite (20%) and the hippuric acid metabolite (19%) and O- and N- glucuronide formation accounted for approximately 5% of the excreted drug **[9, 10]**. Similar results were reported for the structurally-similar compound, mabuterol **[11]**.

For the phenolic-type β-agonists, conjugate formation has been reported. Two main types of hydrolysis procedures, namely enzyme and acid hydrolysis, have been used prior to sample clean-up. Enzyme hydrolysis of salbutamol conjugates was carried out in urine and liver homogenate; the samples were adjusted to pH 5.0 and incubated with a dilute solution of enzyme (glucuronidase/ sulphatase) at 37°C for 16-18 h **[4, 5, 12]**. The method of Montrade *et al.* **[12]** is particularly informative in this regard, as the authors carried out hydrolysis studies on urine and a number of bovine tissues (e.g. liver, kidney, lung and brain). Their results showed that the sulphate conjugate occurs to a greater extent than the glucuronide conjugate. Interestingly, no glucuronide conjugation was found in the heart tissue.

Other authors maintain that glucuronides are present in the higher percentage (and hence only use glucuronidase for hydrolysis purposes) **[13, 14]**. The best compromise is to use a combination of both types of enzyme (e.g. as contained in suc d' helix pomatia). An EIA procedure for β-agonists has been described by Degand *et al.* **[15]** which by-passes both sample clean-

up and enzyme hydrolysis steps; the urine samples were simply diluted to reduce the effects of interferences, and the antibody in the kit, directed against the aliphatic part of the molecule, did not distinguish between free and conjugated forms of salbutamol. A method by Howells *et al.* for salbutamol in liver did not use a hydrolysis procedure, and hence the results of analysis of incurred samples (by EIA) were probably underestimated by approximately 40-50% **[16]**. Acid hydrolysis procedures have also been reported; Selinger *et al.* found that the incubation time required for enzymatic hydrolysis of conjugated metaproterenol was lengthy (> 180 h) and did not lead to complete deconjugation. The alternative acid hydrolysis procedure solved both these problems; plasma supernatants were incubated with 0.2 ml 2 M hydrochloric acid at 65°C for 90 min to deconjugate the O-sulphate ester of metaproterenol **[17]**. A similar procedure was adopted for the hydrolysis of isoproterenol sulphate **[18]**.

This study describes the development of a multi-β-agonist extraction procedure using MSPD. Extracts from the procedure were incubated at optimum hydrolysis conditions and the determination of the individual β-agonists was carried out by immunoassay.

4.2 EXPERIMENTAL

4.2.1 Reagents and Equipment

Hexane and ethanol ('Extra Pure' grade) from Merck (Darmstadt, Germany), dichloromethane and methanol (both 'Hypersolv' grade) and diethyl ether from BDH (Poole, UK) and double-distilled water were used. Other chemicals used were Analar grade from BDH, or equivalent. Suc d'helix pomatia (containing 100,000 units β-glucuronidase and 1,000,000 units of sulphatase per ml) was supplied by Sepracor (Villeneuve la Garenne, France). Clenbuterol hydrochloride, salbutamol and terbutaline were obtained from Sigma (St. Louis, MO, USA). Cimaterol and mabuterol were supplied by Laboratoire d'Hormonologie (Marloie, Belgium). Bond-Elut SPE C_{18} cartridges (1 ml) were supplied by Analytichem International (Harbor City, CA, USA).

Immunoassay kits used for determination of β-agonists were the following: (a) radioimmunoassay kit, supplied by Laboratoire d'Hormonologie (Marloie, Belgium), with antiserum which had been raised against salbutamol and had principal cross-reactivities relative to salbutamol (100%) of 118% to clenbuterol, 78% to mabuterol, 29% to terbutaline and 11.6% to cimaterol and (b) enzyme immunoassay kit supplied by R-Biopharm (Germany), with antiserum which had been raised against clenbuterol and had

principal cross-reactivities relative to clenbuterol (100%) of 71% to mabuterol, 11% to salbutamol, 10% to terbutaline and 6% to cimaterol.

For the radioimmunoassay, 0.01 M phosphate buffer (pH 7.0), containing 1 g/l gelatin and 0.1 g/l thiomersal, was used. Dextran-coated charcoal was prepared by adding 2 g activated charcoal (Sigma) and 0.25 g dextran T70 (Pharmacia, Uppsala, Sweden) to 500 ml double-distilled water. The scintillation cocktail was Cocktail T (BDH). Preparative grade Sepralyte C_{18} (40 μm, irregular size) and Isolute C_{18} (50 μm) packing materials were supplied by Jones Chromatography (Mid-Glamorgan, UK). Preparative grade Bondesil C_{18} (40 μm) material was supplied by Analytichem (Harbor City, CA). The C_{18} material was prepared by placing it in a plastic syringe barrel (10 ml) and washing sequentially with two column volumes each of hexane, dichloromethane and methanol and drying by vacuum aspiration. Extraction columns were prewashed plastic syringe barrels (10 ml) and qualitative filter paper discs (No. 1, Whatman, Maidstone, UK) were used as frits.

4.2.2 Apparatus

A LKB Wallac 1219 Rackbeta Liquid Scintillation counter was used to count the radioactivity present in extracts from the MSPD procedure. Absorbance measurements on samples determined by EIA were carried out using a Dynatech Minireader with a 410 nm filter.

4.2.3 Methods

4.2.3.1 Samples

All samples of bovine liver were stored frozen until analysis. Liver samples from animals certified as not treated with β-agonists were used in fortification studies. Liver samples from salbutamol-treated animals were provided by the Central Veterinary Laboratory, Weybridge, UK and were used for studies on determination of conjugated residues.

4.2.3.2 Fortification

The method was validated by analysis of samples fortified with salbutamol; levels of 0, 0.5, 1.0 and 2.5 ng of salbutamol, in 10 μl ethanol, were added to 0.5 g tissue. Fortification was carried out by pipetting the material on to the tissue and leaving for 10 minutes prior to extraction.

4.2.3.3 MSPD Extraction

0.5 g liver was weighed into a glass mortar and 2 g of C_{18} packing material was added to the mortar and blended with the tissue, using a glass pestle, for 40 seconds. The mixture was removed from the mortar and

transferred to a 10 ml syringe barrel containing two filter paper discs and with a 100 μl pipette tip attached to its outlet. The syringe barrel was tapped to settle the mixture and two filter paper discs were placed at the head of the column. The mixture was compressed to a volume of 4.5 ml with a syringe plunger (from which the rubber seal and pointed plastic retainer had been removed). Two types of elution procedures were adopted; either: (a) water wash clean-up prior to methanol elution; or (b) no water wash, but further clean-up by C_{18} solid-phase extraction.

For (a), the column was washed with 8 ml hexane and 4 ml double-distilled water. After each wash solvent had flowed through the column, positive pressure was applied with a pipette bulb to remove surplus solvent. 9 ml of methanol was added to the column, the first 1 ml of eluate (mainly water) was discarded and the remaining eluate collected. For (b), the column was washed with 8 ml hexane: diethyl ether (6: 4), after which the analytes of interest were eluted with 12 ml of methanol and this extract was vortexed and centrifuged (2000 rpm, 10 min) to remove precipitated proteins.

The methanolic extracts produced from (a) and (b) were both evaporated to dryness under a stream of nitrogen at 60°C on a sample concentrator and subjected to enzyme hydrolysis

4.2.3.4 Enzyme Hydrolysis

Dried extracts from the MSPD procedure were redissolved in 0.4 ml phosphate-gelatin buffer (pH 5.0), 0.1 ml of suc d'helix pomatia solution (diluted to an appropriate concentration with buffer) was added, and the solution vortexed for 60 seconds. A study was carried out to determine the optimum and most practical hydrolysis conditions for salbutamol residues in incurred samples. Extracts produced from the MSPD column were incubated at 37°C for periods of 2 or 16 h and with enzyme concentrations of 500 U glucuronidase/5000 U sulphatase or 2500 U glucuronidase/25,000 U sulphatase. The tubes were cooled to room temperature prior to SPE clean-up (MSPD procedure (a)) or direct analysis (MSPD procedure (b)).

4.2.3.5 C_{18} SPE procedure

C_{18} cartridges were solvated with 2 ml methanol and conditioned with 2 ml water. The enzyme-treated MSPD extract, to which was added 0.5 ml water and 0.08 ml sodium hydroxide (1 M), was added to the column and allowed to flow under gravity. The column was washed twice with 1 ml water and dried thoroughly under vacuum. The column was eluted with 2 ml methanol, the extract evaporated under nitrogen at 60°C and redissolved in 0.5 ml phosphate-gelatin buffer, pH 7.0.

4.2.3.6 Enzyme immunoassay

The clenbuterol standard supplied with the EIA kit (and used over the range of 0-162 pg/well) was replaced by salbutamol, used at 0-1000 pg/well to allow for the relatively low cross-reactivity for salbutamol. The following solutions were added to the wells; 0.1 ml of enzyme conjugate, 0.02 ml of standard salbutamol or prepared sample and 0.1 ml of antiserum. The microtiter plate was shaken and incubated overnight at 5°C. After emptying and washing the wells, 0.05 ml aliquots of substrate and chromogen were added and the microtiter plate incubated at room temperature for 30 min. Finally, 0.1 ml of stop solution (1.0 M sulphuric acid) was added and the absorbance measured.

