APPLICATION OF SOLID-PHASE EXTRACTION TECHNIQUES TO THE EXTRACTION AND CLEAN-UP OF β-AGONISTS FROM BIOLOGICAL MATRICES

by

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A thesis submitted for the degree of
Master of Science

Dublin City University

July 1995

Declaration

I hereby certify that this material, which is submitted for assessment on the programme of study leading to the award of Master of Science is entirely Sean Collin's 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 this work.

Signed: Michael O'Keeffe Date: 14/9/95

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Dedicated to my parents, Cyril and Teresa

Acknowledgements

(prepared on Sean's behalf by his supervisor at the National Food Centre, Dr Michael O'Keeffe)

The contributions of the following organisations and individuals are acknowledged

Teagasc and the Irish American Partnership for financial support,

International Sorbent Technology Ltd , and the Central Veterinary Laboratory, UK, for generous supply of materials,

The staff at the National Food Centre, particularly Sean's colleagues in the Food Analysis Department, for their assistance, support and friendship,

Sean's supervisor at Dublin City University, Prof Malcolm Smyth, for his guidance, encouragement and advice,

Dr Orla Kennedy is thanked for assisting with the final preparation of this thesis

Abstract

Solid-phase extraction techniques were evaluated for the extraction of β-agonists from biological matrices prior to determination by radioimmunoassay

Clenbuterol was extracted from aqueous liver homogenates using a combination of on-column liquid/liquid partitioning coupled to a silica solid-phase extraction step following initial enzymatic digestion of the liver sample The procedure was validated at fortification levels of 0 2 and 0 4 ppb clenbuterol Recovery of clenbuterol was greater than 60% at both concentrations

Four β-agonists were extracted from bovine urine and liver samples using mixed-mode solid-phase extraction columns The procedure was validated for clenbuterol, mabuterol, salbutamol and terbutaline at levels of 2 and 5 ppb The mean recovery for the β-agonists from liver and urine was greater than 75% and 85%, respectively The limit of detection was 0 13 ng/ml and 0 46 ng/g of clenbuterol in urine and liver, respectively

Clenbuterol and salbutamol were extracted from bovine liver samples using a mixed-mode matrix solid-phase dispersion/ion exchange procedure Four types of sorbent were investigated in order to determine which sorbent combined most effectively with the ion exchange material in retaining the β-agonists Recovery of clenbuterol and salbutamol from liver samples fortified at 50 ppb was greater than 80%, the resulting extracts were insufficiently purified to allow for determination of β-agonists at low or sub-ppb levels An enzymatic hydrolysis step was required for the determination of conjugated residues of salbutamol in incurred liver samples

CHAPTER 1

INTRODUCTION

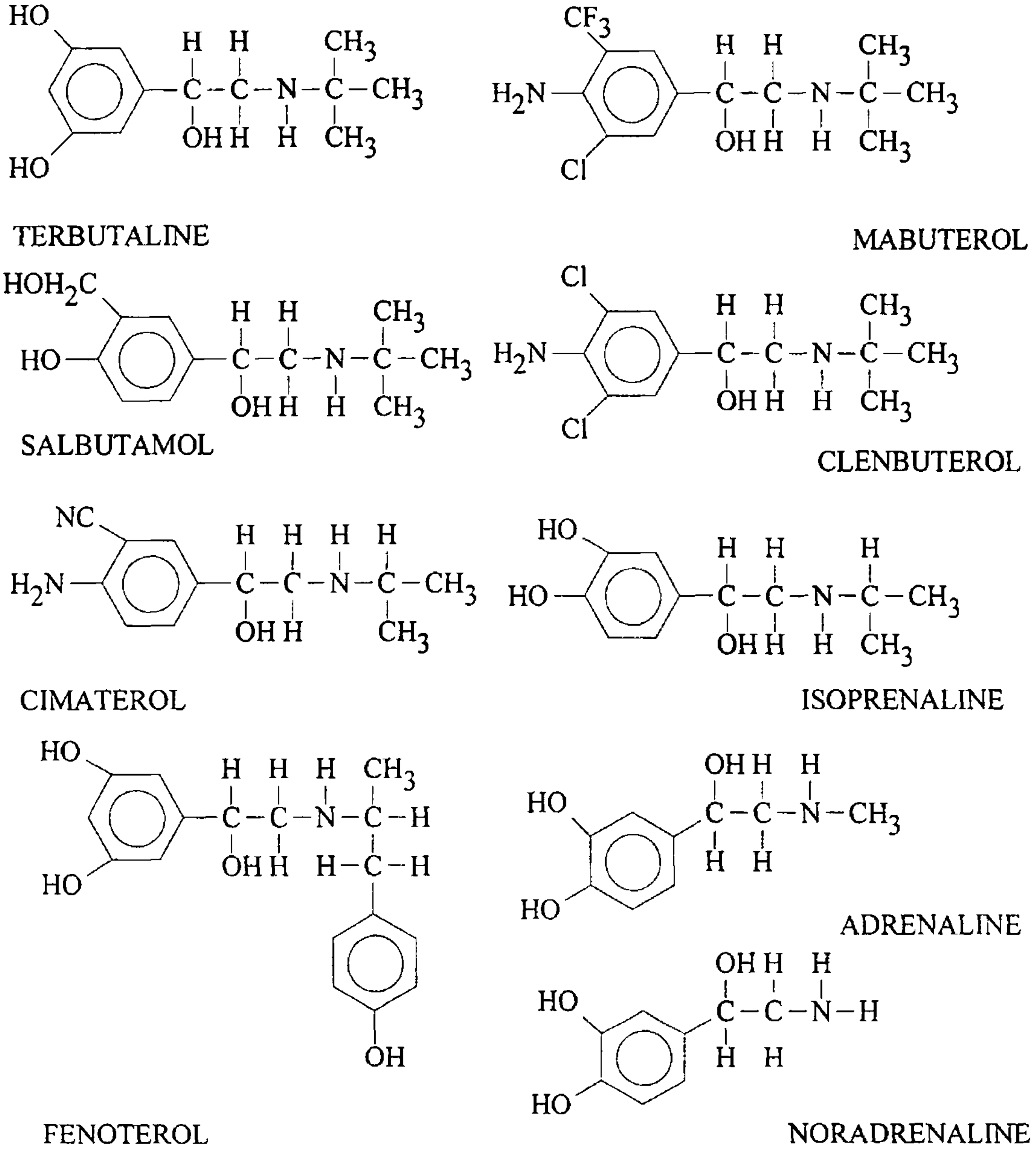
1.1 β-AGONISTS

Noradrenaline is an adrenergic neurotransmitter which is synthesised and stored in peripheral nerve endings. The molecule stimulates adrenergic receptors within the immediate vicinity and does not normally enter the plasma in sufficient quantities to act as a circulating hormone. Adrenaline is a circulating hormone of the adrenal medulla and influences physiological events at adrenergic receptors throughout the body.

The adrenergic receptors have been divided into both α- and β- subtypes in order to describe the ability of adrenergic agonists to cause contraction (α- effect) or relaxation (β-effect) of smooth muscle. The response of a tissue to a particular agonist depends on the presence and proportion of α- and β- receptors and the ability of the agonist to interact with the receptors. β-Receptors can be subdivided further into β1 and β2 receptors. The β-receptors of the heart and intestinal smooth muscle are classified as β1 type, while the receptors of the bronchial, vascular, and uterine smooth muscle are classified as β2 receptors [1].

Synthetically produced β-agonists are close analogues of adrenaline and noradrenaline, and consequently have the ability to influence various metabolic and physiological processes. The structures of seven β-agonists, along with adrenaline and noradrenaline, are shown in Figure 1.1. Synthetic compounds which selectively stimulate β1 or β2 activity have been used in the treatment of cardiac failure and asthma, respectively. The selective nature of these compounds results in reduced side effects particularly in the treatment of bronchial problems where stimulation of cardiac functions is undesirable.

Figure 1 1 Structures of seven β-agonists, adrenaline and noradrenaline



TERBUTALINE

MABUTEROL

SALBUTAMOL

CLENBUTEROL

CIMATEROL

ISOPRENALINE

FENOTEROL

ADRENALINE

NORADRENALINE

1.2 THERAPEUTIC USE OF β-AGONISTS IN HUMAN AND VETERINARY MEDICINE

β-Agonists are used to treat chronic obstructive airway diseases in both humans and animals Another use has been in pregnant mammals for tocolysis These compounds achieve the desired effect through their selective stimulation of the β2 receptors on bronchial and uterine muscles Although most of the β-agonists shown in Figure 1 1 are used in human medicine, only clenbuterol is used in veterinary treatment at a recommended dosage of 0 8 µg/Kg bodyweight twice daily for a maximum period of ten days [2] The selectivity of β-agonists for the β-receptors has been increased significantly based on knowledge that stimulation of a β-receptor is achieved by molecules containing an aromatic group with an ethanolic group at the α-position and a secondary amine with bulky substituents (Figure 1 2)

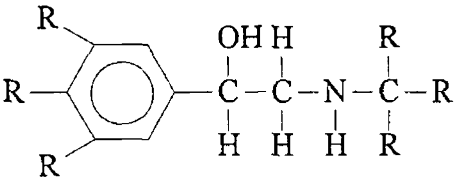


Figure 1 2 "Template" for β-agonist activity

1.3 (RE)-PARTITIONING EFFECTS OF β-AGONISTS IN MEAT PRODUCTION

The first reported effects of treatment with β-agonists to alter fat and muscle deposition in steers [3], lambs [4] and broilers [5] were in 1984, and all results originated from the American Cyanamid Co in Princeton, New Jersey The effects of a variety of β-agonists on the growth performance and carcass quality of sheep, cattle, pigs and poultry have been reviewed extensively elsewhere [6,7] Both reviews have concluded that β-agonists can be used to effect nutrient repartitioning so that the composition of growth is altered to give less fat and more lean in the carcass of meat animals

These compounds are orally active and are effective at a concentration of around 0 25 ppm to 4 ppm in feed depending on the species The repartitioning effect is much greater in ruminants than in pigs or poultry, but nevertheless is commercially important for all species On the negative side, β-agonists have also been found to increase the meat toughness in ruminants and poultry Table 1 1 demonstrates the effects of β-agonists on lean and fat values in cattle, sheep, poultry and pigs [7]

Table 1 1 The effects of β-agonists on the lean and fat values of meat producing animals

Animal	Increase in trimmed lean (%)	Reduction in dissectable fat (%)
Cattle	10	30
Sheep	18	41
Poultry	6	17
Pigs	5-10	27

The level of β-agonists required to promote a repartitioning effect ranges from 0 25 ppm to 4 ppm in feed [7], while the therapeutic level for clenbuterol in cattle is 0 8 μg/Kg bodyweight [2] The therapeutic level differs, therefore, from the repartitioning level by an order of magnitude, for example, 0 32 ppm clenbuterol in sheepfeed, given an average weight of sheep of 40 Kg and an average daily consumption of 1 Kg feed, represents 8 μg/Kg bodyweight, which is 10 times the therapeutic level

1.4 β-AGONIST RESIDUES IN TISSUES OF TREATED ANIMALS

The use of compounds with hormonal or thyreostatic action was prohibited within the EU in 1986, except under veterinary prescription **[8]** Nevertheless, several cases of food poisoning were reported in Spain when bovine liver containing high levels of clenbuterol was consumed **[9]** The results of two pharmacokinetic studies on residues of clenbuterol present in the liver, kidney and urine of sheep and veal calves after treatment at levels in excess of the recommended therapeutic dose are shown in Table 1 2 The levels of clenbuterol determined in kidney and liver indicate the potential danger of eating tissues from animals which have been treated illegally with clenbuterol Both of these studies have shown that urine (and other biological fluids such as plasma and bile) contain appreciable levels of clenbuterol during the treatment period, but once a withdrawal period is initiated, the levels of clenbuterol rapidly diminish, and residues only remain detectable in organs and tissues after a three day withdrawal period Urine, and other biological fluids, are useful therefore as analytical samples where "on-farm" sampling is possible and where no withdrawal period has been observed The bovine eye has also been shown to accumulate residues of clenbuterol at levels greater than those found in the liver **[11,12]**, but no pharmacokinetic data are available which cover the accumulation of other β-agonists in this tissue Salbutamol also has been shown to accumulate in the liver of treated animals **[13]**, which would indicate that this organ is a useful matrix for residue analyses The high accumulation of clenbuterol in the eye is offset by the small sample size available (1-2 g), which may not provide enough sample for both screening and confirmatory work

Table 1 2 Concentration of clenbuterol in liver, kidney and urine of ovine and bovine species treated above the recommended therapeutic dose and with no withdrawal period

Animal	Organ	Therapeutic dose (x times)	Clenbuterol (ng/g)	Ref
Lambs	Liver	1	20 2	[10]
		10	162 0	
		25	302 4	
	Urine	1	3 6	
		10	118 7	
		25	141 2	
	Kidney	1	16 4	
		10	83 9	
		25	267 2	
Calves	Liver	6 25	39 0	[11]
	Kidney	6 25	33 0	
	Urine	6 25	44 9	

1.5 CONCLUSION

β-Agonists have both a therapeutic and repartitioning effect in meat producing animals depending on the concentration used Although the use of these compounds as (re)-partitioning agents in the rearing of animals has been banned within the EU, their growth promoting properties ensure that their illegal usage must be continuously monitored Pharmacokinetic studies have revealed that residues of β-agonists accumulate in both the liver and eyes of meat producing animals, and that these residues persist when the treatment programme has ceased These tissues are therefore of major interest in the detection of β-agonists residues, particularly the liver, due to its size and relative accessibility at time of slaughter

1.6 REFERENCES

1 Timmerman, H , in “Beta-agonists and their effects on animal growth and carcass quality”, (ed J P Hanrahan), Elsevier Applied Science, London, 1987, 13

2 Boenisch, B and Quirke, J F , in “Proceedings of the FLAIR Workshops held in Ghent and Thessaloniki, 1992”, (eds H A Kuiper and L A P Hoogenboom, RIKILT-DLO, Wageningen, The Netherlands) ISBN 90-5601-003-4, 102

3 Ricks, C A , Dalrympl, R H , Baker, P K and Ingle, D L , Journal of Animal Science, **59**, 1984, 1247

4 Baker, P K , Dalrymple, R H , Ingle, D L and Ricks, C A , Journal of Animal Science, **59**, 1984, 1256

5 Dalrymple, R H , Baker, P K , Gingher, P E , Ingle, D L , Pensack, J M and Ricks, C A , Poultry Science, **63**, 1984, 2376

6 J P Hanrahan (ed), “Beta-agonists and their effects on animal growth and carcass quality”, Elsevier Applied Science, London, 1987

7 Moloney, A P and Allen, P , in “Proceedings of the FLAIR Workshops held in Ghent and Thessaloniki, 1992”, (eds H A Kuiper and L A P Hoogenboom, RIKILT-DLO, Wageningen, The Netherlands) ISBN 90-5601-003-4, 89

8 Council of the European Communities, Off J Eur Commun , **L275**, 1986, 336

9 Martinez-Navarro, J F , Lancet, **336**, 1990, 1311

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

11 Meyer, H D and Rinke, L M , J Anim Sci , **69**, 1991, 4358

12 Sauer, M J , Pickett, R J H , and MacKenzie, A L , Anal Chim Acta, **275**, 1993, 195

13 Montrade M -P , Riverain S , Le Bizec B and Andre F , in “Proceedings of the FLAIR Workshops held in Ghent and Thessaloniki, 1992”, (eds H A Kuiper and L A P Hoogenboom, RIKILT-DLO, Wageningen, The Netherlands) ISBN 90-5601-003-4, 143