4.2.3.7 Radioimmunoassay

0.1 ml aliquots of ethanolic standards (standard curve 0-1000 pg salbutamol/tube) were added to culture tubes, evaporated to dryness under a stream of nitrogen and redissolved in 0.5 ml phosphate gelatin buffer (pH 7.0). 0.1 ml of sample extracts were added to 0.4 ml of phosphate gelatin buffer. After vortexing and incubating for 15 minutes at 37°C, 0.1 ml [^{3}H]-clenbuterol and 0.1 ml antiserum were added. The tubes were incubated at 37°C for 15 minutes and overnight at 4°C. 0.5 ml of dextran-coated charcoal

suspension was added and the tubes centrifuged at 2000 rpm for 10 minutes. The supernatant was decanted into scintillation vials, 10 ml of scintillation cocktail added and the radioactivity counted for 5 minutes.

4.3 RESULTS AND DISCUSSION

4.3.1 Direct MSPD using a water wash clean-up step

4.3.1.1 Method Optimisation

The MSPD procedure used for clenbuterol analysis (see Chapter 3) required a water wash (8 ml) to remove polar interferences. However, because of the hydrophilic nature of salbutamol, losses could be expected with 8 ml water; hence lower volumes were evaluated. The minimal volume of water that could remove the red-coloured polar components from the MSPD column was 4 ml, and this was used in all further work.

Three different C_{18} materials were evaluated for this modified MSPD method, namely Bondesil, Isolute and Bondesil-Sepralyte C_{18} materials. Table 4.1 shows the comparison between the three C_{18} types at salbutamol fortification levels of 2 and 5 ng/g. Isolute material gave rise to relatively poor recovery of salbutamol (47-55%) followed by Bondesil (60%), but

Salbutamol added (ng/g)	Salbutamol determined (ng/g)		
	(a) Mean ± SD	(b) Mean ± SD	(c) Mean ± SD
2	1.20 ± 0.05	1.20 ± 0.15	1.59 ± 0.08
5	2.31 ± 0.60	2.70 ± 0.19	4.32 ± 0.42

Table 4.1 Direct MSPD of 2 and 5 ng/g salbutamol in liver with (a) Isolute (n = 3) (b) Bondesil (n = 5) and (c) Sepralyte (n = 4) C_{18} materials.

extraction with Sepralyte material resulted in superior recoveries (approximately 80%). This suggests that the drug attachment on the tissue C_{18} mixture is stronger for the Sepralyte material than for the other materials, and the water (4 ml) does not elute the compound from the column. For the other materials there was partial elution of salbutamol in the water wash. For this reason Sepralyte C_{18} material was chosen for all further work involving a water wash step.

4.3.1.2 Enzyme Hydrolysis

Table 4.2 shows the salbutamol levels determined in incurred liver samples extracted by the MSPD procedure and subjected to enzyme hydrolysis under various conditions. Results for analysis by RIA showed a significant increase in the level of drug determined after enzyme hydrolysis for 2 h with low enzyme concentration (500/5000 U), but there was little further increase in residue level with extended incubation and/or higher enzyme concentration (2500/25000 U). In contrast, the salbutamol levels determined by EIA decreased with enzyme hydrolysis, and the extent of the decrease was related to the length of the incubation period and the enzyme concentration. The difference in results may be explained by differences in the antisera: the antiserum in the RIA kit, raised against salbutamol-hemisuccinate-BSA, and the antiserum in the EIA kit, raised against clenbuterol-diazo-BSA, have lower

Enzyme hydrolysis		Salbutamol (ng/g)	
Glucuronidase/ sulphatase (U)	Incubation period (h)	MSPD/RIA Mean ±SD	MSPD/EIA Mean ± SD
0	0	7.5 ± 0.6	26.5 ± 6.2
500/ 5000	2	13.5 ± 1.1	19.1 ± 6.7
	16	15.0 ± 1.7	16.0 ± 6.3
2500/ 25000	2	15.9 ± 3.7	15.9 ± 5.5
	16	14.7 ± 2.2	12.6 ± 2.7

Table 4.2 Salbutamol levels determined in liver samples from treated animals after hydrolysis with glucuronidase/sulphatase at 37°C (n = 6)

and higher cross-reactivity, respectively, to conjugated forms of salbutamol compared with free salbutamol. RIA provided better reproducibility (reflected in the lower coefficients of variation obtained) and the higher cross-reactivities of this antiserum to other β-agonists make it more suitable for multi-residue determination. RIA, therefore, was the technique selected for further validation of the MSPD procedure. The final hydrolysis conditions selected were 1000 U glucuronidase/10,000 U sulphatase per 0.5 ml final extract and incubation for 2 h at 37°C. A more concentrated enzyme activity gave rise to a slightly higher free salbutamol concentration, but there was a colouration of the extract which caused a higher background signal in the immunoassay.

4.3.1.3 Method Validation

The inter- and intra-assay variations of the method are shown in Tables 4.3 and 4.4. Good recovery of residue was determined at the 1 ng/g (97%), level but lower recoveries were determined for the 2 ng/g (87%) and the 5 ng/g (65%) levels. These apparent low recoveries are due to inaccuracy of the radioimmunoassay procedure rather than to losses in the extraction procedure; a relatively high non-specific binding (NSB) value occurs in extracts prepared by the MSPD procedure and its negative influence on quantitation increases with concentration of analyte in the extract. A similar effect was observed for the assay of clenbuterol by MSPD (see Chapter 3),

Salbutamol added (ng/g)	Salbutamol determined (ng/g)	
	Mean ± SD	CV (%)
1	0.97 ± 0.09	8.8
2	1.73 ± 0.25	14.4
5	3.27 ± 0.62	18.9

Table 4.3 Inter-assay variation for MSPD/RIA determination of salbutamol in liver (n = 5); Extracts measured against a salbutamol standard curve without control tissue extract.

Salbutamol added (ng/g)	Salbutamol determined (ng/g)	
	Mean ± SD	CV (%)
1	0.89 ± 0.12	14.0
5	3.50 ± 0.60	17.0

Table 4.4 Intra-assay for MSPD/RIA determination of salbutamol in liver (n = 6).

and could be overcome by use of a standard curve containing tissue extract for the radioimmunoassay. The intra-assay results showed acceptable variation within a single assay for samples fortified at 1 and 5 ng/g (Table 4.4).

4.3.1.4 Limit of detection

A tentative limit of detection for the MSPD/RIA procedure was determined by analysis of liver samples free of salbutamol residues. The mean value for these samples (n = 8) was 0.35 ng/g ($\pm$ 0.11 ng/g, n = 8). The limit of detection, calculated as the mean + 3 times the standard deviation, was found to be 0.69 ng/g.

4.3.1.5 Incurred sample analyses

Liver samples from animals treated with salbutamol were analysed using the developed procedure. Table 4.5 shows the salbutamol levels determined in incurred samples before and after enzyme hydrolysis. The results show that at the three dose levels used with a withdrawal period selected to give residue-positive samples, 40-45% of the residue occurs in the liver in a conjugated form. These results are consistent with a salbutamol deconjugation study carried out by Montrade *et al.* **[12]**; using an extraction procedure involving "mixed phase" SPE sample clean-up on a liver

Sample	Treatment			Salbutamol determined (ng/g)	
	No.	Dose (IM, µg/kg)	Withdrawal period (h)	(a) Mean ± SD	(b) Mean ± SD
1	I	0.2	18	0.88 ± 0.05	1.54 ± 0.18
2		1.0	18	7.95 ± 0.89	13.68 ± 1.57
3		5.0	18	26.38 ± 6.50	44.40 ± 8.74
4	II	2.0[1]	168	0.23 ± 0.08	0.26 ± 0.17
5		2.0[1]	168	0.16 ± 0.08	0.29 ± 0.13

[1] Dose given twice daily for 4 days

Table 4.5 Salbutamol levels determined in liver samples from treated animals by MSPD/RIA before (a) and after (b) hydrolysis using glucuronidase/ sulphatase (n = 4).

homogenate and GC-MS analysis, these authors found that 42-50% of the salbutamol residues occur as conjugates. Table 4.5 also shows the results for analysis of liver samples of animals which had longer withdrawal periods of 7 days. No residues were detectable above the limit of determination of the method. This was to be expected as salbutamol, possessing a catechol structure, is metabolized by the COMT pathway (catechol O-methylation transferase reaction), resulting in a short half-life **[8]**.

4.3.1.6 Multiresidue analysis

The developed method was applied to the analysis of other β-agonists. The method was tested for clenbuterol, mabuterol, terbutaline and cimaterol using liver samples fortified with these drugs at 2 ng/g. Samples were fortified, also, with terbutaline and cimaterol at 10 ng/g because of their relatively low cross-reactivities to the RIA antiserum. Table 4.6 shows results for analysis of these fortified samples. Residue levels were determined, also, using clenbuterol and mabuterol standard curves. Salbutamol (or mabuterol) standard curves are most suitable for a multi-residue procedure with clenbuterol, salbutamol and mabuterol detectable at 1 ng/g or less and terbutaline detectable at about 2 ng/g. Cimaterol, because of its low cross-reactivity to the antiserum, gave a result below the limit of detection of the method for fortification level of 2 ng/g. Cimaterol would be detectable at a

β-agonist added	Amount added (ng/g)	Amount determined (ng/g)		
		Clenbuterol standard curve Mean ± SD	Salbutamol standard curve Mean ± SD	Mabuterol standard curve Mean ± SD
Clenbuterol	2	1.36 ± 0.21	2.37 ± 0.28	2.30 ± 0.10
Salbutamol	2	0.80 ± 0.11	1.33 ± 0.05	1.46 ± 0.10
Mabuterol	2	0.98 ± 0.23	1.66 ± 0.28	1.73 ± 0.14
Terbutaline	2	0.38 ± 0.07	0.60 ± 0.01	0.76 ± 0.04
Cimaterol	2	0.24 ± 0.02	0.38 ± 0.08	0.51 ± 0.09
Terbutaline	10	1.32 ± 0.05	2.25 ± 0.11	2.25 ± 0.34
Cimaterol	10	1.03 ± 0.04	1.73 ± 0.20	1.83 ± 0.34

Table 4.6 Determination of various β-agonists, by MSPD/RIA using clenbuterol, salbutamol and mabuterol standard curves prepared without control tissue extract..

level of about 4 ng/g. Taking into consideration the differing cross-reactivities of the β-agonists to the assay antiserum, the MSPD extraction procedure gives reasonable recovery for all 5 substances tested and is appropriate as a multiresidue extraction method.