CHAPTER 2

REVIEW OF CURRENT METHODS FOR THE DETERMINATION β-AGONISTS IN BIOLOGICAL MATRICES

2.1 INTRODUCTION

This review focuses on the main stages of a residue detection method, namely,

1 sample pretreatment,

2 clean-up techniques,

3 chromatographic separations (optional),

4 determination

Each of these stages will be discussed with specific reference to the determination of β-agonist residues in biological fluids and tissues

2.2 SAMPLE PRETREATMENT

Sample pretreatment involves the techniques used with biological matrices in order to present them in a suitable format for residue extraction The techniques used in sample pretreatment depend on the matrix under consideration, liquids requiring generally less pretreatment than tissues The tissues are normally first homogenised, and after this step the tissues may be enzymatically digested or sonicated in order to liberate residue from the sample matrix Enzymatic digestion techniques were used initially by Degroodt et al [1] in the analysis of clenbuterol by high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC) In their method a 5 g portion of tissue was incubated with 5 mg subtilisin A and 10 ml 0 2 mol/l Tris buffer, pH 8, containing 0 1 mol/l $CaCl_2$ in a closed 30 ml centrifuge tube for one hour This sample pretreatment technique has since been used in the preparation of clenbuterol extracts prior to enzyme immunoassay (EIA) [2,3,4] and gas chromatography coupled with mass spectrometry (GC-MS) [5]

A sample pretreatment technique using subtilisin A has also been described by Leyssens et al [6] for the analysis of seven β-agonists in liver, the sample extracts being finally analysed by GC-MS Sample pretreatment involving the sonication of homogenised tissue samples in an acidic environment, in order to precipitate proteins, has been described for the analysis of clenbuterol and salbutamol with determination by HPTLC [7], HPLC with fluorescence detection [8], HPLC with ultra violet (UV) detection [1,9] and EIA [10] Other sample pretreatment procedures merely involve homogenisation of the tissue in perchloric acid [11], Sorensen buffer [12] or potassium carbonate [13] prior to further sample cleanup Urine samples do not require an extensive pretreatment step, as centrifugation of the sample [14] or filtration [15,16] generally results in samples suitable for the cleanup procedure

2 2 1 DECONJUGATION OF β-AGONIST RESIDUES PRIOR TO RESIDUE EXTRACTION AND CLEAN-UP

Many drugs and metabolites are present in urine and tissues as conjugates, such as glucuronides or sulphates, which are very polar and essentially not directly extractable into organic solvents Generally it is necessary to hydrolyse these conjugates so as to release the parent molecule for extraction and determination This is usually achieved chemically with hydrochloric acid or sodium hydroxide, or enzymatically with enzymes such as β-glucuronidase, sulphatase or suc d'Helix pomatia (SHP), which contains both β-glucuronidase and sulphatase enzymes

Haasnoot et al [15] showed that clenbuterol is mainly present in urine as the free molecule, and that a deconjugation step involving SHP resulted in lower levels of clenbuterol being determined, probably due to the degradation of clenbuterol during the two hour incubation at 37^0C Both glucuronide and sulphate conjugates of salbutamol have been determined in the urine [17] and tissues [18] of treated calves The use of a mixed enzyme preparation of glucuronidase and sulphatase, such as SHP, is required, therefore, for deconjugation of salbutamol The conditions used for hydrolysis with SHP vary from 2 hours at 37^{0}C [19] to 20 hours at 50^{0}C [18], but most methods use an overnight deconjugation step at 37^{0}C [17,20,21] Metaproterenol conjugates have been chemically hydrolysed in human plasma samples by incubation for 90 minutes at 65^{0}C in an acidic environment [22] Following deconjugation of the β-agonists, the various sample extraction and clean-up techniques discussed below can be applied

2.3 SAMPLE EXTRACTION AND CLEAN-UP TECHNIQUES

Sample extraction and clean-up techniques can be divided into solvent extraction, liquid/liquid partitioning, solid-phase extraction and immunoaffinity chromatography

2 3 1 SOLVENT EXTRACTION

Extraction of the aniline type β-agonists (clenbuterol and mabuterol), which lack phenolic groups on the benzene ring, has been undertaken using polar solvents under alkaline pH conditions Salbutamol, terbutaline and other β-agonists, which have aromatic phenolic groups, are not efficiently extracted with this procedure due to their high polarity Tert-butylmethylether has been used to extract clenbuterol from animal feed, tissues and urine [23], biological fluids [24] and faeces [25] Extraction of clenbuterol from urine [2] and tissues [5,11] using diethyl ether has been reported Mabuterol, cimaterol and clenbuterol were extracted from urine samples with diethyl ether prior to determination by a radioreceptor assay [26] Mixtures of organic solvents, such as diethyl ether *n*-butanol (9 1), have been used to extract clenbuterol from urine [27,28] and mabuterol from plasma samples [29] The increased polarity of the solvent resulted in recoveries of greater than 90% for both compounds Some extractions involve an initial "washing" of the samples at an acidic pH, where clenbuterol is charged and will not leave the aqueous phase The aim is to remove interferences, such as lipids, in the first extraction, after which the pH is adjusted to alkaline conditions and clenbuterol is extracted Petroleum ether [30,10] and tert-butylmethylether [27] have both been used as "washing solvents" After the extraction, the solvent is evaporated and the sample extract is reconstituted in an appropriate solvent prior to analysis by thin layer chromatography (TLC) [31], HPLC with electrochemical detection [29,30] or by immunoassay [24,25] A back extraction step involving the addition of hydrochloric acid to the organic phase is often used to effect clean-up and preconcentration of clenbuterol into a small volume prior to analysis [1,11,32] or further clean-up Further clean-up may involve washing the acidic extract with ethyl acetate hexane (2 1) [12] or hexane and tert-butylmethylether [33] prior to the re-extraction of clenbuterol into an organic solvent under alkaline conditions

2 3 2 ION-PAIR EXTRACTIONS AND SALTING OUT TECHNIQUES

A useful approach to dealing with a highly polar drug such as salbutamol or terbutaline, is to convert it into a neutral ion-associating complex by addition of an excess of suitable ions of opposite charge and extract the analyte into an organic solvent Di(2-ethylhexyl)phosphate (DEHP) has been used to extract salbutamol from plasma and serum as an ion-pair complex into chloroform [34,35] and ethyl acetate [36] The addition of hydrochloric acid to all three organic extracts was used to back-extract salbutamol into a small volume prior to analysis by HPLC or further clean-up [36]

In some cases, a salting out procedure (addition of an excess of salt to the sample before extraction) can shift the partition equilibrium in favour of the extraction Leyssens et al [6] used a mixture of tert-butanol/ethyl acetate to extract seven β-agonists, including clenbuterol and salbutamol, from a liver digest saturated with sodium chloride, prior to determination by GC-MS Salbutamol has also been extracted from human plasma saturated with sodium chloride (at pH 10 6) into ethyl acetate, with determination by GC-MS [37]

2 3 3 ON-COLUMN LIQUID/LIQUID EXTRACTION

Columns containing diatomaceous earth may be used to perform "on-column" liquid/liquid extractions (LLE) The aqueous sample is usually poured into the column, and after an equilibration period, the organic extracting solvent is added to the column and the eluate containing the analyte is collected This form of LLE benefits from having no shaking or centrifugation steps ‘ChemElut’ and ‘Extrelut’ columns are examples of two commercially available materials Sample clean-up using these columns in the extraction of β-agonists involves adjusting the pH of the tissue homogenate or urine sample to pH 10 or above prior to its application to the column

Organic solvents such as hexane [1,38], dichloromethane [39], toluene dichloromethane [32], and tert-butylmethylether [40] have been used to extract clenbuterol from biological fluids and tissues Clenbuterol can be back-extracted from the resulting eluate with hydrochloric acid prior to analysis by HPLC [1,32,38] or further clean-up by solid-phase extraction [40] Alternatively, the eluate has been evaporated, followed by derivatisation of clenbuterol prior to analysis by HPLC [39] Van Ginkel et al [20] used an ‘Extrelut’ column in a sample clean-up step for the analysis of five β-agonists in biological tissues, fluids and animal feed, but poor recoveries of salbutamol from liver samples (40-50%)

were reported This relatively low recovery was probably due to the on-column extraction step which used ethyl acetate as the extracting solvent, in this case the organic solvent did not have the required polarity to extract salbutamol from the hydrophilic environment

2 3 4 SOLID-PHASE EXTRACTION

Solid-phase extraction (SPE) is frequently used in the extraction and clean-up of β-agonists from biological matrices As a fluid sample or sample extract is passed through a SPE column, the β-agonists may be extracted from the sample onto the sorbent material in the column Using a suitable wash, interferences can be removed before the β-agonists are recovered from the column with an elution solvent Four types of SPE, namely reversed-phase, normal phase, ion exchange and mixed-mode, have been applied to the extraction of β-agonists

2 3 4 1 REVERSED-PHASE SOLID-PHASE EXTRACTION

These columns, which contain a non-polar stationary phase and depend on Van der Waals forces, have been used to retain β-agonists from aqueous samples Sample clean-up steps involving C18 reversed-phase SPE as the sole purification step in the analysis of clenbuterol in biological fluids have been reported, with determination by EIA **[10,41,42]** and GC-MS **[43]** Clenbuterol-containing liver samples have also been cleaned-up by C18 SPE following an initial sonication step in hydrochloric acid to precipitate proteinaceous matter **[44,45]**

C18 SPE has been used to clean-up plasma and serum samples containing salbutamol prior to HPLC analysis with fluorescence **[46]** and electrochemical detection **[47]** These columns were used for the extraction of fenoterol, terbutaline and salbutamol from plasma samples with determination by GC-MS **[48]**

Reversed-phase SPE columns have often been used in conjunction with other clean-up techniques Urine samples containing salbutamol have been cleaned-up using C18 SPE prior to LLE with tert-butylmethylether [36] Similarly, both fenoterol and salbutamol were extracted into ethyl acetate using DEHP following preliminary clean-up of a serum sample with a C18 cartridge [48] SPE clean-up steps have been reported also for the purification of clenbuterol-containing sample extracts following on-column LLE [40] and immunoaffinity column clean-up [49], or prior to immunoaffinity column clean-up in the analysis of liver samples for four β-agonists (clenbuterol, salbutamol, terbutaline and mabuterol) with determination by EIA [50] The broad applicability of reversed-phase SPE for the clean-up of β-agonists in various biological matrices is reflected in its use both as the unique, and also as a preliminary or final, clean-up step

2 3 4 2 NORMAL PHASE SOLID-PHASE EXTRACTION

Silica SPE columns, for which the major retention mechanism is based on hydrogen bonding and dipole-dipole interaction, have been used to extract salbutamol [51] and terbutaline [52] from plasma samples, with determination by HPLC with fluorescence detection Fuerst et al [23] used a silica SPE column to clean-up tert-butylmethylether extracts of animal feed, tissues and urine containing clenbuterol, while Schmitz et al [53] also used a silica column to further purify tissue and urine extracts containing clenbuterol prior to HPLC analysis

2 3 4 3 ION EXCHANGE SOLID-PHASE EXTRACTION

Ion exchange SPE columns, such as the 'XAD-2' variety, have been used for the extraction of salbutamol from plasma [54] and urine [55] prior to determination by mass spectrometry Lindberg et al also used ion exchange columns for the clean-up of plasma samples containing terbutaline [56] and salbutamol [57] In general, these columns are used for the extraction of polar molecules such as salbutamol and terbutaline, while samples containing the less polar β-agonists are usually cleaned up using reversed-phase SPE

2 3 4 4 MIXED-MODE SOLID-PHASE EXTRACTION

Mixed-mode SPE columns have been designed to provide two or more primary interactions for retention of β-agonists from biological matrices These columns have both ion exchange and reversed-phase retention characteristics, and allow for the use of stronger washes The dual retentive character of these columns has been exploited successfully in the retention of both types of β-agonists (aniline and phenolic types) from biological fluids and tissue extracts Both 'BondElut Certify' and 'Clean Screen DAU' mixed-mode SPE columns were used by Dumasia et al [58] in the analysis of clenbuterol in horse urine GC-tandem mass spectrometry was used to analyse for seven β-agonists in bovine urine and liver extracts following a solid-phase extraction clean-up step using 'BondElut Certify' columns The liver samples, following enzymatic digestion, were cleaned-up by liquid/liquid and solid-phase extraction prior to their application to the mixed-mode SPE columns Poor recoveries for salbutamol (<35%) and terbutaline (<25%) from urine and liver matrices were reported, but higher recoveries of clenbuterol (>50%) and mabuterol (>80%) were achieved from both matrices [6] Thirteen β-agonists were determined at the low ng/ml level in bovine urine by GC-MS using 'Clean Screen DAU' mixed-mode SPE columns as the clean-up step The recoveries of salbutamol and terbutaline were 22% and 36%, respectively, while higher recoveries were achieved for clenbuterol (68%) and mabuterol (73%) [14] These columns were also used in a study of salbutamol metabolism in calves to purify both urine and tissue samples prior to analysis by GC-MS [18] High recoveries of terbutaline (66%), clenbuterol (90%), and salbutamol (85%) were reported by Gambiola et el [59] for extraction from urine using 'BondElut Certify' columns with determination by EIA The suitability of mixed-mode solid-phase extraction columns for the extraction of both types of β-agonists from biological tissues and fluids requires the problem of low recovery for some of the more polar β-agonists to be addressed However, the multi-residue extraction capability of these columns, coupled with the potential for extensive washing, indicates their potential in the development of multi-residue extraction techniques for β-agonists

2 3 5 IMMUNOAFFINITY COLUMN CHROMATOGRAPHY

Immunoaffinity column chromatography (IAC) has been applied to the determination of residues of β-agonists in biological extracts Antibodies raised against the β-agonists, when immobilised on a solid support and packed into a precolumn, can be used for the selective extraction of the analytes Antibodies may be immobilized on solid supports in different ways, such as by means of physical adsorption, affinity adsorption or covalent binding to a stationary phase The β-agonists may be eluted with either buffers or water-organic modifier mixtures Buffers change the ionic retention interactions, while organic modifiers denature the antibodies, with consequent release of the β-agonists Urine samples containing clenbuterol have been cleaned up using IAC prior to EIA analysis, recovery of clenbuterol was 80% and a limit of detection of 0 2 ng/ml was reported [16] Liver, kidney and bile samples were analysed also using this column [3] and with a commercially available IAC kit [4] which achieved a limit of detection of 0 5 ng/g Confirmatory analysis of clenbuterol in urine was carried out by GC-MS following a combined clean-up using IAC and SPE (C18) techniques [49] Salbutamol also has been extracted from lyophilized rat tissue [60] and liver samples [8] using an IAC purification step yielding recoveries of greater than 60%

Van Ginkel and co-workers [20] used polyvalent antibodies raised against clenbuterol and cimaterol in an IAC column to retain terbutaline, salbutamol, clenbuterol, mabuterol and cimaterol from tissues, urine and animal feed Two antibodies were used on the column, one retained β-agonists with tert-butyl end groups, while the other retained cimaterol which has an iso-propyl end group The recovery for salbutamol from liver (40-50%) was less than the recovery for the other four β-agonists (65 ± 8%) Schilt et al [71] used an IAC column containing anti-clenbuterol antibodies with relative cross-reactivities of 3 3%, 3 3% and 2 8% for salbutamol, cimaterol and terbutaline, respectively The lowest detection levels for a positive identification of the analytes in feed, urine and liver samples were 10 ng/g, 3 ng/ml and 1 ng/g, respectively A combination of C18 SPE and IAC has been used to extract four β-agonists from urine with good recoveries for clenbuterol (75%) and salbutamol (65%) being achieved [50] From these examples it can be seen that IAC columns are suitable for extraction of both aniline and phenolic type β-agonists from both biological fluid and tissue samples The selectivity of the retention mechanism, which relies on a highly specific antibody-antigen interaction, is an important feature of IAC, as opposed to other clean-up techniques such as solvent extraction or SPE, which are dependent on the affinity of the analyte(s) for one phase (solid or liquid) over another