4.3.2 Linkage of MSPD with a C_{18} SPE clean-up step

4.3.2.1 Method validation

An inter-assay variation of salbutamol, clenbuterol and mabuterol, at 1 ng/g and 2 ng/g levels, measured using salbutamol standard curves with and without tissue extract was carried out. Table 4.7 shows the results of the the former option. The table also provides comparisons between the two main C_{18} material brands (Bondesil and Isolute). The use of an extract containing standard curve gives the best recovery data, as was found for the clenbuterol assay in Chapter 3. For either C_{18} material type, the measured recovery for salbutamol is 79% or greater, for clenbuterol greater than 100% and for mabuterol 64% or greater; these results indicate high recovery of the β-agonists by the MSPD/SPE procedure with the relatively high and low recoveries obtained for clenbuterol and mabuterol, respectively, being explained by the cross-reactivity of the antiserum towards these compounds (for clenbuterol it is 118% and for mabuterol it is 78%).

The two grades of C_{18} material gave comparable results, for example

β-agonist added (ng/g)	n	β-agonist determined (ng/g)			
		(a) Bondesil C_{18}		(b) Isolute C_{18}	
		Mean ± SD	CV(%)	Mean ± SD	CV(%)
1 (S)	5	0.79 ± 0.11	13.9	0.85 ± 0.22	26.1
2	5	1.80 ± 0.27	15.0	1.79 ± 0.21	11.7
1 (C)	5	1.15 ± 0.22	19.1	1.10 ± 0.21	19.1
2	5	3.19 ± 0.82	25.7	2.42 ± 0.51	21.1
1 (M)	5	0.69 ± 0.06	8.7	0.69 ± 0.11	15.9
2	5	1.32 ± 0.19	14.4	1.27 ± 0.17	13.4

Table 4.7 Inter-assay variation of salbutamol (S), clenbuterol (C) and mabuterol(M) at 1 and 2 ng/g levels in liver extracts from MSPD/SPE/RIA. The performance of the two C_{18} brands was evaluated and the β-agonists were determined against a salbutamol standard graph containing tissue extract.

0.79 ng/g (Bondesil) and 0.85 ng/g (Isolute) for salbutamol at 1 ng/g recovery. There was a difference, however, between C_{18} materials for the clenbuterol 2 ng/g level due mainly to a highly inflated result being obtained for one sample (4.58 ng/g, Bondesil material). These results show that the method works well for different brands of C_{18} material.

The inter-assay data using standard curve without tissue extract (results not shown) demonstrates that lower recovery values were being obtained. However, taking into account the cross-reactivity of the antiserum, the mean measured recovery for all three compounds was $> 65\%$. Hence, a curve without tissue extract may be used for routine work. The intra-assay variation data for liver samples fortified with β-agonists at the 2 ng/g level is provided in Tables 4.8 (salbutamol standard curve with added tissue extract). The coefficients of variation were always less than 20%. This is considered acceptable in view of the nature of the sample matrix. For the Isolute material, the recovery of mabuterol was low (mean recovery, corrected for cross-reactivity, of 52%) compared to that obtained consistently in the inter-assay study. For the other compounds the recovery was always greater 80%, irrespective of the C_{18} material used.

β-agonist added (ng/g)	n	β-agonist determined (ng/g)			
		(a) Bondesil C_{18}		(b) Isolute C_{18}	
		Mean ± SD	CV(%)	Mean ± SD	CV(%)
2 (S)	4	2.24 ± 0.17	7.8	1.87 ± 0.21	11.2
2 (C)	4	2.54 ± 0.48	18.9	1.99 ± 0.17	8.5
2 (M)	4	1.51 ± 0.26	17.2	0.82 ± 0.12	14.6

Table 4.8 Intra-assay variation of 2 ng/g salbutamol (S), clenbuterol (C) and mabuterol (M) in liver extracts from MSPD/SPE/RIA using either Bondesil or Isolute C_{18} material. The extracts were determined against a salbutamol standard curve containing tissue extract.

4.3.2.2 Limit of detection

Limits of detection for the MSPD/RIA method were determined by analysis of liver samples (n = 7), free of β-agonist residues. The limits, calculated as the mean + 3 times the standard deviation, were determined depending on the brand of C_{18} material used and on the type of curve (without or with tissue extract) used:

(a) Bondesil/with tissue extract curve:

0.17 ng/g (mean response + SD = 0.0260 ng/g + 0.048 ng/g)

(b) Bondesil/without tissue extract curve:

0.16 ng/g (mean response + SD = 0.029 ng/g + 0.045 ng/g)

(c) Isolute/with tissue extract curve:

0.57 ng/g (mean response + SD = 0.071 ng/g + 0.165 ng/g)

(d) Isolute/without tissue extract curve:

0.28 ng/g (mean response + SD = 0.037 ng/g + 0.082 ng/g)

Apart from the results for the Isolute material determined by a curve without tissue extract (c), the limits fall well below the maximum residue limit (MRL) of 0.5 ng/g **[10]**. For (c) there was one negative control value giving a high background level (0.4 ng/g), resulting in a high standard deviation and consequently a high limit of detection.

4.4.2.3 Incurred sample analyses

Liver samples from animals treated with salbutamol and clenbuterol were analysed using the developed procedure. Again, the method was evaluated with respect to C_{18} material brand and curve type. Table 4.9 shows the results of these analyses. As for the inter-assay analysis, there was no significant difference between the results obtained using Bondesil or Isolute C_{18} materials. For the incurred salbutamol sample (at approx. 1.2 ng/g) a lower result was obtained with this method than was obtained by direct MSPD extraction (1.54 ng/g). The values obtained for the clenbuterol incurred sample (i.e. 4.34 and 3.96 ng/g) were somewhat inflated over that obtained before (3.60 ng/g) using a clenbuterol standard curve containing tissue extract; the inflated results are probably due to the higher cross-reactivity to clenbuterol (118%) in this assay.

	Residue determined (ng/g)		
		MSPD/SPE/RIA method (n = 5)	
Sample	Previous methods	Bondesil C_{18}	Isolute C_{18}
		Mean ± SD	Mean ± SD
A	1.54[1]	1.14 ± 0.31	1.17 ± 0.19
B	3.60[2]	4.34 ± 1.55	3.96 ± 1.43

Table 4.9 Results for residue-positive samples containing salbutamol (sample A) and clenbuterol (sample B) determined by the developed procedure. Results are compared to previous methods, either direct MSPD/RIA ([1]) or solvent extraction/RIA ([2]).

4.5 CONCLUSIONS

The ease of extraction of clenbuterol by MSPD (Chapter 3) indicated that the technique might be suitable, also, for other β-agonist compounds; compounds similar in structure to clenbuterol like mabuterol and cimaterol were obvious candidates. The phenolic β-agonists, being more polar and ionizable were, however, liable to be removed in the water wash. The present study demonstrates that good recovery of salbutamol may be obtained, depending on the C_{18} material used. Of the three C_{18} materials tested Sepralyte retains salbutamol most strongly and allows the use of a water wash. However, to provide a more universal procedure, i.e. a procedure which could extract a range of β-agonists irrespective of the material brand, the water wash step was eliminated and replaced by solid-phase extraction. Using this purifying step, recoveries of 80% or better were achieved for all three compounds tested. Moreover, the procedure has the capability to detect residues below the 1 ppb level.

The inclusion of a hydrolysis step was necessary for residues which occur as conjugates. Results from other studies show that, for salbutamol, two main conjugates are present in liver samples, namely glucuronides and sulphate esters **[12]**. The incorporation of a post-MSPD enzyme hydrolysis step into the procedure deconjugates the residues to give an accurate value for total β-agonists present. A short hydrolysis time (2 h) coupled with a low

enzyme concentration was found to be sufficient to achieve deconjugation. The results show that 40-45% of salbutamol residues occur in the liver in a conjugated form.

4.6 REFERENCES

1. A.P. Moloney and P. Allen, in Proceedings of the FLAIR Workshop, Thessaloniki, Greece (Eds; H.A. Kuiper and LA.P. Hoogenboom), Oct 30-31, 1992, p. 89.
2. P. Furst, C. Furst and W. Groebel, *Dtsch. Lebensm.-Rdsch.*, **85**, 1989, 341.
3. L. Leyssens, C. Driessen, A. Jacobs, J. Czech and J. Raus, *J. Chromatogr (Biomed. Applic.)*, **564**, 1991, 515.
4. L.A. van Ginkel, R.W. Stephany and H.J. van Rossum, *J. Assoc. Off. Anal. Chem.*, **75**, 1992, 554.
5. M.-P. Montrade, B. Le Bizec, F. Montreau, B. Siliart and F. Andre, Anal. Chim. Acta, **275**, 1993, 253.
6. S.A. Barker, A.R. Long and C.R. Short, *J. Chromatogr.*, **475**, 1989, 353.
7. F.J. Schenk, S.A Barker and A.R. Long, *J. Assoc. Off. Anal. Chem.*, **75**, 1992, 655.
8. R.F. Witkamp and A.S.J.P.A.M. van Miert, in Proceedings FLAIR Workshop, Thessaloniki, Greece, (eds; H.A. Kuiper and L.A.P. Hoogenboom, Oct 30-31, 1992, p.75.
9. H. Hooijerink, R. Schilt, W. Haasnoot and D. Coortheijn, *J. Pharm. Biomed. Anal.*, **9**, 1991, 485.