2.4 CHROMATOGRAPHIC SEPARATION TECHNIQUES

2 4 1 HPLC SEPARATION SYSTEMS

The majority of HPLC separation techniques for β-agonists utilise reversed-phase (RP) columns, with some use of normal phase columns Chicken liver [9] and rat brain [11] extracts containing clenbuterol have been separated from interferences on reversed-phase C8 and C18 columns, respectively, while plasma [61] and liver [8] samples containing salbutamol have been separated from interferences on reversed-phase columns prior to fluorescence detection

Ion pairing agents such as sodium heptane-sulfonic acid have been used in conjunction with RP columns for the analysis of clenbuterol in plasma [29] and pharmaceutical gels [62], and also for the analysis of salbutamol in serum [36] and plasma [63] samples HPLC mobile phases containing perchloric acid were used to separate salbutamol and terbutaline from plasma on a cation exchange column [52] The perchlorate ion, which forms an ion-pair with the β-agonists, was also used in a column switching assay to separate salbutamol on a RP C2 column following its elution from a cation exchange preconcentration column [64]

A cyano (CN) loop column on a rheodyne valve was used to purify plasma extracts containing salbutamol and fenoterol through a 1 ml water wash prior to separation on a C18 analytical column with a perchlorate ion-containing mobile phase [65] Ion pair buffers containing sodium dodecyl sulphate and acetic acid (pH 3 5) have been used with acetonitrile as the organic modifier to separate clenbuterol and cimaterol in tissues, animal feed and faeces extracts [32] and clenbuterol in urine extracts [40] A liquid chromatographic column switching system for automated sample pretreatment and determination of clenbuterol in calf urine, consisting of an IAC precolumn, a C18 preconcentration column and a RP analytical column, also used a sodium dodecyl sulphate

(SDS)/acetic acid ion pair buffer in the elution step from the C18 column and in the analytical separation step **[15]** Plasma samples containing terbutaline and salbutamol at overdose levels have been analysed recently with sample clean-up in a multidimensional column chromatographic method The sample was extracted, purified and concentrated on the C18 precolumn prior to separation on the analytical column using a mobile phase containing 6 5 mM SDS **[66]**

Normal phase retention mechanisms have been used to separate salbutamol in rat tissue **[60]** and plasma extracts **[34,51]** with fluorescence detection Enantiomeric forms of salbutamol, following derivatisation **[68]**, have been separated using chiral HPLC phases **[67]** and RP columns but it is important to note that most residue analysis schemes are concerned with the detection and quantification of β-agonists in a biological sample and that the isomeric form of the analyte is of secondary importance

2 4 2 GAS CHROMATOGRAPHIC SEPARATION SYSTEMS

Due to the low volatility of β-agonists and the possibility of adsorption of these polar compounds to the GC column, derivatisation is required prior to any gas chromatographic step Silylating agents such as N-O-Bis(trimethylsilyl)triflouro-acetamide (BSTFA), N-methyl-N-Trimethylsilyltrifluroacetamide (MSTFA), N-methyl-N(tert-butyldimethylsilyl) trifluroacetamide (MTBSTFA), chloromethyldimethyl-chlorosilane (CMDMCS) and hexamethyldisilazane (HMDS) have been used to derivatise β-agonists Other derivatisation agents, such as pentafluropropionic anhydride (PPA), phosgene, and boronic acids, have been applied successfully to GC separation of β-agonists Table 2 1 lists the numerous derivatisation agents used for gas chromatographic separation of β-agonists extracted from biological fluids and tissues The determination technique most commonly used in combination with gas chromatographic separation is mass spectrometry The fragmentation patterns of the various derivatives and their suitability to mass spectrometric determinations are discussed in the section GC-MS determination (2 5 4 1)

Table 2 1 References to derivatisation agents used in the analysis of β-agonists by GC separation

Derivatisation agent	Clenbuterol	Salbutamol	Multi-residue
BSTFA	[9,49,69]	[18,48,54]	[14,20,53,70,71]
MSTFA	[72]		[6,58,59]
MTBSTFA	[49]		
CMDMCS			[14,58]
HMDS	[23,33]	[73]	
PPA	[12]		
Phosgene	[13]		
Boronic acid	[5,27]		[74]

2 4 3 THIN LAYER CHROMATOGRAPHIC SEPARATIONS

TLC and HPTLC methods for the separation of β-agonists have all used chromatographic plates coated with silica gel 60 HPTLC has been used to separate clenbuterol and cimaterol from biological fluid and tissue extracts [32,44] and clenbuterol from urine and tissue extracts [1], while conventional TLC has been applied to clenbuterol analysis in urine [31,75] Extracts of liver [7] and urine [76] containing salbutamol have been separated using HPTLC techniques with quantification by densitometry

2.5 DETERMINATION TECHNIQUES

The determination methods which can be applied to cleaned up extracts comprise the following:

1. spectroscopic determination,
2. electrochemical determination,
3. immunoassay determination,
4. mass spectrometric determination.

2.5.1 SPECTROSCOPIC DETERMINATION

2.5.1.1 APPLICATIONS OF HPTLC AND TLC

Absorption microdensitometry has been used to detect an indoaniline derivative of salbutamol in liver [7] and urine [7,76] extracts at 650 nm with limits of detection of 2 ng/ml and 1 ng/g in urine and liver, respectively. The indoaniline dye was formed by the reaction of salbutamol with dimethyl-p-phenylenediamine to form 4-{[4-(dimethylamino)phenyl]imino}-2(hydroxy-methyl)2,5 cyclohexadien-2one which was separated from interferences on a silica gel 60 TLC plate prior to quantification (Figure 2.1). Detection of clenbuterol and cimaterol residues in animal tissues and feed using absorption microdensitometry, after the TLC plates were sprayed with modified Ehrlichs reagent, have resulted in detection limits of 0.25 ng/g and 0.5 ng/g for cimaterol and clenbuterol, respectively, in liver extracts [44] and an even lower detection limit for clenbuterol in animal tissues and feed of 0.2 ng/g [32].

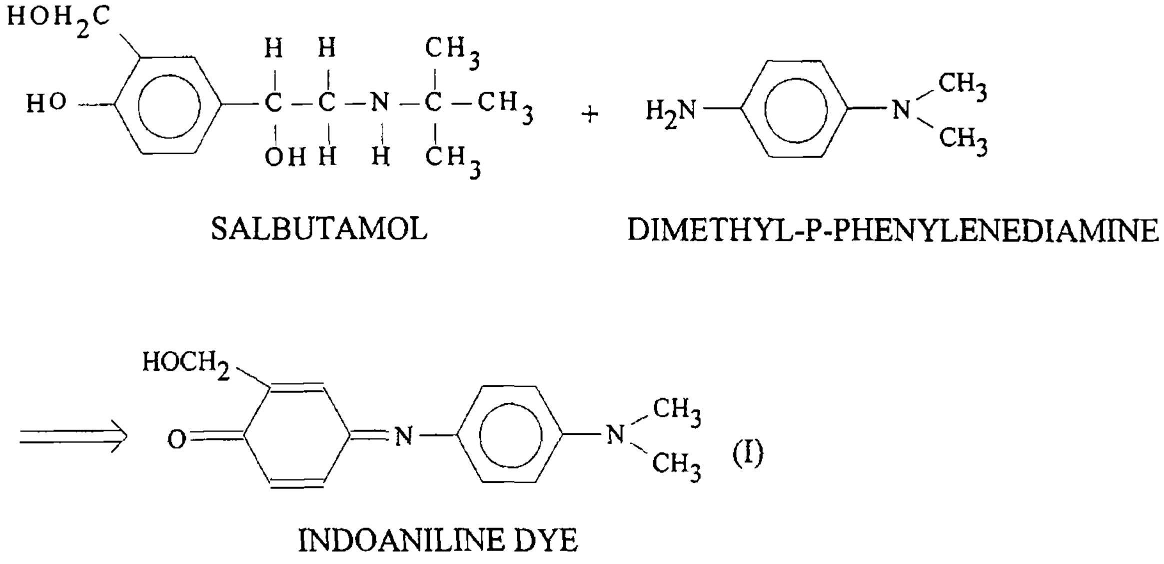


Figure 2.1 Reaction of salbutamol with dimethyl-p-phenylenediamine to form an indoaniline dye

2.5.1.2 APPLICATIONS OF HPLC WITH UV DETECTION

UV absorption spectrophometry has been applied to the quantification of β-agonists both directly and after derivatisation. Direct UV detection has been applied to clenbuterol determination in urine and liver extracts. Although limits of detection of 0.5 ng/ml [15,40] have been achieved for urine analysis, the reported detection limits for muscle of 33 ng/g [11] and for liver extracts of 2 ng/g [9], which are above the 0.5 ng/g maximum residue limit (MRL) for clenbuterol, lack the required sensitivity for residue analysis. The poor sensitivity in conventional HPLC-UV determination of β-agonists has led to the development of derivatisation procedures which have decreased the limits of detection of clenbuterol by an order of magnitude. A pre-HPLC derivatisation procedure, involving the reaction of clenbuterol with N,N-dimethylaniline to form dimethylaniline-clenbuterol (Figure 2.2), has been used to detect clenbuterol in urine and vitreous humour extracts. The detection limit of the initial method for clenbuterol in urine was 1 ng/ml [77] but this limit was further decreased to 0 4 ng/ml in the analysis of both vitreous humour and urine extracts [39]

Figure 2 2 Clenbuterol-dimethylaniline derivative

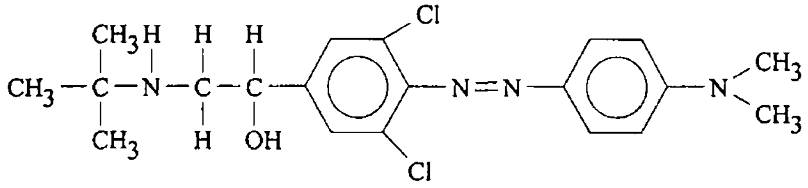
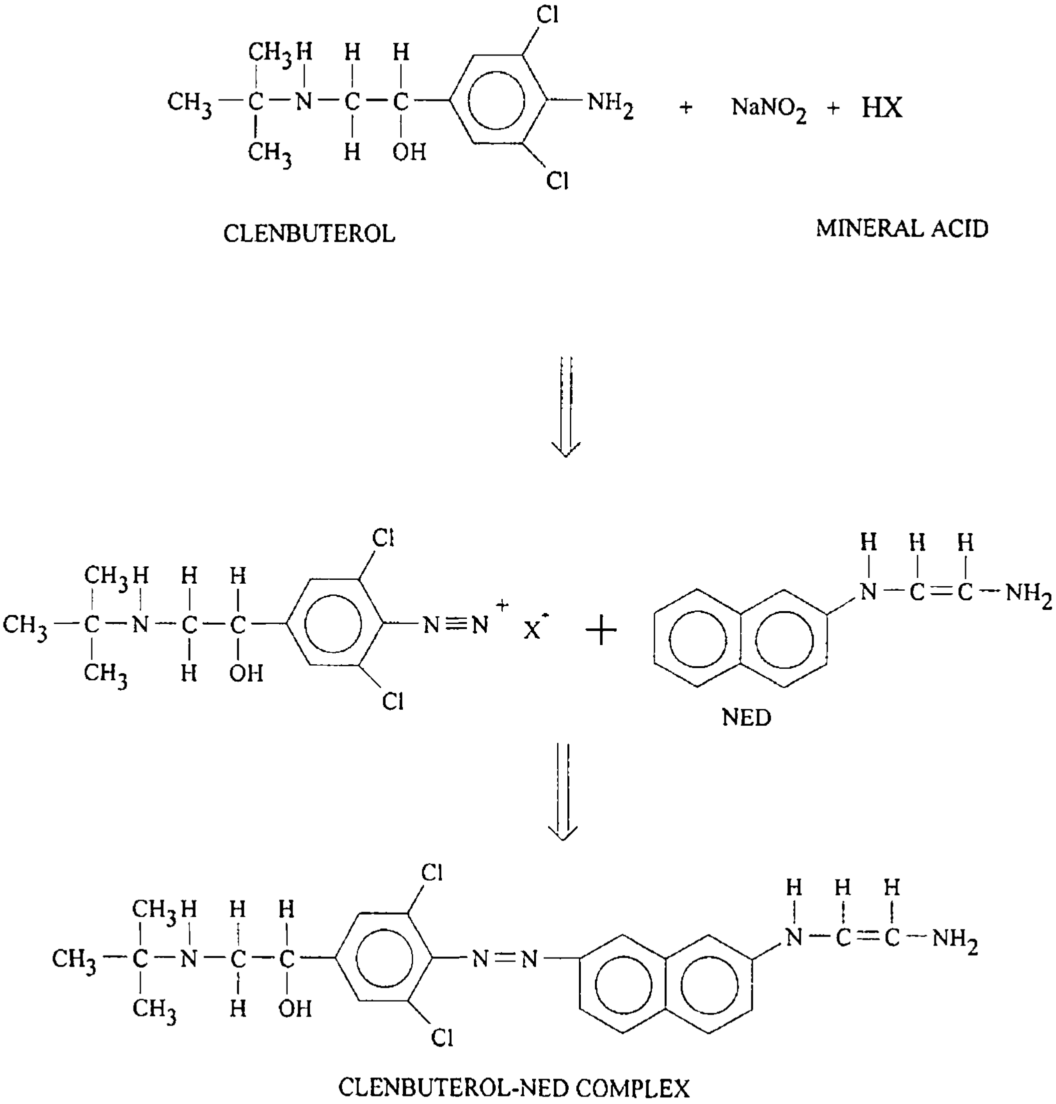


Figure 2 3 Formation of a clenbuterol diazonium salt



Post-column derivatisation reactions have been used also to analyse for both clenbuterol and cimaterol residues in biological fluids and tissue extracts The derivatisation reaction involves the coupling of clenbuterol and cimaterol to *N*-1-napthylethylene diamine (NED) This reaction, which is based on the formation of a diazonium salt of clenbuterol and/or cimaterol under acidic conditions in the presence of nitrite ions prior to the coupling stage, is shown in Figure 2 3 The derivatised analytes result in very low limits of detection being achieved (Table 2 2)

Table 2 2 Limits of detection for clenbuterol and cimaterol in biological fluids and tissue extracts by UV detection following post column derivatisation with *N*-1-naphthylethylene diamine

Matrix	β-agonist	Detection wavelength (nm)	Limit of detection
Tissues, feed, faeces and biological fluids [32]	Clenbuterol Cimaterol	493 537	0 1 ng/ml (liquid) 0 2 ng/g (solid)
Liver [44]	Clenbuterol Cimaterol	496 536	0 5 ng/g 0 25 ng/g
Urine and tissues [1]	Clenbuterol	500	0 25 ng/ml (liquid) 0 5 ng/g (solid)

2 5 1 3 APPLICATIONS OF HPLC WITH FLUORESCENCE DETECTION

Spectrofluorometric methods of analysis have been applied to the determination of many β-agonist residues in biological samples Fluorescence is one of several mechanisms by which a molecule returns to the ground state after it has been excited by absorption of radiation Fluorescence is expected from molecules which contain an aromatic ring, however, the presence of electron-withdrawing groups on the aromatic ring such as chlorine (clenbuterol) and flourine (mabuterol) decrease fluorescence capability dramatically. This explains why most HPLC-fluorescence detection systems have been applied to salbutamol, which contains no electron-withdrawing groups.

Fluorescence detection of salbutamol in plasma extracts has achieved limits of detection ranging from 1 ng/ml **[35,51,78]** to sub ng/ml levels **[34,46,61]**. Salbutamol in liver extracts, following IAC clean-up and separation on a C18 column, has been determined with a limit of detection of 1 ng/g **[8]**.

2.5.2 ELECTROCHEMICAL DETERMINATION

Electrochemical detection, in combination with HPLC, has been used to detect several β-agonists, mainly in biological fluids, at the low and sub ng/ml level. Both fenoterol and salbutamol were determined in plasma extracts using a dual carbon electrode system **[65]**. The first electrode, operating at 900 mV, was used to oxidise and detect both β-agonists, while the response of the second electrode operating at 500 mV was subtracted from the response of the first electrode in order to enhance the signal to noise ratio of the system. Limits of detection of 0.5 ng/ml and 1.25 ng/ml were reported for salbutamol and fenoterol, respectively. A similar method has also been applied to salbutamol determination in serum samples with a limit of detection of 1ng/ml **[47]**. Detection methods involving a single carbon electrode have also been successfully applied to the determination of salbutamol in serum **[36]**, plasma **[66]** and lyophilized rat carcass **[60]** with limits of detection of 0.8 ng/ml, 1 ng/ml and 0.1 ng/g, respectively.

An interesting combination of both UV and ECD detection systems following HPLC separation of clenbuterol was recently reported **[40]**. The ratio of electrochemical to UV absorbance signals was used to increase specificity, but due to the progressive decrease of the electrochemical response, ECD detection could only be applied for a few consecutive analyses Clenbuterol and mabuterol have been analysed also in equine plasma using a carbon electrode operated at 750 mV with limits of detection of 0 5 and 2 ng/ml, respectively [29]

2 5 3 IMMUNOASSAY DETERMINATION

Both radioimmunoassay (RIA) and enzyme immunoassay (EIA) techniques have been applied to the determination of β-agonists in biological matrices

The principle of radioimmunoassay is based on competition between a fixed amount of radiolabelled antigen and the analyte (present in standards or sample extracts) for binding to a limited amount of antibody After incubation, the free and antibody-bound fractions are separated and the concentration of radiolabelled material in either the antibody-bound or free fraction is determined If a quantity of analyte is present in either a standard solution or sample extract, it will compete with the labelled antigen for the limited number of binding sites on the antibodies present A standard curve is derived and the concentration of analyte in sample extracts is determined by interpolation

EIA techniques typically involve the coating of a β-agonist specific antibody on microplate wells, followed by competition between the β-agonist (as analyte standard or in sample extract) and enzyme-labelled β-agonist for binding to the antibody After a washing step, the enzyme substrate is added, which forms a coloured reaction product The amount of antibody-bound enzyme-labelled β-agonist is quantified by absorption spectrophotometry at a suitable wavelength and this quantity is inversely related to the amount of analyte present as a standard or in a sample extract

Salbutamol has been quantified in biological fluids using both EIA and RIA techniques Adam et al used monoclonal salbutamol antibodies to quantify salbutamol in urine by RIA [79] and later by EIA [80] Polyclonal antibodies have also been used to determine salbutamol in both urine [17] and plasma [81] samples by RIA The former method used ^{3}H-clenbuterol as the tracer, while the latter used ^{3}H-salbutamol Zimmer et al [93] were the first to use a radioimmunoassay, with a standard curve of 0-40 ng/tube, in pharmacokinetic studies on clenbuterol that had been administered to rats and dogs Delahaut et al determined clenbuterol at the sub ng/ml range in plasma, urine and faeces using a radioimmunoassay specific for clenbuterol [25] The antibody for this procedure was developed using the method of Yamamoto et al [83] and involved the immunisation of rabbits with diazotized clenbuterol human serum albumin conjugate The cross-reactivity of the harvested antibody relative to clenbuterol (100%) was 9%, 8% and 3% for terbutaline, salbutamol and cimaterol, respectively This radioimmunoassay was used in our laboratory to determine clenbuterol in liver samples following a matrix solid-phase dispersion clean-up step [82] This procedure was later adapted into a multi-residue method capable of determining salbutamol, clenbuterol, mabuterol, terbutaline and cimaterol at levels of less than 1 ng/g [19]

The literature describes a number of clenbuterol specific enzyme immunoassays, consisting of assays involving one immunogenic recognition and those involving two immunogenic recognitions The latter method, referred to as a double antibody technique, was initially used by Yamamoto and Iwata [83] for the analysis of clenbuterol in plasma with determination by fluorescence spectrometry at 450 nm The double antibody technique involves the competitive binding of clenbuterol and enzyme-labelled clenbuterol to antibody in solution A second antibody which binds the clenbuterol-specific antibodies is coated on the microplate wells and retains the clenbuterol antibodies (together with any bound clenbuterol or enzyme-labelled clenbuterol), while the unbound clenbuterol and enzyme-labelled clenbuterol are eliminated in the washing step Enzyme-labelled salbutamol has also been used as the competing enzyme-labelled β-agonist in the determination of clenbuterol in urine [41], liver [86] and animal tissues [10] The use of enzyme-labelled salbutamol as opposed to enzyme-labelled clenbuterol resulted in lower limits of detection being attained

Enzyme immunoassays, using a single antibody, have been used to determine clenbuterol in urine using either clenbuterol phosphatase [16,42] or clenbuterol horseradish peroxidase enzyme labels [2,24,87] The same enzyme-labelled β-agonists have been used also in the analysis of liver and tissue extracts for clenbuterol, using colour forming substrates such as tetramethylbenzidine [2,86] and o-phenylene diamine perhydrol [24] Bucknall et al [86] used a salbutamol horseradish peroxidase label for the analysis of liver and urine extracts for clenbuterol, the lower cross-reactivity of the salbutamol horseradish peroxidase label, compared to the clenbuterol analyte, allowed for use of a larger sample size in the assay giving a low limit of detection of 0 25 ng/ml

Multi-residue β-agonist enzyme immunoassays have been applied to the analysis of urine samples No clean-up steps were required prior to the EIAs, apart from dilution of the urine samples [87,88,89] Haasnoot et al [88] determined five β-agonists in an enzyme immunoassay which used salbutamol horseradish peroxidase as the enzyme label and tetramethylbenzidine as the colour forming substrate Clenbuterol and mabuterol could be determined at the 1 ng/ml level, while terbutaline, cimaterol and salbutamol were detectable at the 5 ng/ml level The antibody employed in a multiresidue technique must have a high cross-reactivity for several β-agonists, the higher the cross-reactivity the more sensitive the assay The importance of the cross reactivity of antibodies in a multi-residue determination is shown by the limits of detection achievable for the various β-agonists (Table 2 3)

Table 2 3 Cross-reactivities of antibodies used in β-agonist enzyme immunoassays and the corresponding limits of detection (LOD) for the various β-agonists

β-agonist	CROSS-REACTIVITY OF ANTIBODY USED					
	Anti-salb[87]	LOD	Anti-salb[88]	LOD	Anti-salb[89]	LOD
clenbuterol	100%	0 14 ng/g	100%	1 ng/ml	100%	0 3 ng/ml
salbutamol	115%		5%	5-10 ng/ml	28%	0 9 ng/ml
mabuterol	65%		140%	1 ng/ml	95%	0 3 ng/ml
tertbutaline	31%		4%	5-10 ng/ml	21%	1 3 ng/ml
cimaterol	13%		4%	5-10 ng/ml		

A radioreceptor assay for the detection of clenbuterol, mabuterol and cimaterol in bovine urine samples based on competition for binding to β-adrenergic plasma membrane receptors and using a radio-labelled tracer (^{3}H-dihydroalprenolol) has been described and has a limit of detection of 2 4 ng/ml for clenbuterol [26]

2 5 4 MASS SPECTROMETRIC DETERMINATION

2 5 4 1 APPLICATIONS OF GC-MS

In comparison to classical chromatographic detection systems which rely largely on retention time and to immunoassays which rely on antibody analyte interaction, mass spectrometry provides unambiguous confirmation of the identity of the analyte(s) in a sample due to its ability to detect molecular ions and related fragments Combination of the resolving power of chromatography with mass spectrometry gives a uniquely effective tool for the identification and quantitation of analytes in sample extracts Trimethylsilyl derivatives of clenbuterol **[9,49,69]** and salbutamol **[48,54]** have been analysed by GC-MS in both electron impact (EI) and positive ion chemical ionization (CI) modes N-O-Bis(trimethylsilyl)-trifluroacetamide, which was used in preparing the trimethylsilyl derivatives, has also been used in multi-residue GC-MS analysis of β-agonists **[14,20,59,70,71]** Figure 2 4 shows the fragmentation patterns observed in the analysis of TMS derivatives of β-agonists

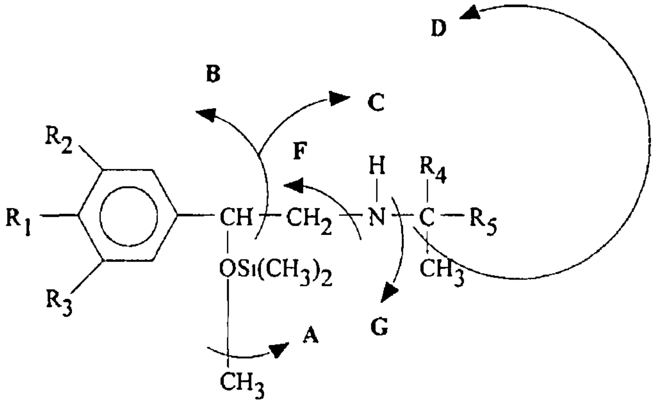


Figure 2 4 Main fragmentations observed in mass spectra of β-agonist TMS derivatives

The m/z weight of fragment C is either 86 or 72 depending on whether the β-agonist contains a tert-butyl end group or an isopropyl end group (cimaterol) Several multi-residue methods focus on the detection of fragments B and C for screening purposes **[14,20,59,71]** and confirmation is based on the detection of four fragment ions **[20,59,70]** One interesting method uses a combination of results from GC-MS (EI) and GC-MS (CI) analyses in order to confirm β-agonist presence in sample extracts if the GC-MS (EI) procedure cannot alone provide sufficient structural information **[71]** The limits of detection achieved with this method for feed, urine and liver were 10 ng/g, 3 ng/ml and 1 ng/g, respectively

N-methyl-N(tert-butyldimethylsilyl)-trifluroacetamide (MSBSTFA) has also been used to generate a tert-butyldimethylsilyl derivative of clenbuterol which was analysed simultaneously with a TMS derivative of clenbuterol by GC-MS in both the EI and CI mode The simultaneous analysis of two different derivatives allowed for the determination of four diagnostic ions at levels of 10 ng/g and above The limit of detection based on the m/z 86 fragment was 0 5 ng/g [49]

Hexamethyldisilazane, another TMS derivatising agent, has been used in the analysis of clenbuterol and salbutamol using GC-MS in the CI mode Ammonia was used as the reagent gas for the analysis of clenbuterol in urine samples Single ion monitoring (SIM) was used to detect the clenbuterol ion $[M+H]^+$ at m/z 349, while the internal standard ion D_9-clenbuterol was monitored at m/z 358 [33] The same ions were monitored in a method for clenbuterol determination in animal tissue and feed, as well as in urine, by Furst et al with sub ng/g limits of detection [23] Using methane as the reagent gas, Furst and co workers also determined salbutamol in urine samples The salbutamol-TMS ions monitored were m/z 440 4, 441 4, 456 4 and 366 3 An internal standard, D_9-clenbuterol-TMS, was monitored at m/z 358 2 and 360 2 [73]

Trimethylsilyl derivatives of clenbuterol formed using N-methyl-N-trimethylsilyl-trifluroacetamide, have been used in the GC-MS (EI mode) for the analysis of biological tissues and feed [53] and horse urine [58] Again, the most abundant fragment ion was m/z 86, but fragment ions of higher mass were monitored at m/z 334, 335, 337 and 405 for confirmation purposes [53] Leyssens and co workers analysed trimethylsilyl derivatives of eight β-agonists by GC-tandem mass spectrometry [6] Identification was based upon (1) retention time, (2) the presence of a $[M+H]^+$ ion for each β-agonist and (3) the ratio of two or three fragment (daughter) ions generated by collision-induced dissociation of the corresponding $[M+H]^+$ ion GC-MS-MS techniques such as this benefit from lower background signals during analysis and consequently have lower limits of detection The disadvantage of TMS derivatives of β-agonists is that the most abundant ions attained with TMS derivatives are also the ions of lowest molecular weight and this imposes limitations on the capacity to provide confirmatory analysis

Cyclic 2-(dimethyl)silamorpholine (CDMS) derivatives of β-agonists have been analysed by GC-MS in EI mode **[58]** and in both EI and CI modes **[14]** The derivatising agent, chloromethyldimethylchlorosilane, involves an intra-molecular alkylation and the formation of a heterocycle which is stable for over one week at 4^0C The use of cyclic DMS derivatives allowed for the detection of a large number of characteristic ions for thirteen β-agonists The fragmentation patterns, using EI ionization techniques, were very specific giving M^+, $[M-15]^+$, $[M-29]^+$ and $[M-57]^+$ ions and provided more informative data than TMS derivatives Figure 2 5 shows a cyclic DMS clenbuterol derivative

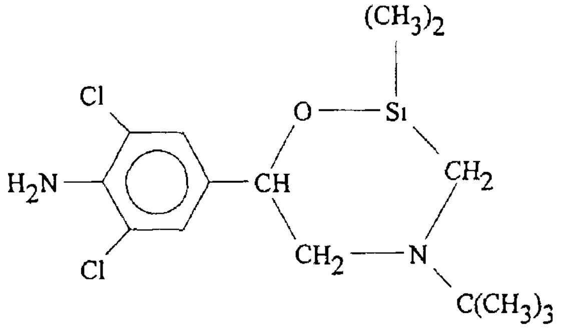
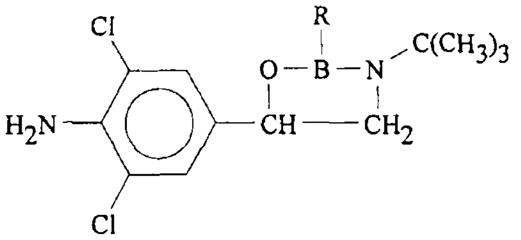


Figure 2 5 Cyclic DMS derivative of clenbuterol

Boronic acid derivatives of clenbuterol have been analysed by GC-MS in EI mode (Figure 2 6) Polettini and co workers used 1-butane boronic acid for the analysis of clenbuterol in urine samples **[27]** The most abundant ions were in the high mass range at m/z 243, 327 and 342 and the limit of detection was 0 5 ng/ml Determination of clenbuterol in liver, muscle, retina and urine extracts by GC-MS (EI mode) was achieved using a methylboronic acid derivative of clenbuterol **[5]** The most prominent ions were m/z 243, 285 and 300 The m/z 300 ion is the molecular ion and the m/z 285 fragment is the molecular ion minus a methyl group The m/z 243 fragment is due to the additional loss of $OBCH_3$. The closeness of the three ions and their relatively high mass allowed for monitoring of clenbuterol residues using single ion monitoring. The detection limit for the method was reported at 0.05 ng/g using high resolution mass spectrometry.