10. B. Boenisch and J.F. Quirke, in Proceedings of the FLAIR Workshop, Thessaloniki, Greece (Eds: H.A. Kuiper and L.A.P. Hoogenboom), Oct 30-31, 1992, p.102.

11. M. Horiba, T. Murai, K.Nomura, T. Yuge, K. Sarai and E. Osada, *Arzeim.-Forschung,* **34**, 1984, 1668.

12. M.-P. Montrade, S. Riverain, B. Le Bizec and F. Andre, in Proceedings FLAIR Workshop, Thessaloniki, Greece (Eds; H.A. Kuiper and LA.P. Hoogenboom), Oct 30-31, 1992, p. 143.

13. R. Angeletti, M. Paleologo-Oriundi, R. Piro and R. Bagnati, *Anal. Chim. Acta,* **275**, 1993, 215.

14. Ph. Delahaut, M. Dubois, Y. Colemonts, A. Boenke and G. Maghuin-Rogister, in Proceedings of the EuroResidue 2 Conference, Veldhoven, The Netherlands, May 21-23, 1990, p. 257.

15. G. Degand, A. Bernes-Duyckaerts, Ph. Delahaut and G. Maghuin-Rogister, *Anal. Chim. Acta,* **275**, 1993, 241.

16. L. Howells, M. Sauer, R. Sayer and D. Clark, *Anal. Chim. Acta,* **275**, 1993, 275.

17. K. Selinger, H.M. Hill, D. Matheou and L. Dehelean, *J. Chromatogr. (Biomed. Applic.)*, **493**, 1989, 230.

18. R.C. Causon, R. Desjardins, M.J. Brown and D.S. Davies, *J. Chromatogr.*, **306**, 1984, 257.

CHAPTER 5

SUPERCRITICAL FLUID EXTRACTION AND CHROMATOGRAPHY OF β-AGONISTS

5.1 INTRODUCTION

A supercritical fluid (SF) may be described as a dense gas which possesses physico-chemical properties intermediate between those of liquids and gases. This state is reached by bringing the substance above its critical temperature (T_c) and pressure (P_c). A SF may be defined from the pressure-temperature phase diagram shown Figure 5.1 (supercritical fluid, carbon dioxide). The solvent strengths of SFs approach those of liquid solvents as their density is increased, thereby having the capacity to dissolve a variety of solutes, even those of high molecular weight and low volatility. In addition, because SFs have solute diffusivities an order of magnitude higher, and viscosities an order of magnitude lower, than liquid solvents they have much better mass transfer characteristics. Table 5.1 shows a comparison in properties between a gas, a SF and a liquid. The SF possesses the most ideal properties for use as a mobile phase in chromatograpic science; its low viscosity characteristic allows a lower pressure drop over a column than for a liquid for a given flow rate. This low column pressure drop results in the column having apparently more theoretical plates per meter and increased chromatographic efficiency. Its higher analyte diffusivity causes narrower chromatographic peaks and hence better sensitivity **[1]**.

The solvent strength of a SF can be easily controlled, unlike a liquid where the solvent strength is essentially constant irrespective of the

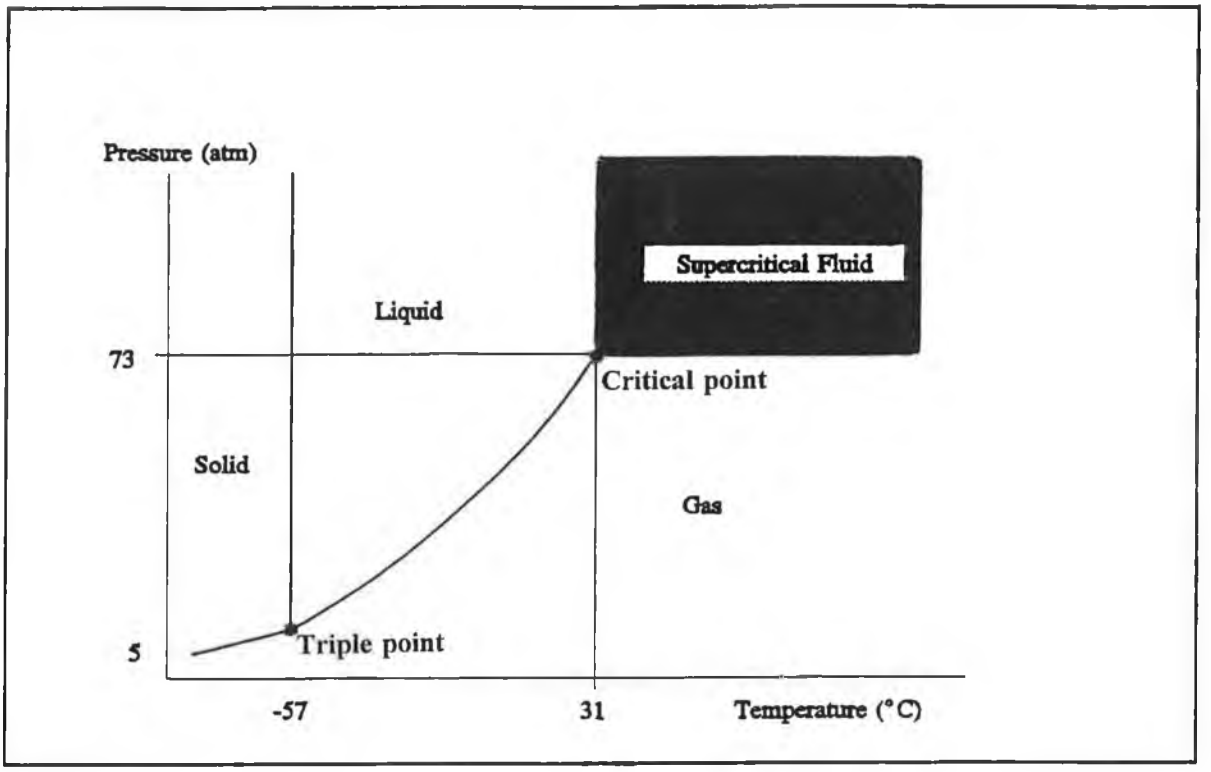


Figure 5.1 Pressure-temperature phase diagram for supercritical carbon dioxide.

Mobile phase	Density ($g\ ml^{-1}$)	Viscosity ($g\ cm^{-1}\ s^{-1}$)	Diffusivity ($cm^2\ s^{-1}$)
Gas	10^{-3}	$0.5\text{-}3.5 \times 10^{-4}$	0.01-1.0
SF	0.2-0.9	$0.2\text{-}1.0 \times 10^{-3}$	$3.3\text{-}0.1 \times 10^{-4}$
Liquid	0.8-1.0	$0.3\text{-}2.4 \times 10^{-2}$	$0.5\text{-}2.0 \times 10^{-5}$

Table 5.1 Comparison in physical properties, from a chromatographic point of view, for gas, liquid and supercritical fluid (SF) mobile phases.

Compound	Boiling point (°C)	T_{cr} (°C)	P_{cr} (bar)	Density (g/ml)
Carbon dioxide	-78.5	31.3	72.9	0.45
Nitrous oxide	-89.0	36.5	71.4	0.46
Ammonia	-33.4	132.3	111.3	0.24
Xenon	-107.3	16.6	58.4	1.10

Table 5.2 The physical properties, relevant to SFE and SFC, of various SF substances.

extraction conditions. The solvent strength of the SF depends upon the temperature and pressure applied to it. At a constant temperature the application of a low pressure produces a low density, and hence relatively non-polar, SF and which will result in the extraction of non-polar analytes. Increasing the pressure to different points allows the selective fractionation of analytes according to their solubility in the SF. Figure 5.2 shows how supercritical carbon dioxide is affected by various temperature/pressure conditions.

A wide variety of substances may be used as SFs but, practically, only three or four have been found suitable. Table 5.2 shows the characteristic physical properties of these substances. Carbon dioxide is by far the most used. It is inert, inexpensive and non-toxic and has low T_c and P_c values. Nitrous oxide possesses similar T_c and P_c values but there have been some serious accidents in its usage **[2, 3]** due to its oxidising properties. Xenon, with T_c and P_c values of 16.6°C and 58.4 bar has almost ideal properties but it is too expensive for routine use in analysis. Supercritical ammonia is applicable to polar analytes but has higher T_c and P_c values and is toxic and corrosive. Other substances have also been used (for example chlorodifluorocarbon **[4]**) but the vast majority of applications report the use of supercritical carbon dioxide (SC-CO_2).