$R=C_4H_9$ butane boronic acid derivative, $R=CH_3$ methyl boronic acid derivative

Figure 2.6 Boronic acid derivatives of clenbuterol

Methylboronic acid and N-butylboronic acid derivatives of salbutamol were also analysed using GC-MS in EI mode [74]. The methylboronic acid derivative was preferred as it did not degrade the GC column and was easier to make. The ions monitored were m/z 287, 272 and 230, the first being the molecular ion while the other two ions resulted from the loss of a methyl $[M-15]^+$ group and of methyl and $OBCH_3$ groups $[M-57]^+$, respectively. Girault et al. achieved very low limits of detection for clenbuterol in bovine plasma and tissue extracts [12]. Following derivatisation with pentafluoropropionic anhydride, clenbuterol and the internal standard D_9-clenbuterol were measured by GC-MS in negative ion chemical ionization mode using methane as the reagent gas. The mass spectrometer was set to monitor m/z 368 and m/z 377 ions of the pentafluracyl derivatives. For tissue samples the limit of detection was 10 pg/g. Phosgene has been used also to derivatise clenbuterol to form a cyclic oxazolidine derivative [13]. The ions monitored were the molecular ion at m/z 302 and two fragments, $[M-59]^+$ and $[M-101]^+$. The confirmation criteria for the presence of clenbuterol in extracts were based upon the retention time and the ion ratios for samples being comparable to those for the standard material A limit of detection of 0 25 ng/g was achieved

2 5 4 2 APPLICATIONS OF LC-MS

Liquid chromatography coupled to mass spectrometry has been used to determine clenbuterol and salbutamol-sulphate in urine samples In the former case, the LC-MS was operated in the positive ion acquisition mode, monitoring the molecular ion at m/z 277, and a limit of detection of 1 ng/ml was obtained **[90]** Fast atom bombardment (FAB) and thermospray mass spectroscopy were used in the latter analysis, both in the negative ion monitoring mode Only thermospray proved successful, however, as too many interfering peaks were present with the FAB technique The ions monitored were m/z 318 and 321 representing the $[M-H]^-$ ion for the salbutamol-sulphate and its deuterated analogue respectively **[55]**

Debrauwer et al have investigated the fragmentation patterns for clenbuterol **[28]** and other β-agonists **[91]** in LC-MS systems using an electrospray interface with collision-induced disassociation of the charged ions formed Fragmentation patterns could be controlled through adjusting the voltage difference between the glass capillary transfer and the skimmer region The fragmentations observed mainly involved a loss of water from the side chain hydroxy group and cleavages at the secondary amine function

A thermospray interface was used by Van Rhijn et al in the LC-MS-MS analysis of clenbuterol and other β-agonists **[92]** This method monitored both the molecular ions of the β-agonists and also a common fragmentation giving rise to the loss of 74 mass units The analysis was performed by flow injection, without a chromatographic step, resulting in a rapid method with simultaneous detection of a range of β-agonists

2.6 CONCLUSION

A variety of extraction and clean-up techniques may be used in the analysis of individual β-agonists in biological matrices, but most multi-residue techniques rely on either immunoaffinity chromatography, mixed-mode solid-phase extraction or matrix solid-phase dispersion to efficiently extract both the phenolic and aniline type β-agonists simultaneously

Due to differences in the spectroscopic properties of the more commonly used β-agonists, (clenbuterol and mabuterol are detectable by UV spectrophometry while salbutamol and terbutaline are detected using spectrofluorometry), immunoassays and MS determination techniques are the preferred multi-residue detection methods for the analysis of β-agonists in biological matrices The good sensitivity associated with immunoassay techniques has resulted in their widespread use in multi-residue determination procedures Immunoassays can determine a number of β-agonists, depending on the cross-reactivity of the antiserum used, but these techniques are not confirmatory In order to provide sufficient information to satisfy confirmation requirments, GC-MS and LC-MS(-MS) techniques are used

2.7 REFERENCES

1 Degroodt, J M , Wyhowski de Bukanski, B , Beernaert, H and Courtheyn, D , Z Lebensm Unters Forsch , **189,** 1989, 128

2 Elliott, C T , McCaughey, W J and Shortt, H D , Food Addit Contam , **10**, 1993, 231

3 Sauer, M J , Pickett, R J H , Dixon, S N and Jackman, R in "Proceedings of the EuroResidue II Conference, Veldhoven, The Netherlands, May 3-5, 1993" (eds N Haagsma, A Ruiter and P B Czedik-Eysenberg), CIP-Gegevens Koninklijke Bibliotheek, Den Haag, ISBN 90-6159-016-7, 622

4 Mc Connell, R I , Lamont, J V , Campbell, J and Fitzgerald, S P in "Proceedings of the EuroResidue II Conference, Veldhoven, The Netherlands, May 3-5, 1993" (eds N Haagsma, A Ruiter and P B Czedik-Eysenberg), CIP-Gegevens Koninklijke Bibliotheek, Den Haag, ISBN 90-6159-016-7, 485

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

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

7 De Groof J , Degroodt J -M , Wyhowski de Bukanski, B and Beernaert, H , Z Lebensm Unters Forsch , **193**, 1991, 126

8 Degroodt, J M , Wyhowski de Bukanski, B and Srebrnik ,S , Z Lebensm Unters Forsch, **195**, 1992, 566

9 Moretti, V M , Pieramati, C ,Valfre, F and Olivieri, O , in "Proceedings of the EuroResidue II Conference, Veldhoven, The Netherlands, May 3-5, 1993" (eds N Haagsma, A Ruiter and P B Czedik-Eysenberg), CIP-Gegevens Koninklijke Bibliotheek, Den Haag, ISBN 90-6159-016-7, 499

10 Meyer, H H D and Rinke, L , J Anim Sci , **69**, 1991, 4538

11 Botterblom, M H A , Feenstra, M G P and Erdtsieck-Ernste, E B H W , J Chromatogr (Biomed Applic), **613**, 1993, 121

12 Girault, J and Fourtillan, J B , J Chromatogr , **518**, 1990, 41

13 Wilson, R T , Groneck, J M , Holland, K P and Henry, A C , J Assoc Off Anal Chem , **77**, 1994, 917

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

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

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

17 Delahaut, Ph , Dubois, M , Colemonts, Y , Boenke, A and Maghuin-Rogister, G , in "Proceedings of the EuroResidue II Conference, Veldhoven, The Netherlands, May 3-5, 1993" (eds N Haagsma, A Ruiter and P B Czedik-Eysenberg), CIP-Gegevens Koninklijke Bibliotheek, Den Haag, ISBN 90-6159-016-7, 257

18 Montrade, M -P , Riverain, S , Le Bizec, B and Andre, F , in "Proceedings of the FLAIR Workshops held in Ghent and Thessaloniki 1992", (eds H A Kuiper and L A P Hoogenboom), ISBN 90-5601-003-4, 143

19 Boyd, D , O'Keeffe, M and Smyth, M R , Analyst, **119**, 1994, 1467

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

21 R J Heitzman (ed), "Veterinary Drug Residues-Residues in food-producing animals and their products Reference Materials and Methods", 2nd edition, Blackwell Scientific Publications, Oxford, 1994, Cy 2 1

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

23 Furst, P , Furst, C and Groebel, W , Deutsch Lebensm Rundsch, **85**, 1989, 35

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

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

26 Helbo, V , Vandenbroeck, M and Maghuin-Rogister, G , in “Proceedings of the EuroResidue II Conference, Veldhoven, The Netherlands, May 3-5, 1993” (eds N Haagsma, A Ruiter and P B Czedik-Eysenberg), CIP-Gegevens Koninklijke Bibliotheek, Den Haag, ISBN 90-6159-016-7, 362

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

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

29 Qureshi, G Ali and Eriksson, A , J Chromatogr , **441**, 1988, 197

30 Rinke, L , Meyer, H H D and Rapp, M , Chromatogram, **11**, 1990, 6

31 Brunn, H , Fleischwirtsch , **68**, 1988, 1476

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

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

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

35 Hutchings, M J , Paul, J D and Morgan, D J , J Chromatogr (Biomed Applic), **277**, 1983, 423

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

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

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

39 Brambilla, G F , Agrimi, U and Pierdominici, E , Ital J Food Sci , **4**, 1991, 303

40 Hooijerink, H , Schilt, R , Haasnoot, W and Courtheyn, D , J Pharm Biomed Anal , **9**, 1991, 485

41 Meyer, H H D , Rinke, L and Dursch, I , J Chromatogr (Biomed Applic), **564**, 1991, 551

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

43 Schmid, J and Bucheler, A , Biomed Environ Mass Spectrom , **17**, 1988, 415

44 Degroodt, J -M , Wyhowski de Bukanski, B , De Groof, J and Beernaert, H , Z Lebensm Unters Forsch , **192**, 1991, 430

45 Ramos, F , Castilho, C and Noronha da Silveira, I , in "Proceedings of the EuroResidue II Conference, Veldhoven, The Netherlands, May 3-5, 1993" (eds N Haagsma, A Ruiter and P B Czedik-Eysenberg), CIP-Gegevens Koninklijke Bibliotheek, Den Haag, ISBN 90-6159-016-7, 563

46 Li, K , Yuan, Y S , Zhang, J H , Tu, X and Lu, W L , Yaowu Fenxi Zazhi, **13**, 1993, 389

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

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

49 van Rhijn, J A , Traag, W A and Heskamp, H H , J Chromatogr , **619**, 1993, 243

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

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

52 McCarthy, P T , Atwal, S , Sykes, A P , Ayres, J G , Biomed Chromatogr , **7**, 1993, 25

53 Schmitz, P , Pelzer, G , Degraeve, J , Degand, G , Gaspar, P and Maghuin-Rogister, G , in "Proceedings of the EuroResidue Conference, Noordwijkerhout, The Netherlands, May 21-23, 1990" (eds N Haagsma, A Ruiter and P B Czedik-Eysenberg), CIP-Gegevens Koninklijke Bibliotheek, Den Haag, ISBN 90-6159-011-6, 326

54 Tanner, R J N Martin, L E and Oxford, J , Anal Proc , **20**, 1983, 38

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

56 Lindberg, C and Jonsson, S , Biomed Mass Spectrom , **9**, 1982, 493

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

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

59 Gabiola, C , in "Proceedings of the EuroResidue II Conference, Veldhoven, The Netherlands, May 3-5, 1993" (eds N Haagsma, A Ruiter and P B Czedik-Eysenberg), CIP-Gegevens Koninklijke Bibliotheek, Den Haag, ISBN 90-6159-016-7, 289

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

61 Ong, H , Adam, A , Perreault, S , Marleau, S , Bellemare, M , Du-Souich, P and Bealieu, N , J Chromatogr (Biomed Applic), **89**, 1989, 213

62 Hamann, J A , Johnson, K , and Delbert, T J , J Chromatogr Sci , **23**, 1986, 34

63 Kurosawa, N , Morishima, S , Owada, E and Ito, K , J Chromatogr (Biomed Applic), **30**, 1984, 485

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

65 Tamisier-Karolak, T , Delhotal-Landes, B , Jolliet-Riant, P , Milliez, J , Jannet, D , Barre, J and Flouvat, B , Therap Drug Monitor , **14**, 1992, 243

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

67 Adams, A G and Stewart, J T , J Liq Chromatogr , **16**, 1993, 3863

68 He,L and Stewart, T J , Biomed Chromatogr , **6**, 1992, 291

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

70 Debrauwer, L , Delous, G and Bories, G , Chromatographia, **36**, 1993, 218

71 Schilt, R , Haasnoot, W , Jonker, M A , Hooijerink, H and Paulussen, R J A , in “Proceedings of the EuroResidue Conference, Noordwijkerhout, The Netherlands, May 21-23, 1990”(eds N Haagsma, A Ruiter and P B Czedik-Eysenberg), CIP-Gegevens Koninklijke Bibliotheek, Den Haag, ISBN 90-6159-011-6, 320

72 Xu, Y X , Shen, L , Liu, X , Cui, K R , Wu, Y , Wang, S and Zhag, C J , Fenxi Huaxue, **21**, 1993, 1432

73 Furst, P , Furst, C , and Groebel, W , Deutsch Lebensm Rundsch , **85** , 1989, 341

74 Zamenick, J , J Anal Toxic , **14**, 1990, 132

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

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

77 Biondi, P A , Guidotti, L , Montana, M , Manca, F , Brambilla, G and Lucarelli, C , J Chromatogr Sci , **29**, 1991, 90

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

79 Adam, A , Ong, H , Sondag ,D and Rapaille, A , J Immunoassay, **11**,1990, 329

80 Adam, A , Ong, H , Granel, A , Messier, J , Bellemare, M , Lantin, F , Sauve, G and Tyssen, P , J Immunoassay, **12**, 1991, 207

81 Loo, J C K , Beaulieu, N , Jordan, N , Brien, R and McGilveray I J , Res Comm Chem Path Pharm , **55**, 1987, 283

82 Boyd, D , Shearan, P , Hopkins, J P , O' Keeffe, M and Smyth, M R , Anal Chim Acta, **275**, 1993, 221

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

84 Haasnoot, W , Hamers, A R M , van Bruchem, G D , Schilt, R and Frijns, L M H , in "Food Safety And Quality Assurance - Applications of Immunoassay Systems", (eds M R A Morgan , C J Smith and P A Williams), Elsevier Applied Science, London, 1992, 185

85 Delahaut, P , Degand, G , Dubois, M , Schmitz, P and Maghuin-Rogister, G in "Proceedings of the EuroResidue Conference, Noordwijkerhout, The Netherlands, May 21-23, 1990"(eds N Haagsma, A Ruiter and P B Czedik-Eysenberg), CIP-Gegevens Koninklijke Bibliotheek, Den Haag, ISBN 90-6159- 011-6, 149

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

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

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

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

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

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

92 van Rhijn, J A , Tragg, W A , Heskamp, H H and Zuidema, T , in "Proceedings of the EuroResidue II Conference, Veldhoven, The Netherlands, May 3-5, 1993" (eds N Haagsma, A Ruiter and P B Czedik-Eysenberg), CIP-Gegevens Koninklijke Bibliotheek, Den Haag, ISBN 90-6159-016-76, 572

93 Zimmer, A , Drug Research, **26**, 1976, 1442

CHAPTER 3

APPLICATION OF SOLID-PHASE EXTRACTION TO THE EXTRACTION AND CLEAN-UP OF CLENBUTEROL FROM LIVER WITH DETERMINATION BY RADIOIMMUNOASSAY

3.1 INTRODUCTION

A workshop, held in Brussels in 1987 under the auspices of the European Community, reviewed the use of β-agonists in animal production [1] The species of importance were cattle, sheep, pigs and poultry and it was concluded that β-agonists could be used to effect repartitioning so that carcass composition was altered to give less fat and more lean

A review by Moloney and Allen [2] found a typical increase of 10% in trimmed lean and a 25-30% reduction in dissectable fat in the carcasses of cattle treated with β-agonists during the finishing period A similar effect was seen in sheep with growth rate increasing by up to 29% while the percentage protein had increased by up to 18% and the amount of dissectable fat was reduced by up to 41% β-agonist treatment of pigs had little effect on carcass weight, but increased muscle growth was reported along with a 10% increase in trimmed lean Similarly, the effect of β-agonists on weight gain in broilers was low (<5%) but the fat content was reduced by 8-10% The review concluded that β-agonists improve the efficiency of carcass lean in all major meat-producing species and that, while the repartitioning effect is expressed to a greater extent in ruminants than in pigs or poultry, it is commercially important for all species This commercial importance has huge implications for the livestock trade and, even though the use of compounds with hormonal or thyreostatic action was prohibited within the European Community in 1986 [3], the financial benefits to livestock producers has resulted in the illegal use of clenbuterol and other β-agonists The illegal use of clenbuterol was evident in an outbreak of food poisoning from liver containing clenbuterol residues in Spain In the 135 cases reported clenbuterol was detected in the urine of two patients, while the concentration of clenbuterol in bovine liver samples was found to be as high as 291 ppb [4] Incidents such as these require the availability of sensitive methods capable of determining clenbuterol residues in edible tissue in order to protect the consumer

The persistence of residues of clenbuterol in tissues and body fluids of veal calves which had been treated with a dose of 5 µg/Kg bodyweight twice daily for three weeks was studied by Meyer et al [5] The highest residue concentration of clenbuterol was found in the eye (118 ng/g) when no withdrawal period had been observed and, even after a two week withdrawal period, 15 ng/g was still remaining Although clenbuterol was observed in most other tissues immediately after termination of treatment, the only edible tissue showing appreciable levels of clenbuterol after a two week withdrawal period was the liver (0 58 ng/g), indicating this organ to be a suitable tissue for residue analysis Elliott et al [6] studied the residues of clenbuterol in tissues of sheep treated with both therapeutic and growth enhancing concentrations and found that liver samples consistently contained the highest concentrations of clenbuterol