The two major analytical applications which utilise SFs are supercritical fluid extraction (SFE) and supercritical fluid chromatography (SFC). The

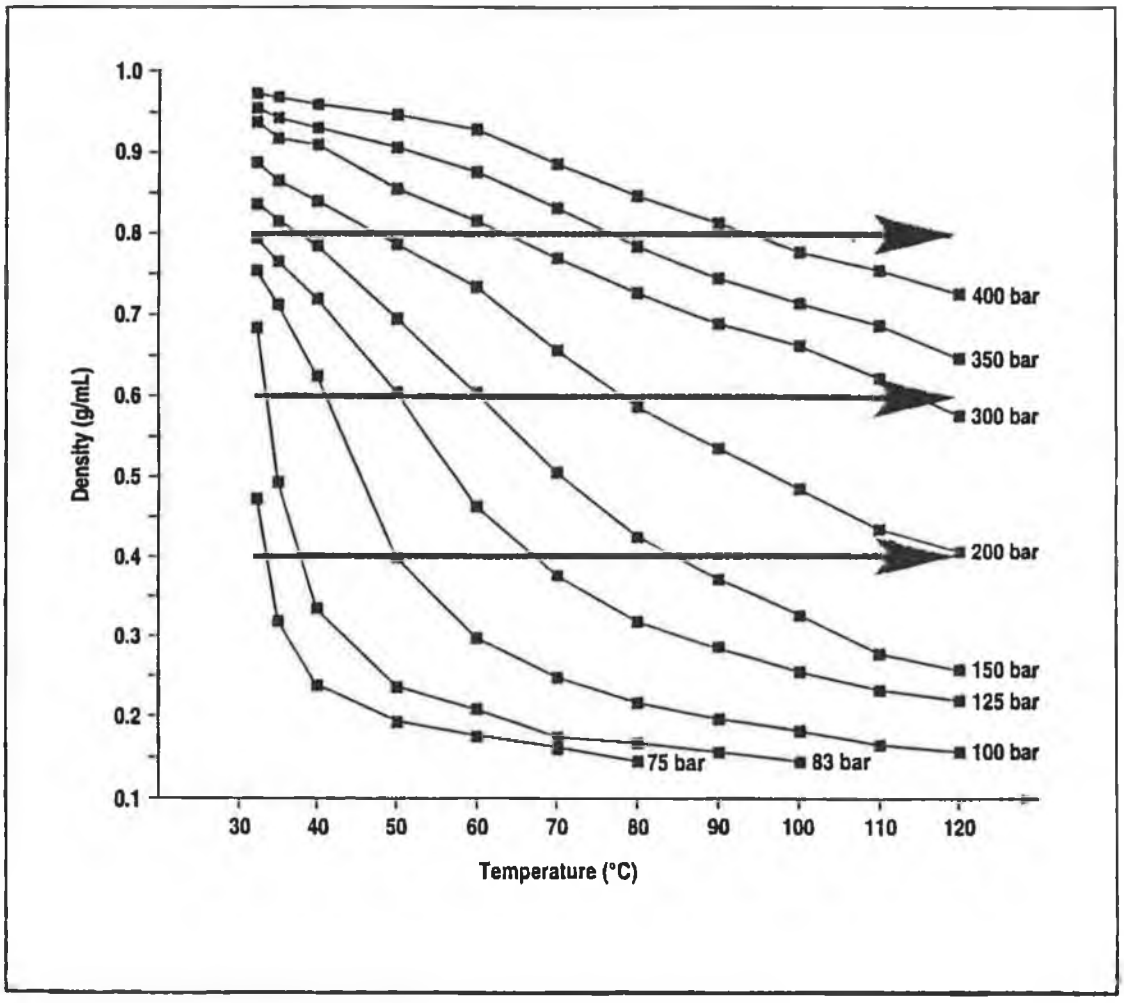


Figure 5.2 Plot of carbon dioxide density vs temperature at various pressures.

former technique may be viewed as an analyte isolation procedure analogous to liquid-liquid extraction whereas the latter is a chromatographic technique with the combined separation properties of both HPLC and GC. A combination of both techniques (i.e. SFE/SFC) is a possible sample clean-up option **[5]**. Extracts from SFE may be analysed by SFC but in most cases HPLC or GC is preferred **[6-8]**. A SFE apparatus consists of a pump which directs the SF through a heated sample vessel where the analytes are extracted and then swept to a collection device via a flow restrictor from which the depressurised SF is vented. The analytes may be rinsed from the collection device with an organic solvent (normally methanol). SFE can be performed in dynamic mode (continuous flow of SC-CO_2 through the extraction cell) or static mode (fixed amount of SC-CO_2 in the extraction cell) or even a combination of both. A schematic diagram of the SFE apparatus is shown in Figure 5.3.

SC-CO_2 is relatively non-polar hence its extracting capacity for medium to high polar analytes depends on the application of high temperature/pressure conditions **[9]** or the introduction of a modifier solvent in a low percentage **[10, 11]**. Many reviews have appeared which focus on the merits of SFE and ways of optimising extraction conditions for various analytes **[12, 13]**. The bulk of the literature reporting SFE deals with SF-CO_2 extractions of relatively non-polar components like pesticides **[14-16]**, environmental contaminants (e.g. PCBs, PCHs) **[17-22]** and fats/oils **[23-26]**. For more polar compounds

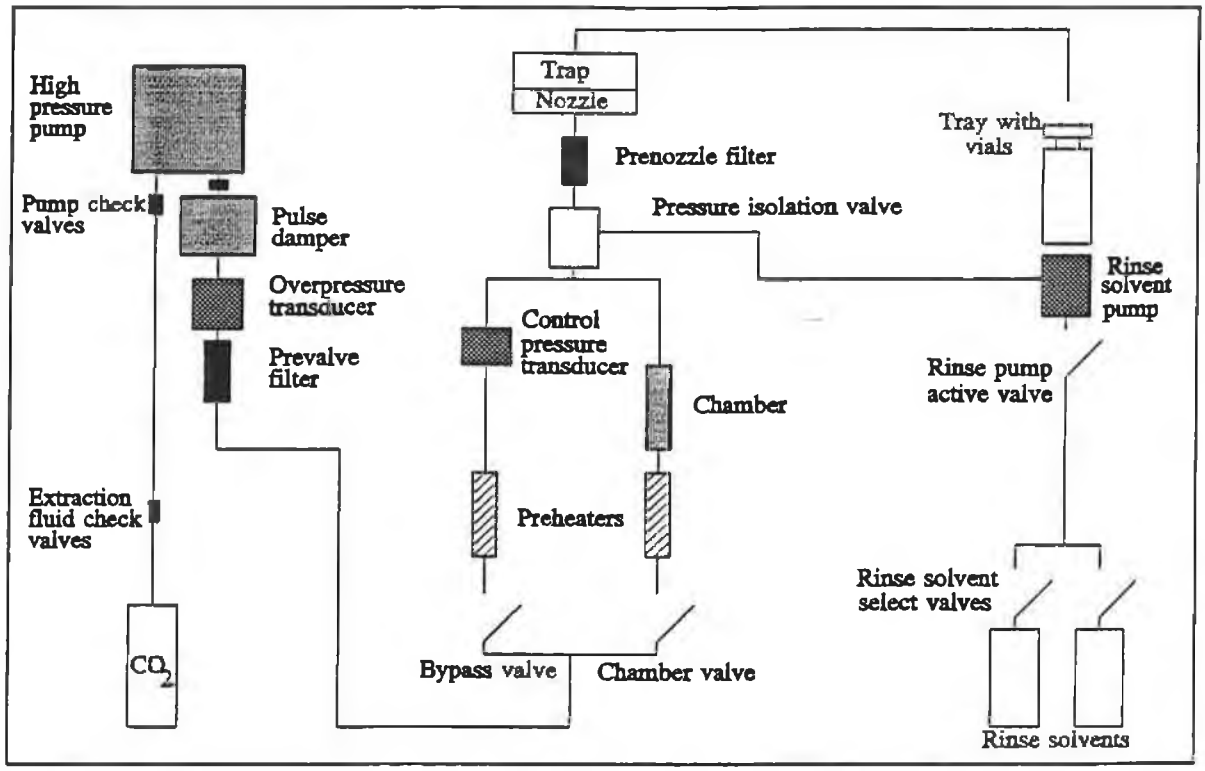


Figure 5.3 Schematic of the HP-7680T supercritical fluid extractor.

the addition of a modifying solvent was required; for instance Eureby *et al.* found that extracting tipredane (a corticosteroid) with pure supercritical CO_2 gave low recovery whereas addition of a modifier (ethanol, up to 33% v/v) gave rise to recoveries of greater than 80% **[6]**. Similar experiences were reported by other authors **[10, 11]**. Although extracts from SFE need not be confined to SFC analysis, an "in-line" SFE-SFC linkage is advantageous as the injection solvent is the same as the mobile phase, i.e. the output characteristics from the first instrument and the input characteristics of the second instrument are compatible.