Even after a fifteen day withdrawal period, the liver samples of the three treatment groups (therapeutic level, 10 x therapeutic level, 25 x therapeutic level) had mean concentrations of 0 59, 3 17 and 3 63 ng/g respectively, all of which are above the 0 5 ng/g maximum residue limit specified in the UK for clenbuterol The relatively fast rate of elimination of clenbuterol from other organs, such as the lungs and kidneys, in the treated sheep precluded these organs from being considered as suitable samples for residue analysis In this study the eye tissues were not analysed, but both studies showed that the highest accumulation of clenbuterol in edible tissues occured in the liver and that even after a two week withdrawal period the level of clenbuterol found in this tissue was above the UK maximum residue limit The high accumulation of clenbuterol in the eye tissues is offset by the limited sample size available for analysis (1 g) A review of the literature relating to the analysis of liver samples for clenbuterol shows several references to the extraction of clenbuterol from homogenised liver samples using solvent extraction techniques Solvents such as diethyl ether **[6,7]** tert-butylmethylether **[8,9]** and ethyl acetate **[10,11]** have been used successfully to extract clenbuterol from samples adjusted to alkaline pH The disadvantages of classical liquid/liquid extraction (LLE) procedures for liver extractions, such as emulsion formation, difficulty in automation and the need for centrifugation, have prompted the introduction of on-column LLE procedures on diatomaceous earth Extractions are carried out under gravity with the aqueous liver digest or fluid sample being poured onto the column After an equilibration time, the organic extracting solvent is poured into the column and the analyte is collected On-column LLE procedures have already been used for the extraction of clenbuterol from various animal tissues and biological fluids Clenbuterol has been extracted from urine [12] and animal tissues [13] at alkaline pH on 'ChemElut' columns using hexane as the extracting solvent In both cases hydrochloric acid was added to the hexane eluate in order to back extract and preconcentrate the analyte into a small volume prior to determination Courtheyn et al [14] used an organic mixture of toluene dichloromethane (3 1) to extract both clenbuterol and cimaterol from animal tissues, faeces, feed, urine, plasma, blood and bile after the samples at pH 12 had been applied to 'ChemElut' columns The analytes were back extracted from the organic mixture using hydrochloric acid prior to their determination by HPLC using a post-column derivatisation step An interesting combination of on-column LLE of clenbuterol from urine at pH 10-11 with tert-butylmethylether followed by further clean-up and concentration on a C2 SPE cartridge, after the organic eluate had been evaporated and the sample reconstituted in borate buffer at pH 9 5, was reported by Hooijerink et al [15] This procedure was the first attempt to combine on-column LLE with a SPE clean-up and concentration step, with the only disadvantage of the procedure being the need for evaporation of the tert-butylmethylether eluate prior to the SPE step All the other on-column LLE methods discussed relied on a back extraction step with hydrochloric acid in order to extract and concentrate clenbuterol from the organic eluate

The aim of this study was to develop a method which used an on-column LLE step to extract clenbuterol from the primary extract and to further clean-up and concentrate the sample directly on a SPE column prior to determination by radioimmunoassay

3.2 EXPERIMENTAL

3 2 1 REAGENTS AND MATERIALS

Toluene ('Aristar' grade), dichloromethane ('HiPerSolv' grade) and diethylether ('GPR' grade) were supplied by BDH (Poole, UK) Hexane ('extra pure') and Ethanol ('absolute GR') were obtained from Merck (Darmstadt, Germany) Other chemicals used were AnalaR grade from BDH, or equivalent Tris(hydroxymethyl)methylamine buffer (0 1 M, pH 8 0), containing 14 8 g/l calcium chloride was used for the liver digestion and extraction step Protease enzyme, type XXIV (bacterial) was obtained from Sigma (St Louis, MO, USA)

'ChemElut' LLE columns (20 ml sample capacity) were obtained from Varian (California, USA) and Sep-Pak silica SPE columns were supplied by Waters (Massachusetts, USA) Phosphate buffer (0 01 M, pH 7 0), containing 1 g/L gelatin and 0 1 g/L thiomersal, was used for the radioimmunoassay Dextran-coated charcoal was prepared by adding 2 g activated charcoal (Sigma) and 0 25 g dextran T70 (Pharmacia, Uppsala, Sweden) to 500 ml doubly-distilled water The immunoassay kit used for the determination of clenbuterol was obtained from Laboratoire d'Hormonologie (Marloie, Belgium) The scintillation cocktail was Cocktail T (BDH)

3 2 2 APPARATUS

A LKB Wallac 1219 Rackbeta liquid scintillation counter was used to count the radioactivity present in extracts from the on-column LLE and silica SPE procedure during method development and for radioimmunoassays

3 2 3 LIVER EXTRACTION PROCEDURE

Liver (1 25 g) was weighed into a 60 ml centrifuge tube 25 ml TRIS buffer (0 2 M, pH 10) containing protease enzyme (0 33 mg/ml) and calcium chloride (0 1 M) was added Following homogenisation and digestion for one hour at 55°C with periodic mixing, the digest was immersed in boiling water for 15 minutes to deactivate the enzyme The sample was centrifuged at 2000 rpm for 20 minutes and 20 ml of the resulting supernatant was collected

3 2 4 ON-COLUMN LIQUID/LIQUID EXTRACTION

The pH of the supernatant was adjusted to pH 12 using 1 6 ml 1 M NaOH The primary extract was applied to the 'ChemElut' column and allowed to equilibrate for 20 minutes Clenbuterol was eluted from the column with 3 x 20 ml toluene

3 2 5 SILICA SOLID-PHASE EXTRACTION

The collected toluene eluate was applied to a silica solid-phase extraction column which had been conditioned with 10 ml toluene The eluate was allowed to pass through the silica column under gravity The column was washed with 4 ml hexane and 4 ml acetonitrile and dried for ten minutes under vacuum Clenbuterol was eluted with 9 ml ethanol The collected eluate was evaporated at 40^0C under a gentle stream of nitrogen and resuspended in 0 5 ml ethanol

3 2 6 RADIOIMMUNOASSAY

0 2 ml aliquots of the liver extracts were added to culture tubes Tubes containing 1000, 250, 100, 40, 10, 0 pg clenbuterol were prepared for the standard curve The samples and standards were evaporated to dryness at 40^0C under a stream of nitrogen and redissolved in 0 5 ml phosphate gelatin buffer (pH 7) After vortexing, the culture tubes were incubated at 37^0C for 10 minutes 0 1 ml ^{3}H clenbuterol (3000 cpm) and 0 1 ml antiserum were added and the culture tubes were incubated at 37^0C for a further 15 minutes and overnight at 4^0C 0 5 ml dextran-coated charcoal was added and, after shaking, the tubes were centrifuged at 2000 rpm The supernatants were decanted into scintillation vials and 10 ml cocktail T was added The vials were counted for 5 minutes

3 2 7 METHOD VALIDATION

Bovine liver samples analysed as free of β-agonist residues were used for fortification studies To liver samples (1 25 g) 25 μl and 50 μl volumes of an ethanolic solution containing clenbuterol at 10 ng/ml were added to give fortification levels of 0 2 and 0 4 ppb, respectively The fortified liver samples were left to equilibrate for 10 minutes before extraction

The interassay variation for determination of clenbuterol in liver was determined by analysis of samples, fortified at 0 2 and 0 4 ppb, on 5 different occasions The intra assay variation for determination of clenbuterol in liver was determined by analysis of replicate samples (n=6), fortified at 0 2 and 0 4 ppb, in a single assay

3.3 RESULTS AND DISCUSSION

3 3 1 ON-COLUMN LIQUID/LIQUID EXTRACTION

Tritiated clenbuterol was used to determine which elution solvent gave the highest recovery of clenbuterol from the LLE column The primary extract was applied at two pH levels, pH 10 and pH 12, both of which are above the pKa of clenbuterol The organic solvents evaluated were hexane, toluene and a mixture of toluene dichloromethane (3 1) 3 x 20 ml of each solvent were used to elute clenbuterol from the primary extract which had been applied to the column and allowed to equilibrate for 20 minutes The tritiated clenbuterol present in the eluates was back-extracted into 0 2 ml hydrochloric acid (0 1 M) and, following the addition of 0 01 ml of 10 M NaOH, clenbuterol was re-extracted into ether (6 ml) The ether samples were evaporated at 40^{0}C under a stream of nitrogen and resuspended in 1 ml methanol The methanol fractions were decanted into scintillation vials and counted following the addition of Cocktail T (10 ml) The recoveries of clenbuterol are shown in Table 3 1 The highest recoveries were achieved from the 'ChemElut' column when the primary extract was adjusted to pH 12 and toluene was used as the extraction solvent The low recoveries obtained with hexane are due to the very non-polar nature of this solvent, relative to toluene, which renders it unsuitable for extraction of clenbuterol, even at pH 12 The low recovery at pH 10 with toluene is due to the pKa of clenbuterol (9 3) and the pH of the primary extract being very close At pH 12, 2-3 units above the pKa of clenbuterol, the molecule exists in a non-charged state and is easier to extract from the 'ChemElut' column

Table 3 1

Recovery of tritiated clenbuterol from primary extracts using 'ChemElut' columns under various conditions

Eluent	Hexane		Toluene		Toluene Dichloromethane (3 1)
pH	10	12	10	12	12
Recovery (%)	41	35	38	80	75

3 3 2 SILICA SOLID-PHASE EXTRACTION

The silica SPE step was required to extract clenbuterol from the toluene eluate generated in the on-column LLE step Solid-phase extraction with a C18 type cartridge was not possible due to the noncompatibility of its hydrophobic retention mechanisms with organic solvents Silica SPE cartridges were conditioned with 10 ml toluene prior to the application of 60 ml toluene containing ^{3}H-clenbuterol which was allowed to pass through the column under gravity The column was dried under vacuum for 10 minutes Three organic solvents were evaluated for their capacity to elute clenbuterol and the recoveries are shown in Table 3 2 Ethanol, which yielded a recovery of 88% for clenbuterol, was chosen as a suitable elution solvent for the silica clean-up step The silica silanol groups retain clenbuterol strongly, and neither acetonitrile or methanol are capable of removing clenbuterol from the sorbent Ethanol, however, competes effectively with the silanol groups retentive power Elution with ethanol is considered to be due to a combination of (a) the non-polar interaction of the slightly larger alkyl group of ethanol (compared with methanol) with clenbuterol and (b) competition between the hydroxyl moiety of ethanol and the silanol groups in their polar interactions with the primary and secondary amino groups of clenbuterol The completed extraction and clean-up procedure yielded extracts suitable for determination of clenbuterol by radioimmunoassay

Table 3 2

Recovery of tritiated clenbuterol from silica SPE cartridges using three elution solvents

Eluent	Acetonitrile	Methanol	Ethanol
Elution volume (ml)	9 0	9 0	9 0
Polarity index	6 2	6 6	5 2
Recovery (%)	0 7	13 0	88 0

3 3 3 METHOD VALIDATION

The inter and intra-assay variations for the determination of clenbuterol in fortified liver are shown in Tables 3 3 and 3 4, respectively Both the inter and intra-assay variations have acceptable coefficients of variation The recoveries for clenbuterol at 0 2 ppb are higher than those attained at 0 4 ppb, this may be explained as the differing effect of matrix interference in the sample extracts at different points of the RIA standard curve such that the sample extracts containing lower amounts of clenbuterol are less affected An extract-containing standard curve would remove this effect as the matrix interference present in the sample extracts would also be present in the standard curve An extract-containing standard curve was not used in this experiment because of the large number (i e 7) of additional residue free control liver samples which would require processing for the standard curve This effect has been observed previously, such as for a MSPD method to extract clenbuterol from liver with determination by RIA [16] The authors reported similar recoveries of clenbuterol at three fortification levels when an extract containing standard curve was used, while the recovery for different fortification levels differed by 19% when an external standard curve was used in the radioimmunoassay

Table 3 3 Inter-assay variation for SPE/RIA determination of clenbuterol in liver

Clenbuterol added (ng/g)	n	Clenbuterol determined	
		Mean ± SD (ng/g)	CV (%)
0 2	5	0 16 ± 0 01	9 3
0 4	4	0 25 ± 0 02	6 1

Table 3 4 Intra-assay variation for SPE/RIA determination of clenbuterol in liver

Clenbuterol added (ng/g)	n	Clenbuterol determined	
		Mean ± SD (ng/g)	CV (%)
0 2	6	0 15 ± 0 01	6 1
0 4	6	0 26 ± 0 04	16 4

3 3 4 INCURRED SAMPLE ANALYSIS

Liver samples from animals treated with clenbuterol were analysed by this procedure The concentration of clenbuterol determined by this method compares well with the results obtained with a standard method used in the laboratory The mean concentrations of clenbuterol determined with this procedure and with the standard method were 0 43 ± 0 06 ng/g and 0 36 ± 0 05 ng/g, respectively

3 3 5 LIMIT OF DETECTION

The limit of detection for the procedure was calculated as the mean response for twelve control liver samples plus three times the standard deviation

Mean response for control liver samples (n=12)		0 06 ng/g
Standard deviation about the mean		0 028 ng/g
Limit of detection	0 06 ng/g + 3 x 0 028 ng/g =	0 14 ng/g

The calculated limit of detection is below the UK maximum residue limit for clenbuterol in edible tissue (0 5 ng/g)

3.4 CONCLUSION

The technique developed has several advantages over other methods used to extract clenbuterol from liver These include the possibility of automating the extraction and clean-up procedure once the primary extract has been generated There is no requirement for centrifugation during the extraction and clean-up stage and problems with emulsions are eliminated through the use of 'ChemElut' on-column liquid/liquid extraction columns By developing compatible on-column LLE and silica SPE clean-up and concentration steps the eluate from the LLE column can be transferred directly to the SPE column thereby avoiding time-consuming solvent evaporation between the two clean-up steps

The suitability of this method for residue analysis is indicated by the low limit of detection, calculated to be 0 14 ng/g The similarity of results obtained for incurred liver samples by this method and a standard method indicate the applicability of this procedure to the analysis of clenbuterol residues in liver

The inter-assay and intra-assay variations have acceptable coefficients of variation and the lower recoveries at higher concentration of clenbuterol could be compensated for through the use of an extract-containing standard curve The application of this procedure to other β-agonist residues, such as salbutamol and terbutaline, may result in poor recoveries due to the hydrophilic nature of these compounds which would reduce their recovery from the on-column LLE step Clean-up steps involving more than one retention mechanism, such as mixed phase SPE, may be more appropriate for extracting β-agonists of a range of chemical characteristics and, therefore, be more suited to a multiresidue procedure

3.5 REFERENCES

1 J P Hanrahan, (ed), “Beta-agonists and their effects on animal growth and carcass quality”, Elsevier Applied Science, London, 1987

2 Moloney, A P and Allen, P , in “Proceedings of the FLAIR Workshops held in Ghent and Thessaloniki, 1992,” (eds H A Kuiper and L A P Hoogenboom, RIKILT-DLO, Wageningen, The Netherlands), ISBN 90-5601-003-4, 89

3 Council of the European Communities, Off J Eur Commun , **L275**, 1986, 336

4 Martinez-Navarro, J F , Lancet, **336**, 1990, 1311

5 Meyer, H D and Rinke, L M , J Anim Sci , **69**, 1991, 4358

6 Elliott, C T , McCaughey, W J and Shortt, H D , Food Addit Contam , **10**, 1993, 231

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

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

9 Furst, P , Furst, C and Groebel, W , Deutsch Lebensm Rundsch , **85**, 1989, 35

10 R J Heitzman (ed), “Veterinary Drug Residues - Residues in food-producing animals and their products Reference Materials and Methods”, 2nd edition, Blackwell Scientific Publications, Oxford, 1994, Cy 2 2