SFC provides an alternative to HPLC and GC techniques for the separation and detection of analytes from sample extracts. The advantages of using SFs for separation purposes have already been discussed. Additionally, SFC is compatible with a wide range of detectors in use with both HPLC and GC. The ultraviolet detection option is extremely popular and applicable to most analytes possessing a chromophore **[7]**. The combination of SFC with a mass spectrometer gives additional sensitivity and provides information on compound structure **[5, 27]**. Suitable stationary phases are not unlike those used in other chromatographic techniques; packed columns (as in HPLC) containing a range of functional groups (C_{18}, C_8, NH_2, etc) bonded to silica and open-tubular columns with stationary phases similar to those used in capillary GC except that the inside diameter is smaller and the stationary phase must be immobilised. A schematic diagram of a SFC apparatus is provided

in Figure 5.4. The relative non-polar nature of supercritical CO_2 means that the bulk of extractions/separations have been of relatively non-polar analytes. For SFE/SFC to become a standard sample extraction/analysis techniques, however, their application to polar analytes must be addressed. Most drugs/drug metabolites contain one or more polar functional groups and the extraction methodologies used are usually lengthy requiring for example liquid-liquid or solid phase extraction. With certain modifications these extractions may be performed more efficiently by SFE. Many authors have reported methods applicable to the more polar drug compounds; for instance Cross *et al.* extracted sulphonamides from liver after first dispersing the sample on diatomaceous earth. The extraction was short (5 min) and recovery of the various drug compounds was in the order 53-93% **[28]**. Khundker *et al.* also extracted a drug compound, ibuprofen, using unmodified SC-CO_2 although high temperature (70°C) and high density (0.7 g/ml) conditions were required **[9]**. A home-made SFE apparatus was described and built by Maxwell and co-workers which could apply twice the maximum pressure of its commercial counterpart. Methods were reported for the extraction of ten nitrosamines from meat samples **[29]** and three nitrobenzamide antimicrobial drugs from liver samples **[30]**. For more efficient extraction of polar drugs, an organic modifier must be added to the supercritical fluid. Liu and co-workers found that 5% methanol in SC-CO_2 was sufficiently polar to extract flavone **[10]** and mebeverine **[11]** from blood plasma. Higher methanol

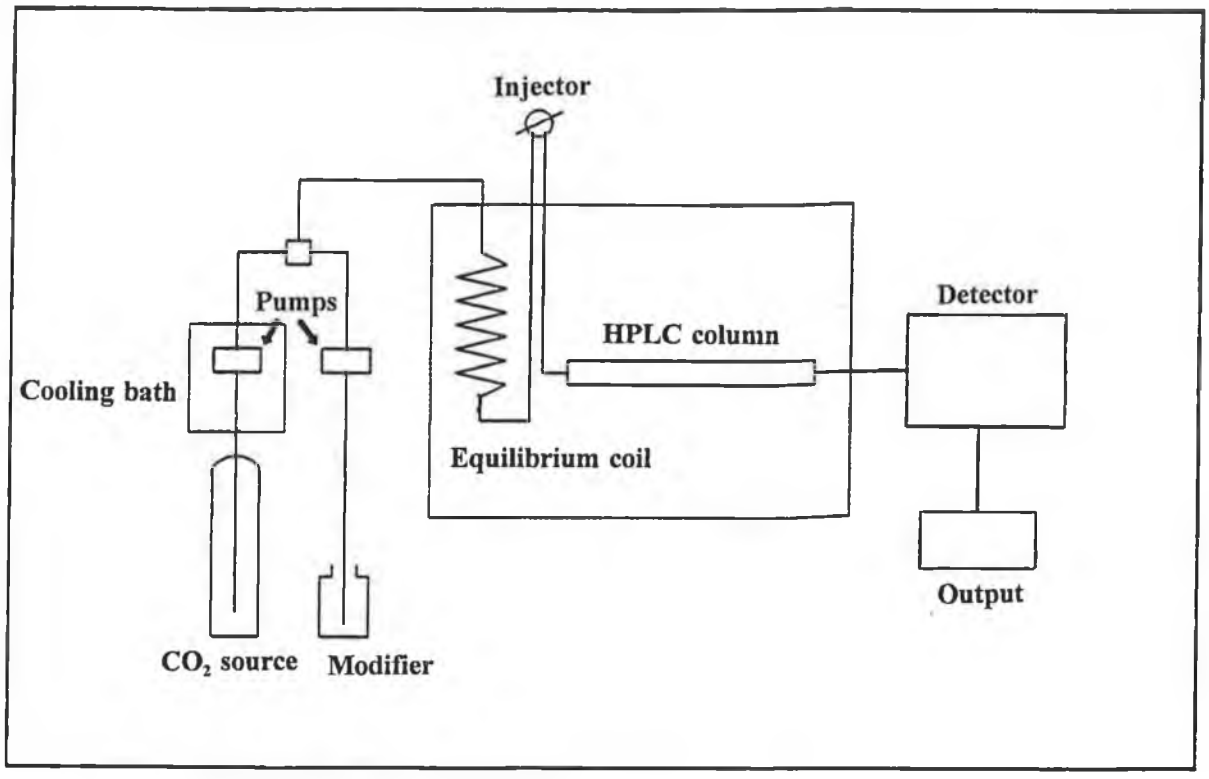


Figure 5.4 Schematic of a packed column SFC system.

concentraions (between 17 and 33%) were necessary to extract tipredane efficiently **[6]**. A novel on-line SFE-SFC-MS-MS technique was described by Ramsey *et al.*; the mainly non-polar components of kidney tissue were washed from the extraction vessel with SC-CO_2, after which the test analytes (hexesterol, diethylstilbesterol) were eluted and separated via gradient elution using SFC to the mass spectrometer for detection **[5]**. Alternative extraction methodologies for pharmaceuticals/polar analytes include the use of supercritical fluid-nitrous oxide extraction **[31]** and in-situ chemical derivatisation **[32]**.

This chapter examines appropriate conditions for the extraction (using SFE) and separation (using SFC) of β-agonists. The β-agonists were first extracted from an inert matrix (filter paper) after optimisation of various parameters. The optimised procedure was applied to the extraction of mabuterol from liver dispersed on both Celite and C_{18} material. The use of a C_{18}/tissue blend was a novel approach to linkage of MSPD with SFE. The extracted samples were assayed by SFC.

5.2 EXPERIMENTAL

5.2.1 Reagents and materials

HPLC-grade methanol was obtained from Rathburn (Walkerburn, UK). Glass wool and triethylamine were obtained from Sigma (Poole, Dorset, UK). Instrument-grade liquid carbon dioxide, supplied in a cylinder with a syphon tube, was obtained from BOC (London, UK). Clenbuterol hydrochloride, salbutamol and terbutaline were purchased from Sigma (St. Louis, MO, USA). Mabuterol and cimaterol were supplied by BGA (Berlin, Germany). Solid support materials were reagent grade Celite (Supelco, Pennsylvania, USA) and octadecylsilane material ("Isolute" $C_{18,}$ end-capped, 40 μm), (International Sorbent Technology (IST), Mid Glamorgan, UK). Qualitative filter paper (No. 1) was supplied by Whatman (Maidstone, Kent, UK).

5.2.2 Apparatus

For supercritical fluid chromatography a Hewlett Packard (HP)-SFC system with UV detection coupled to a HP Series 1050 modifier pump was used. A 3 μm packing, amino-bonded column, 25 cm x 4.6 mm, (Capitol HPLC, Edinburgh, UK) was used to separate the β-agonists. Extractions were performed with a HP Model 7680T supercritical extraction system, also

coupled to a HP Series 1050 modifier pump.

5.2.3 Methods

5.2.3.1 Samples

Samples of bovine liver from animals certified as not treated with β-agonists were homogenised and stored frozen until required for assay.

5.2.3.2 Supercritical fluid chromatography (SFC)

Since previous work had shown that a high % methanol content in the SC-CO_2 was required for the β-blockers, compounds structurally-related to β-agonists **[33]**, methanol-modified SC-CO_2 was evaluated for the β-agonists. A number of HPLC columns (25 cm x 4.6 mm) containing C_{18}, -OH and -NH_2 functional groups bound to silica, were tested for their capacity to separate the compounds. Triethylamine (TEA) was added at a concentration of 1% v/v in methanol to block residual silanol groups and ensure good peak shape. A column temperature of 55°C was maintained to ensure supercritical conditions.

The conditions used for SFC were as follows:

Oven temperature:	55°C
Supercritical CO_2:	250 bar
Mobile phase:	SC-CO_2/methanol/TEA(75%/24.75%/0.25%)
Flow rate:	2.0 ml/min
Detection:	UV at 244 nm
Column:	Amino 3 μm packing, 25 cm x 4.6 mm,

5.2.3.3 Supercritical fluid extraction (SFE)

5.2.3.3.1 Extraction from filter paper

100 μl aliquots of 1 mg/ml solutions of the β-agonists (in methanol) were added to filter paper and, after evaporation of the methanol, the filter paper was placed into the extraction thimble. The following parameters were optimised: fluid density, extraction temperature, extraction time and modifier (methanol) content. The extraction was performed both in dynamic and static modes. The extracted fluid was swept onto a metal (stainless steel) trap which was then rinsed with methanol and the extract was analysed by SFC.

The conditions used for SFE were as follows:

Density:	0.84 g/ml
Chamber temperature:	70°C
Flow rate:	2.0 ml/min
Modifier content:	25%
Extraction time:	30 min

5.2.3.3.2 Extraction from liver

The solid support materials, Celite and C_{18} were evaluated for their capacity to disperse the liver samples. Mabuterol was the test analyte. Liver (0.5 g) was weighed into a glass mortar and fortified with 50 μg (in 50 μl) mabuterol standard. After 10 min, 2 g of C_{18} material or 0.9 g Celite was added and the liver was blended until thoroughly mixed (40-60 s). The mixture was added to the extraction thimble and both ends of the thimble were blocked by glass wool. The extraction conditions described in section 5.2.3.3.1 were then applied.

5.3 RESULTS AND DISCUSSION

A number of stationary phases were tested, but only one, an amino column, gave good separation of the compounds. In fact, for the bulk of applications to pharmaceutical substances, the choice is usually between cyano or amino types of modified silica **[34]**. When all five compounds were injected onto the SFC system, the three least polar compounds (in order of increasing polarity) mabuterol, clenbuterol and cimaterol eluted within the first five minutes and these aniline β-agonists were well separated from each other (Figure 5.5). The phenolic β-agonists, salbutamol and terbutaline did not elute within the first 20 min. It is most likely that these compounds, which are relatively insoluble in the supercritical fluid, are highly retained on the stationary phase. Because of these results, further work was concentrated solely on the separation and extraction of the aniline type compounds.

Extraction in the static mode using a long extraction time (30 min) proved to be more efficient than its dynamic mode counterpart and was thus chosen for all further work. Extraction of the aniline β-agonists from filter paper using pure supercritical CO_2 was totally ineffective due to the compounds' high polarity. The polarity of the supercritical CO_2 was increased by addition of a modifier, methanol. Optimum extraction was achieved with a high methanol content (25%). A high temperature (70°C) coupled with high density supercritical CO_2 (0.84 g/ml) were also necessary conditions to extract

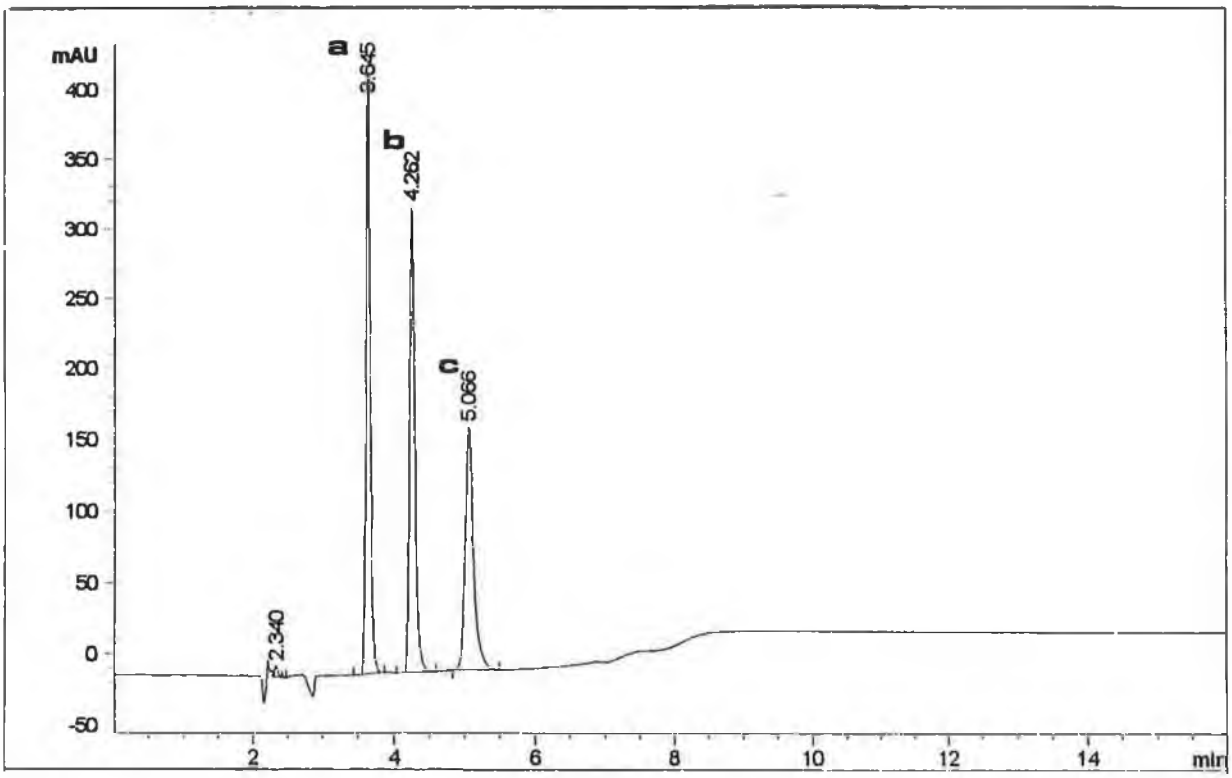


Figure 5.5 Supercritical fluid chromatogram of a mixture of 1 μg (on column) of mabuterol (a), clenbuterol (b) and cimaterol (c). Conditions as explained in text.

the compounds. Figure 5.6 shows an SFE extract from filter paper containing mabuterol and clenbuterol (1 μg on column) using optimum extraction conditions. The extraction efficiency was 89% for mabuterol and 80% for clenbuterol. For evaluation of the solid supports, Celite and C_{18}, mabuterol was chosen as the test analyte. Figure 5.7 shows the chromatograms obtained for Celite and C_{18} based extracts. The negative control liver extract (Figure 5.7 (a)) contains a main interferant at 3.3 min and other smaller interferants (after 5 min) but the area around the retention time for mabuterol (3.8 min) is free from interferences. When the mabuterol-fortified (50 μg/0.5 g) extract was injected, however, the recovery of drug was only 9% (Figure 5.7 (b)).

For C_{18} material better results were achieved: for the mabuterol-fortified extract a recovery of 31% was obtained (Figure 5.7 (c). Similar results were obtained for a repeat of these extractions (Celite-5%, C_{18}-27%). These preliminary results indicate that, with the available equipment, highly modified supercritical fluid is required to extract some of the β-agonists. The level of drug used for this evaluation of SFE (100 ppm) was very high but such fortification was used so that UV detection would not be limiting. The ideal determination technique, in this case, would be immunoassay which could detect β-agonists at less than 1 ppb. When a more comprehensive study is undertaken, in which SFE conditions would be optimised for more efficient extraction, the SFE-immunoassay linkage would be explored.

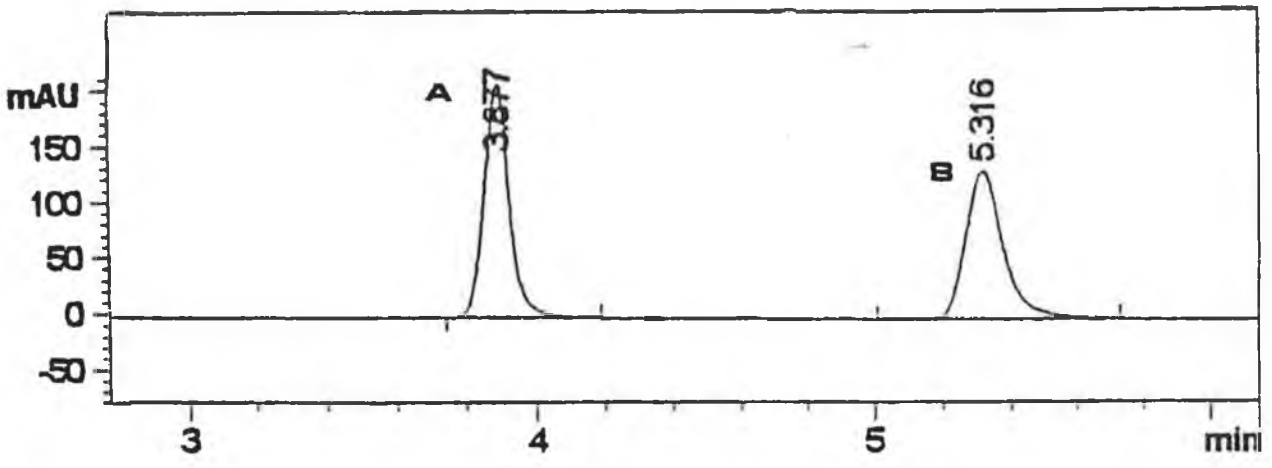


Figure 5.6 Supercritical fluid chromatogram of an extract obtained by SFE from filter paper fortified with 100 μg/ml of 1 mg/ml concentrations of mabuterol (a) and clenbuterol (b).