11 Wilson, R T , Gnneck, J M , Holland, K P and Henry, A C , J Assoc Off Anal Chem , **77**, 1994, 917

12 Eddins, C , Hamman, J and Johnson, K , J Chromatogr Sci , **23**, 1985, 308

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

14 Courtheyn D , Desaever C and Verhe R , J Chromatogr (Biomed Applic),

564, 1991, 537

15 Hooijerink, H , Schilt, R , Haasnoot, W and Courtheyn, D , J Pharm Biomed Anal , **9**, 1991, 485

16 Boyd, D , Shearan, P , Hopkins, S , O'Keeffe, M and Smyth, M R , Anal Chim Acta, **275**, 1993, 221

CHAPTER 4

MULTI-RESIDUE ANALYSIS OF β-AGONISTS IN URINE AND LIVER USING MIXED-MODE SOLID PHASE EXTRACTION WITH DETERMINATION BY RADIOIMMUNOASSAY

4.1 INTRODUCTION

The illegal use of β-agonists in Europe has necessitated the introduction of multi-residue methods capable of determining a range of β-agonists in a single analytical procedure Clenbuterol, salbutamol and mabuterol have been found in samples of urine and liver in The Netherlands [1], while bovine urine samples analysed in Italy were found to contain clenbuterol and, in one case, salbutamol residues [2] High levels of clenbuterol and salbutamol have also been determined in animal feed [3]

The difference in polarity between clenbuterol and salbutamol has resulted in the residue methods available for clenbuterol analysis being unsuitable as multi-residue techniques Methods involving extraction using organic solvents such as tert-butylmethylether [4,5], ethyl acetate [6,7] and diethyl ether [8,9] do not have the required "strength" to extract the more polar β-agonists such as salbutamol and terbutaline from an aqueous environment Similarly, on-column liquid/liquid extraction techniques (discussed in chapter 3) result in low recovery of the more polar β-agonists, while the relatively non-polar β-agonists, such as clenbuterol and mabuterol, are extracted with acceptable recovery Methods reported for the extraction of salbutamol at the low ppb level from biological matrices involve the use of immunoaffinity columns and ion-pair extractions using di(2-ethylhexyl) phosphate (DEHP) The immunoaffinity columns used for the extraction of salbutamol from liver [10], human plasma [11], and lyophilized rat carcasses [12] used anti-salbutamol antibodies bound to sepharose gel, and no information pertaining to their effectiveness in extracting other β-agonists was presented The extraction of polar drugs from biological matrices by converting them into a neutral ion-associating complex by addition of DEHP has proved to be a useful approach in the extraction of salbutamol Hutchings et al [13] initially reported an assay for salbutamol that used an ion-pair extraction and determination by HPLC with detection by means of the drug's inherent fluorescence The ion-pairing agent (DEHP) has since been used to assist the extraction of salbutamol from human serum [14] and plasma with determination by HPLC with fluorescence detection [15,16]

The non-compatibility of the existing analytical techniques used to extract either aniline type or phenolic type β-agonists has resulted in the development of multi-residue techniques capable of extracting both types of β-agonist simultaneously These multi-residue techniques can be divided into three classes matrix solid-phase dispersion (MSPD), immunoaffinity chromatography (IAC), and mixed-mode solid-phase extraction techniques

Matrix solid-phase dispersion, was developed by Barker and co-workers [17,18] for the analysis of pesticide, antibiotic and other veterinary drug residues in biological matrices It involves manual blending of the tissue sample with octadecylsilane (C18) material, packing the resulting material into a column, and washing of the column to remove interferences prior to elution of the residue(s) from the column The technique has been applied to the analysis of clenbuterol in bovine liver with determination by radioimmunoassay [19] and enzyme immunoassay [20] and was successfully applied to the analysis of four β-agonists in liver with detection by radioimmunoassay [21] With this procedure, clenbuterol, salbutamol, and mabuterol were detectable at 1 ng/g and terbutaline was detectable at 2 ng/g The limit of detection was reported to be 0 7 ng/g

Both immunoaffinity chromatography and mixed-mode solid-phase extraction have been discussed in Chapter 2 Previous work with mixed-mode SPE columns have given low recovery, generally, of phenolic type β-agonists compared with aniline type β-agonists [22-25]

The aim of this study was to investigate if equivalent recovery for both types of β-agonists could be obtained using mixed-mode SPE Another mixed-mode SPE column

(‘XtrackT’), previously used in the extraction of cocaine and its major metabolite from neonatal urine [26], was evaluated for the extraction of four β-agonists from urine and liver with determination by radioimmunoassay

4.2 EXPERIMENTAL

4 2 1 REAGENTS AND MATERIALS

Ethyl acetate ('pesticide' grade), methanol ('Hipersolv' grade) and ammonia solution (35%, 'Analar' grade) were from BDH (Poole, UK) 2-Propanol ('HPLC' grade) was supplied by Aldrich (Poole, UK) and ethanol ('absolute GR') was obtained from Merck (Darmstadt, Germany) 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 sulphatase per ml) was supplied by Sepracor (Villeneuve la Garenne, France) Protease enzyme, type XXIV (Bacterial), was obtained from Sigma (St Louis, USA) XtrackT mixed-mode solid-phase extraction columns (500 mg sorbent) were supplied by Technicol (Stockport, UK) The immunoassay kit used for the determination of β-agonists was obtained from Laboratoire d'Hormonologie (Marloie, Belgium), with antiserum which had been raised against salbutamol and had principal cross-reactivities of 100% - salbutamol, 118% - clenbuterol, 78% - mabuterol, and 29% - terbutaline

For the radioimmunoassay, phosphate buffer (0 01 M, pH 7 0), containing 1 g/L gelatin and 0 1 g/L thimerosal 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 doubly-distilled water The scintillation cocktail was Cocktail T (BDH) Clenbuterol hydrochloride, salbutamol and terbutaline were obtained from Sigma (St Louis, MO, USA) Mabuterol was provided as a gift by Laboratoire d'Hormonologie (Marloie, Belgium)

4 2 2 EQUIPMENT

A LKB Wallac 1219 Rackbeta liquid scintillation counter was used to count radioactivity

4 2 3 EXTRACTION, LIVER SAMPLES

Liver (2 g) was weighed into a 60 ml centrifuge tube 10 ml TRIS buffer (0 2 M, pH 10) containing protease enzyme (0 33 mg/ml) and calcium chloride (0 1 M) were added Following homogenisation and digestion for one hour at 55°C with periodic mixing the digest was centrifuged for 20 minutes at 2000 rpm 5 ml of the supernatant was collected, 1 8 g sodium chloride was added and the pH was adjusted to pH 11 5 ml ethyl acetate isopropanol (6 4) was added to the liver extract and the solution was mixed on a horizontal shaker for 15 min The sample was centrifuged (2000 rpm, 15 min) and a 4 ml aliquot of the organic phase was collected This step was repeated and the combined organic phases (8 ml) were evaporated at 40°C under a gentle stream of nitrogen

4 2 4 MIXED-MODE SOLID-PHASE EXTRACTION

6 ml phosphate buffer (0 1 M, pH 6 0) was added to the urine sample (5ml) and to the dried liver extract (equivalent of 0 67g liver) and, where necessary, the pH was adjusted to pH 6 0 The XtrackT column was conditioned by the addition of 5 ml methanol, 5 ml doubly-distilled water and 2 ml phosphate buffer (0 1 M, pH 6 0) The sample was applied at a rate of 2 ml/min and the column was dried for 10 minutes under vacuum The column was washed with 5 ml water and with 5 ml methanol, and the column was dried under vacuum for 15 minutes The β-agonists were eluted from the column using 5 ml methanol containing 2% ammonia The collected eluate was evaporated at 40°C under a

gentle stream of nitrogen and the residue was resuspended in 2 ml ethanol (liver) or 5ml ethanol (urine)

4 2 5 RADIOIMMUNOASSAY

0 2 ml aliquots of liver and urine extracts were added to culture tubes 0 2 ml aliquots of control (residue-free) liver or urine extracts were added to culture tubes containing a clenbuterol standard at 0, 10, 40, 100, 250, 1000 pg for the standard graph and to culture tubes containing 500 and 200 pg (liver extract) and 1000 and 400 pg (urine extract) each of clenbuterol, salbutamol, mabuterol or terbutaline The samples, standards and β-agonist recovery standards, prepared in duplicate, were evaporated to dryness under a stream of nitrogen and redissolved in 0 5 ml phosphate gelatin buffer (pH 7 0) After vortexing, the culture tubes were incubated at 37°C for 10 minutes 0 1 ml ^{3}H-clenbuterol (7500 cpm) and 0 1 ml antiserum were added, and the culture tubes were incubated at 37°C for a further 15 minutes and overnight at 4°C 0 5 ml of dextran-coated charcoal was added and the tubes were centrifuged for 10 min at 2000 rpm The supernatants were decanted into scintillation vials and 10 ml cocktail T added The vials were counted for 5 minutes

4 2 6 METHOD VALIDATION

Bovine urine and liver samples assayed as free of β-agonist residues were used for fortification studies To urine samples (5 ml) 10 μl and 25 μl volumes of an ethanolic solution containing clenbuterol, salbutamol, mabuterol or terbutaline at 1 μg/ml were added to give fortification levels of 2 ppb and 5 ppb, respectively To liver samples (2 g) 10 μl and 25 μl volumes of an ethanolic solution containing the four β-agonists at 0 4 mg/ml were added to give fortification levels of 2 ppb and 5 ppb, respectively The fortified samples were left to equilibrate for 10 minutes before extraction

4.3 RESULTS AND DISCUSSION

4 3 1 MIXED-MODE SPE COLUMNS

These columns contain two retention mechanisms based on octyl and benzene sulphonic acid groups bound to the silica particles The cation exchange properties of the column coupled with its hydrophobic retention characteristics allow for retention of a range of β-agonists The optimum pH for the extraction of β-agonists was found to be pH 6 0, and the elution of these compounds occurred when methanol containing 2% ammonia was applied to the column An important characteristic of this column is that a pre-elution wash with 100% methanol is possible which results in cleaner sample extracts, a methanol wash on a conventional reversed-phase (C18) SPE column would elute the analytes together with interferences

4 3 2 CLEAN-UP OF LIVER EXTRACTS

The application of liver extracts, following protein precipitation with HCl and centrifugation, directly to the mixed-mode SPE column was not successful due to the amount of matrix interferences still present Studies using liver extracts fortified with ^{3}H-clenbuterol and ^{3}H-salbutamol yielded recoveries of less than 30% in the elution step from the SPE columns Two published solvent extraction procedures for β-agonists were evaluated, tert-butanol ethyl acetate (3 7) **[24]** and ethyl acetate isopropanol (6 4) **[26]**, the latter solvent mixture was chosen because of the higher recoveries achieved The effect of pH and ionic strength of the aqueous liver extract on the efficiency of the extraction of salbutamol was investigated (Table 4 1) The saturation of the aqueous layer with sodium chloride prior to the extraction with ethyl acetate isopropanol resulted in good recovery of salbutamol at all pH values Saturation of the aqueous layer also resulted in the formation of a well-defined interface during the extraction procedure particularly for the enzymatically-digested liver extracts The use of HCl as an alternative to enzymatic digestion for the preparation of liver extracts resulted in emulsion formation at the solvent extraction stage

Table 4 1 Extraction of Salbutamol (5 ppb) from an aqueous solution at different pH and sodium chloride levels

β-agonist	pH	Sodium Chloride concentration (g/ 5 ml)	Recovery (%)
Salbutamol	2	0 0	38
		0 8	77
		1 8	91
	7	0 0	90
		0 8	70
		1 8	91
	10	0 0	74
		0 8	70
		1 8	88
	12	0 0	58
		0 8	64
		1 8	74

4 3 3 METHOD VALIDATION

An increase in the non-specific binding (NSB) in the radioimmunoassay is caused by sample extract, particularly liver extract even after the mixed-mode SPE clean-up Mean NSB figures for extract-free buffer, urine extract in buffer and liver extract in buffer are 130 cpm (n=5), 183 cpm (n=8) and 672 cpm (n=9), respectively Good quantitive measurement was obtained, therefore, by preparing the standard curve (clenbuterol) for both urine and liver assays in residue-free sample extract

To allow for the variation in specificity of the antibody for the four β-agonists, individual standards at an appropriate concentration for each analyte were prepared in residue-free sample extract and assayed with the samples

The inter and intra-assay variation of the method for urine and liver are shown in Tables 4 2 to 4 5 Good recoveries for all four β-agonists are achieved from both urine and liver at fortification levels of 2 and 5 ng/g (Tables 4 2, 4 4) The high variability for recovery of mabuterol and terbutaline in urine (Table 4 2) may be due to the relatively lower cross-reactivity of the antibody for the β-agonist The cross-reactivity of the antiserum for terbutaline (29%) determined the lowest fortification level at which the method could be validated for all four β-agonists under identical experimental conditions The method is sensitive to lower levels of clenbuterol, salbutamol and mabuterol The intra-assay variations for urine and liver demonstrate good repeatability of the method for urine and liver assays (Tables 4 3, 4 5)

Table 4 2 Inter-assay variation for recovery of clenbuterol, salbutamol, mabuterol and terbutaline in urine (n=5)

β-agonist	Concentration (ng/ml)	Range	Recovery (%)	
			Mean ± SD	CV
Clenbuterol	2	75-92	84 ± 6 0	15 2
	5	77-106	92 ± 10 4	11 3
Salbutamol	2	73-93	85 ± 8 3	9 7
	5	62-86	76 ± 10 8	14 3
Mabuterol	2	46-95	79 ± 20 6	26 2
	5	89-102	98 ± 5 3	5 5
Terbutaline	2	48-108	81 ± 27 2	33 4
	5	64-106	82 ± 18 0	21 0

Table 4 3 Intra-assay variation for the recovery of clenbuterol, mabuterol, salbutamol, and terbutaline in urine (n=4)

β-agonist	Concentration (ng/ml)	Range	Recovery (%)	
			Mean ± SD	CV
Clenbuterol	2	73-80	77 ± 3 6	4 6
	5	86-89	87 ± 1 3	1 4
Salbutamol	2	55-68	65 ± 7 3	11 4
	5	70-86	77 ± 6 8	8 8
Mabuterol	2	84-91	90 ± 4 6	5 1
	5	87-92	91 ± 2 5	2 8
Terbutaline	2	48-78	69 ± 14 0	20 0
	5	85-100	94 ± 7 0	7 4

Table 4 4 Inter-assay variation for the recovery of clenbuterol, mabuterol, salbutamol, and terbutaline in liver (n=5)

β-agonist	Concentration (ng/ml)	Range	Recovery (%)	
			Mean ± SD	CV
Clenbuterol	2	86-116	92 ± 14 1	15 0
	5	70-108	93 ± 14 3	15 4
Salbutamol	2	80-124	108 ± 17 7	15 4
	5	99-120	109 ± 8 0	7 3
Mabuterol	2	85-116	100 ± 11 2	11 2
	5	82-108	94 ± 10 4	11 1
Terbutaline	2[1]	78-119	100 ± 15 0	15 0
	5	80-103	96 ± 9 2	9 6

[1] n=4

Table 4 5 Intra-assay variation for the recovery of clenbuterol, salbutamol, mabuterol, and terbutaline in liver (n=4)

β-agonist	Concentration (ng/ml)	Range	Recovery (%)	
			Mean ± SD	CV
Clenbuterol	2	87-99	92 ± 5 9	6 4
	5	81-99	90 ± 8 2	9 0
Salbutamol	2	77-117	93 ± 17 7	18 0
	5	89-94	93 ± 2 5	2 7
Mabuterol	2	80-93	86 ± 5 6	6 5
	5	83-97	89 ± 6 0	6 7
Terbutaline	2[1]	88-123	110 ± 17 0	15 3
	5	62-81	73 ± 8 2	11 2