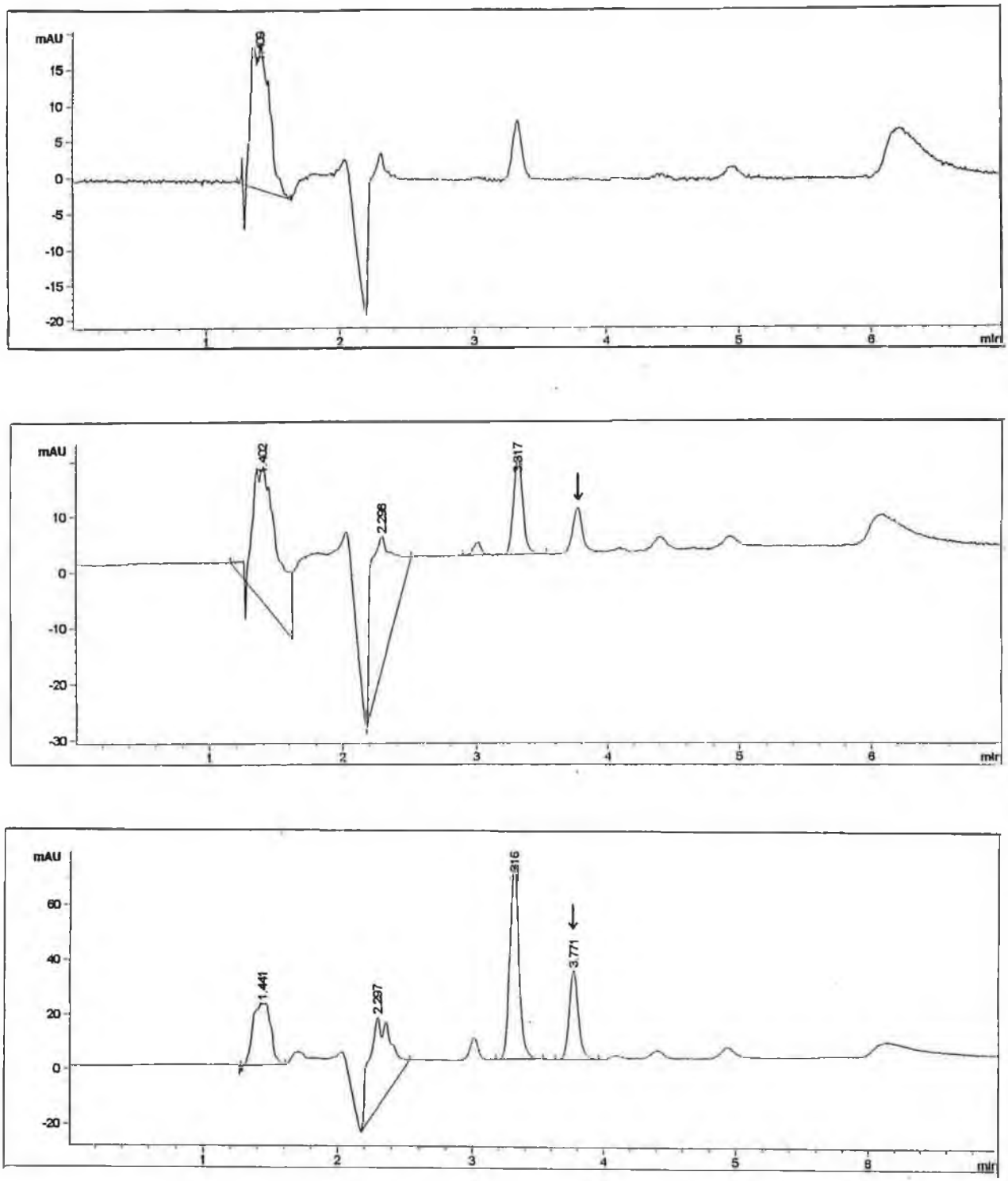


Figure 5.7 Supercritical fluid chromatograms of liver extracts. (a) negative control liver (with Celite) fortified with methanol, (b) negative control liver (with Celite) fortified with 100 µg/ml mabuterol and (c) negative control liver (with C_{18}) fortified with 100 µg/ml mabuterol.

5.4 CONCLUSIONS

The work carried out shows that SFC is a promising technique for the separation of aniline-type β-agonists. The phenolic compounds which are extremely polar, interacted with the column functional groups to the extent that they did not elute in a reasonable analysis time. The conditions for extraction by SFE were extremely harsh and it is questionable whether supercritical conditions were really being applied. However, compounds like mabuterol and clenbuterol may be extracted from an inert matrix (filter paper) at an 80% recovery efficiency. From liver samples the recovery was much lower; mabuterol was extracted at between 5 and 9% when dispersed on Celite and at 27 to 31% when dispersed on C_{18} material. It is interesting to note that samples prepared by an MSPD type procedure gave higher recovery, especially as there are no references in the literature reporting C_{18} as a sample dispersant.

The hydrophilic β-agonists (salbutamol, terbutaline), would appear to be unsuitable candidates for extraction in supercritical CO_2 or even highly-modified supercritical CO_2. Alternative procedures which might be studied include changing to a more polar supercritical fluid (e.g. ammonia) or in-situ derivatisation of the compounds to make them less polar and, therefore, more available to the supercritical fluid.

5.5 REFERENCES

1. C.M. White and R.K. Houck, *J. High Res. Chromatogr. Chromatogr. Comm.*, **9**, 1986, 4.
2. R.E. Sievers and B. Hansen, *B. Chem. Eng. News*, **69**, 1991, 2.
3. D.E. Raynie, *Anal. Chem.*, 65, 1993, 3127.
4. C.P Ong, H.K. Lee and S.F.Y. Li, *J. Chromatogr. Sci.*, **30**, 1992, 319.
5. E.D. Ramsey, J.R. Perkins and D.E. Games, *J. Chromatogr.*, **464**, 1989, 353.
6. M.R. Euerby and R.J. Lewis and S.C. Nichols, *Anal Proc.*, **28**, 1991, 287.
7. L.J. Mulcahy and L.T. Taylor, *Anal. Chem.*, **64**, 1992, 981.
8. L.J. Mulcahy and L.T. Taylor, *J. High Resolut. Chromatogr.*, **13**, 1990, 393.
9. S. Khundker, J.R. Dean and S.M. Hitchen, *Anal. Proc.*, **30**, 1993, 472.
10. H. Liu and K.R. Wehmeyer, *J. Chromatogr. (Biomed. Applic.)*, **115**, 1992, 61.
11. H. Liu, L.M. Cooper, D.E. Raynie, J.D. Pinkston and K.R. Wehmeyer, *Anal. Chem.*, **64**, 1992, 802.
12. D.R. Gere and E. M. Derrico, *LC-GC Int.*, **6**, 1994, 325.

13. D.R. Gere and E. M. Derrico, *LC-GC Int.*, **7**, 1994, 370.

14. K. Schaefer and W. Baumann, *Fres. Z. Anal. Chem.*, **332**, 1989, 884.

15. K.G. Furton and J. Rein, *Anal. Chim. Acta*, **236**, 1990, 99.

16. M.L. Hopper and J.W. King, *J. Assoc. Off. Anal. Chem.*, **74**, 1991, 661.

17. S.B. Hawthorne, M.S. Krieger and D.J. Miller, *Anal Chem.*, **61**, 1989, 736.

18 S.B. Hawthorne, D.J. Miller and M.S. Krieger, *J. Chromatogr. Sci.*, **27**, 1989, 347

19. S.B. Hawthorne, D.J. Miller and M.S. Krieger, *J. High Resolut. Chromatogr.*, **12**, 1989, 714.

20. F.I. Onuska and K.A. Terry, *J. High Resolut. Chromatogr.*, **12**, 1989, 357.

21. J. Rein, C.M. Cork and K.G furton, *J. Chromatogr.*, **545**, 1991, 149.

22. F.M Lancas, M.H.R. Matta, L.J. Hayasida and E. Carrilho *J. High Resolut. Chromatogr. Chromatogr. Comm.*, **14**, 1991, 633.

23. S.B. Hawthorne, M.L. Riekkola, K. Serenius, Y. Holm, R. Hiltunen and K. Hartonen, *J. Chromatogr.*, **634**, 1993, 297.

24. M.K.L. Bicking, T.G. Hayes, J.C. Kiley and S.N. Deming, *J. Chromatogr. Sci.* **31**, 1993, 170.

25. M. Verschuere, P. Sandra and F. David, *J. Chromatogr. Sci.*, **30**, 1992, 388.

26. P. Lembke and H. Engelhardt, *Chromatographia*, **35**, 1993, 509.

27. A.J. Berry, D.E. Games, I.C. Mylchreest, J.R. Perkins and S. Pleasance, *Biomed. Environ. Mass Spectrom.*, **15**, 1988, 105.

28. R.F. Cross, J.L. Ezzell and B.E. Richtor, *J. Chromatogr. Sci.*, **31**, 1993, 162.

29. R.J. Maxwell, J.W. Persabene and W. Fiddler, *J. Chromatogr. Sci.*, **31**, 1993, 212.

30. R.J. Maxwell, O.W. Parks and E.G. Piotrowski, *J. High Resolut. Chromatogr.*, 15, 1992, 807.

31. E. Sauvage, J.L. Rocca and G. Toussaint, *J. High Resolut Chromatogr.*, **16**, 1993, 234.

32. S.B. Hawthorne, D.J. Miller, D.E. Nivens and D.C. White, *Anal. Chem.*, **64**, 1992, 405.

33. P. Jackson, Ph.D Thesis, University of Swansea, 1993.

34. O. Gyllenhaal and J. Vessme, *J. Chromatogr.*, **628**, 1993, 275.

Appendix A: Publications

1. *"Application of Matrix Solid Phase Dispersion for the Determination of Clenbuterol in Liver Samples"*, Damien Boyd, Paula Shearan, Sean Hopkins, Michael O' Keeffe and Malcolm R. Smyth, *Analytica Chimica Acta*, **275**, 1993, 221.

2 *"Matrix Solid Phase Dispersion linked to Immunoassay Techniques for the Determination of Clenbuterol in Bovine Liver Samples"*, Damien Boyd, Paula Shearan, Sean Hopkins, Michael O' Keeffe and Malcolm R. Smyth, *Analytical Proceedings*, **30**, 1993, 156.

3. *"Methodology for Extraction of Salbutamol from Tissue Samples with Determination by Immunoassay"*, Damien Boyd, Michael O' Keeffe and Malcolm R. Smyth, in Proceedings of the FLAIR Workshop on β-agonists and Bound Residues (eds: H.A. Kuiper and L.A.P. Hoogenboom), Thessaloniki, Greece, 1992, p. 133.

4. *"Matrix Solid Phase Dispersion (MSPD) as a Multiresidue Technique for β-Agonists in Bovine Liver Samples"*, Damien Boyd, Michael O' Keeffe and Malcolm R. Smyth, *Analyst*, **119**, 1994, 1467.

5. *"A Voltammetric Study of Salbutamol, Fenoterol and Metaproterenol at Unmodified and Nafion-modified Carbon Paste Electrodes"*, Damien Boyd, Jose Ramon Barrierra Rodriquez, A.J. Miranda Ordieres, Paulino Tunon Blanco and Malcolm R. Smyth, *Analyst* (1994), in press.

6. *"Application of a Nafion-modified Carbon Paste Electrode for the Adsorptive Stripping Voltammetric Determination of Fenoterol in pharmaceutical preparations and Biological Fluids"*, Damien Boyd, Jose Ramon Barrierra Rodriquez, Paulino Tunon Blanco and Malcolm R. Smyth, *Journal of Pharmaceutical and Biomedical Analysis*, (1994), in press.