[1] n=4

4 3 4 LIMIT OF DETECTION

The limit of detection of the procedure was calculated from the mean response for control liver and urine samples plus three times the standard deviation

(a) urine

Mean response for control urine samples (n=5)	0 024 ng/ml
Standard deviation about the mean	0 034 ng/ml
Limit of detection 0 026 ng/ml + 3 x 0 034 ng/ml =	0 13 ng/ml

(b) liver

Mean response for control liver samples (n=8)	0 075 ng/g
Standard deviation about the mean	0 128 ng/g
Limit of detection 0 075 ng/g + 3 x 0 128 ng/g =	0 46 ng/g

The calculated limits of detection for urine and liver samples are at or below the UK maximum residue limit for clenbuterol

4 3 5 INCURRED LIVER ANALYSIS

Incurred liver samples with residue concentrations (determined by alternative methods) of 4 0 ng/g clenbuterol, 3 5 ng/g mabuterol and 8 0 ng/g salbutamol, respectively, were assayed Good agreement was found for concentrations of clenbuterol (4 3 ng/g) and mabuterol (4 7 ng/g) determined by this method but a considerably lower level of salbutamol (1 3 ng/g) was determined The lower level of salbutamol determined is likely due to the occurrence of salbutamol largely as a conjugated residue Studies on the metabolism of salbutamol in farm animals have shown that salbutamol is metabolised into both glucuronide [29] and sulfate conjugates [27] These residues are very polar and essentially not extractable into organic solvents and this would explain the low level of salbutamol determined An enzymic deconjugation step was introduced into the method

Suc d'Helix pomatia was chosen for the hydrolysis step as it contains both β-glucuronidase and sulphatase enzymes which should therefore deconjugate both the glucuronide and sulfate metabolites of salbutamol The deconjugation step involved the addition of 0 66 ml suc d'Helix pomatia (diluted 1 10) to 5ml liver digest supernatant which was adjusted to pH 5 0 and incubated overnight at 37°C, prior to solvent extraction and mixed mode SPE as described With this modification a salbutamol residue concentration of 9 0 ng/g was determined in the sample

4 3 6 CHOICE OF MIXED-MODE SOLID-PHASE EXTRACTION COLUMNS AND IMPORTANCE OF COLUMN PARTICLE SIZE

Due to the hydrophilic character of terbutaline and its biphenol-like structure (Figure 1 1), it was found that the application of terbutaline-containing urine samples to the mixed-mode solid-phase extraction columns at a rate in excess of 0 5 ml/min resulted in significantly reduced recovery of the compound (Table 4 6) The effect of rate of application of samples (1 ml/min) versus recovery was not as pronounced for clenbuterol, salbutamol or mabuterol due to their lower polarities XtrackT columns containing the same material, but of different particle size, were investigated also for their effectiveness in retaining terbutaline in urine samples at an application rate of 0 5 ml/min No significant difference in recovery of terbutaline due to particle size was observed

Table 4.6 Comparison of the recovery of terbutaline from urine using XtrackT columns of different particle size and at different rates of sample application

Terbutaline (ng/ml)	Sample application rate (ml/min)	Xtrack T particle size (μm)	Recovery (%)	
			Mean ± SD	CV
2	0.5	125-210	82^{1} ± 2.7	3.2
	0.5	40-50	69^{1} ± 14.0	20.0
	2.0	40-50	21^{2} ± 14.4	68.5
5	0.5	125-210	93^{1} ± 7.6	8.1
	0.5	40-50	94^{1} ± 7.0	7.4
	2.0	40-50	41^{2} ± 20.5	50.2

[1,2] Within each fortification level, mean recovery values with different superscripts are significantly different ($p<0.01$)

4.4 CONCLUSION

The described method is suitable for the extraction and determination of β-agonists in both bovine urine and liver samples. The recovery of phenolic type β-agonists (salbutamol and terbutaline) from fortified liver samples, at greater than 78%, is substantially higher than the recoveries reported by Leyssens et al. (terbutaline - 20% and salbutamol - 35%), for the analysis of bovine liver [23]. Similarly, the average recovery for salbutamol and terbutaline from fortified urine samples, at greater than 65%, is higher than the recoveries reported by Montrade et al. (terbutaline - 36%, salbutamol - 22%) for the analysis of urine [24]. The high recovery of both types of β-agonists (phenolic and aniline) from both matrices, indicates the suitability of this method as a multi-residue procedure.

The need for an enzymic deconjugation step is shown by the increase in the level of salbutamol determined in an incurred sample after deconjugation with suc d'Helix pomatia. Due to the limited cross-reactivity of the antibody used in the immunoassay step, only four β-agonists could be determined but if a detection technique such as GC-MS was used a wider range of β-agonists could be determined.

4.5 REFERENCES

1 VanGinkel, L A , Stephany, R W and Rossum, H J , J Assoc Off Anal Chem , **75**, 1992, 554

2 Angeletti, R , Oriundi, M P , Piro, R and Bagnati, R , Anal Chim Acta , **275**, 1993, 215

3 Schilt, R , Haasnoot, W , Jonker, M A , Hooijerink, H and Paulussen, R J A , in “Proceedings of the Euroresidue Conference, Noordwijkerhout, The Netherlands, May 21/23, 1990”, (eds N Haagsma, A Ruiter and P B Czedik-Eysenberg), CIP-Gegevens Koninklijke Bibliotheek, Den Haag, ISBN 90-6159-011, 320

4 Degand, G , Duyckaerts, A B and Maghuin-Rogister, G , J Agric Food Chem , **40**, 1992, 70

5 Furst, P , Furst, C and Grobel, W , Deutsch Lebensm Rundsch , **85**, 1989, 35

6 R J Heitzman, (ed), “Veterinary Drug Residues, Residues in food-producing animals and their products Reference Materials and Methods”, 2nd edition, Blackwell Scientific Publications, Oxford, 1994, Cy 2 2

7 Wilson, R T , Grineck, J M , Holland, K P and Henry, A C , J Assoc Off Anal Chem **77**, 1994, 917

8 Elliott, C T , McCaughey, W J and Shortt, H D , Food Addit Contam , **10**, 1993, 231

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

10 Degroodt, J M Wyhowski de Bukanski, B and Srebrnik, S , Z Lebensm Unters Forsch , **195**, 1992, 566

11 Ong, H , Adam, A , Perreault, S , Marleau, S , Bellemare, M , Souich, P and Beaulieu, N , J Chromatogr (Biomed Applic), **89**, 1989, 213

12 Ramos, F , Castilho C , Noronha da Silveira, I , Prates, J A M and Dias Correia J H R , Anal Chim Acta , **275**, 1993, 279

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

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

15 Miller, L G and Greenblatt, D J , J Chromatogr (Biomed Aplic), **381**, 1986, 205

16 Qun Wu, Y , Shi, R , Willams, R L and Lin, E T , J Liq Chromatogr , **14**, 1991, 253

17 Barker, S A , Long, A R and Short, C R , J Chromatogr ,' **475**, 1989, 353

18 Long, A R , Hsieh, L C , Malbrough, M S , Short, C R and Barker, S A , J Agric Food Chem , **38**, 1990, 423

19 Boyd, D , Shearan, P , Hopkins, S , O'Keeffe, M and Smyth, M R , Anal Chim Acta , **275**, 1993, 221

20 Boyd, D , Shearan, P , Hopkins, S , O'Keeffe, M and Smyth, M R , Anal Proc , **30**, 1993, 156

21 Boyd, D , O'Keeffe, M and Smyth, M R , Analyst, **119**, 1994, 1467

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

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

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

25 Montrade, M -P , Le Bizec, B , and Andre, F , in "Proceedings of the FLAIR Workshops held in Ghent and Thessaloniki 1992", (eds H A Kuiper and L A P

Hoogenboom, RIKILT-DLO, Wageningen, The Netherlands, ISBN 90-5601-003-4, 143

26 Moore, C M , Brown, S , Negrusz, A , Tebbett, I , Meyer, W and Jain, L , J Anal Toxicol , 17, 1993, 62

27 Delahaut, Ph , Dubois, M , Colemonts, Y , Boenke, A and Maghuin-Rogister, G , in "Proceedings of the Euroresidue II Conference, Veldhoven , The Netherlands, May 3-5 1993", (eds N Haagsma, A Ruiter, P B Czedik-Eysenberg), CIP-Gegevens Koninklijke Bibliotheek, Den Haag, ISBN 90-6159-011, 257

CHAPTER 5

STUDIES ON THE DEVELOPMENT OF A MIXED-MODE MATRIX SOLID-PHASE DISPERSION PROCEDURE FOR THE EXTRACTION OF β-AGONIST RESIDUES FROM LIVER WITH DETERMINATION BY RADIOIMMUNOASSAY

5.1 INTRODUCTION

Matrix solid phase dispersion (MSPD) has been applied successfully, since its development by Barker et al [1], to the extraction of individual veterinary residues such as ivermectin [2,3], furazolidone [4] and chloramphenicol [5] and also in multi-residue extraction procedures for sulphonamides [6], chlorinated pesticides [7], benzimidazoles [8] and tetracyclines [9] from complex biological matrices such as liver, muscle and milk The technique has been used in our laboratory to extract clenbuterol from bovine liver samples with determination by RIA [10] and EIA [11] and also in the extraction of several β-agonists including clenbuterol, salbutamol, mabuterol, terbutaline and cimaterol from bovine liver samples [12,13] The technique involves a manual blending of tissue sample with octadecylsilane material using a mortar and pestle for a short period of time (typically 40 seconds) The blending disrupts cell membranes in the sample allowing solubilization of the lipophilic regions into the C18 material The resulting mixture is packed into a column and sample components are eluted sequentially using solvents of varying polarity

More recently, mixed-mode solid-phase extraction columns have been used for the extraction of a variety of β-agonists from urine and liver samples [14-17] Mixed-mode SPE columns contain two retention mechanisms based on octyl and cation exchange groups bound to the solid support The cation exchange properties of the column coupled with its hydrophobic retention characteristics allow for retention of a range of β-agonists An important characteristic of these columns is that a pre-elution wash with 100% methanol is possible which results in cleaner sample extracts, a methanol wash on a conventional reversed-phase (C18) SPE column would elute the analytes together with interferences

The aim of this study was to evaluate a similar mixed-mode approach with the MSPD procedure by introduction of an ion-exchange step in order to determine if the combination of both techniques would allow for a more exhaustive clean-up of the liver samples The potential advantage of the combined technique (MSPD/IE) was the speed of a MSPD extraction coupled with the selective retention and clean-up capabilities of the ion exchange step Propylsulphonic acid (PRS) was the ion exchange sorbent chosen as it is a strong cation exchanger and consequently is suited to the extraction of basic compounds such as β-agonists from liver samples The initial method development involved optimising the amount of PRS material to use in the mixed-mode MSPD step, using tritiated clenbuterol and salbutamol The use of tritiated β-agonists allowed for accurate determinations of analyte breakthrough in the washing stages and final recovery in the elution solvents The PRS sorbent was then applied in conjunction with reversed-phase sorbents in the MSPD procedure for the extraction of clenbuterol and salbutamol from liver samples, with determination by radioimmunoassay

5.2 EXPERIMENTAL

5 2 1 REAGENTS AND EQUIPMENT

Ammonia solution, 35% ('AnalaR' grade), methanol ('HiPerSolv' grade) and glacial acetic acid ('AnalaR' grade) were obtained from BDH (Poole, UK) Other chemicals used were of 'Analar' Grade or equivalent

The bulk sorbents used in the MSPD/IE step, endcapped (EC) C18 and C8, monofunctional (MF) C18, non-endcapped C18, and propylsulphonic acid (PRS) were supplied by International Sorbent Technology Ltd , Cambridge, UK

The immunoassay kit used for the determination of β-agonists was obtained from Laboratoire d'Hormonologie (Marloie, Belgium) and the antiserum had principal cross-reactivities of 118%, 100%, 78% and 29% for clenbuterol, salbutamol, mabuterol and terbutaline, respectively Tritiated clenbuterol and salbutamol, also obtained form Laboratoire d'Hormonologie, were used in the method development stages

Phosphate buffer (0 01M, pH 7 0) containing 1 g/l gelatin and 0 1 g/l thimerosal, was used for the RIA Dextran-coated charcoal was prepared by adding 2 g of activated charcoal (Sigma, St Louis, U S A) and 0 25 g of dextran T70 (Pharmacia, Sweeden) to 500 ml of doubly-distilled water The scintillation cocktail was Cocktail T (BDH) Clenbuterol hydrochloride and salbutamol were obtained from Sigma

5 2 2 EQUIPMENT

A LKB Wallac 1219 Rackbeta liquid scintillation counter was used to count radioactivity

5 2 3 EXTRACTION, LIVER SAMPLES

Liver samples (0 5 g), assayed as being free of β-agonist residues, were weighed into glass mortars To the liver samples, 10 μl volumes of ethanolic solutions containing clenbuterol and salbutamol at concentrations of 2 5 μg/ml were added to give fortification levels of 50 ng/g Fortification of liver samples with tritiated clenbuterol and salbutamol was performed by pipetting 10 μl of the relevant ethanolic solution (equivalent to 20,000 cpm) onto the samples All fortified samples were left to equilibrate for 10 minutes before extraction

The samples were blended with 2 g of bulk sorbent (EC C18 or C8, MF C18 or non-EC C18) for forty seconds before the addition of ion exchange material PRS material (0 25, 0 5 and 1 0 g) was added to the sorbent/tissue blend and mixed with a spatula for 30 seconds Any clusters of material were dispersed using a spatula until a homogenous mixture was obtained The resulting blend was transferred to a 10 ml syringe barrel containing two filter paper discs

5 2 4 COLUMN ACTIVATION AND WASHING

Acetic acid, 50 mM (15 ml) was added to the blend and was brought through under vacuum Methanol (8 ml) was applied to the column and brought through under increased vacuum The blend was dried for 15 minutes under vacuum before the elution solvent (8 ml) of 2% to 10% ammonia in methanol was applied

5 2 5 DETERMINATION

(a) Method development, based on fortification with tritiated clenbuterol or salbutamol

For the initial method development 1ml aliquots of the final eluate and of the preceeding washes, from samples which had been fortified with tritiated clenbuterol or salbutamol, were counted in 10ml cocktail T scintillation fluid Control (non-fortified) liver extracts, which had been processed under similar conditions, were spiked with tritiated material immediately before counting and the counts obtained were taken as a reference value for quantification of the breakthrough of analyte in the washing stages and the final recovery in the elution solvent

(a) Method validation, based on fortification with tritiated clenbuterol or salbutamol at 50 ng/g

The MSPD/IE elution solvent was evaporated at 60^0C under a stream of nitrogen and the extracts were reconstituted in 6 25 ml phosphate gelatin buffer Following vortexing and sonication, 0 05 ml of the liver extracts (equivalent to 200 pg of clenbuterol or salbutamol) were added to culture tubes containing 0 45 ml of phosphate gelatin buffer 0 05 ml of control liver extracts were added to culture tubes containing a salbutamol standard at 0, 10, 40, 100, 250 and 1000 pg for the standard curve, and to culture tubes containing 200 pg of clenbuterol and salbutamol standards in 0 05 ml phosphate gelatin buffer (recovery standards) To the salbutamol standard curve and the β-agonist recovery standards, 0 45 ml and 0 40 ml aliquots of phosphate gelatin buffer was added, respectively, bringing the final volume of the sample extracts, standard curve and the β-agonist recovery standards to 0 5 ml After vortexing, the tubes were incubated at 37^{0}C for ten minutes 0 1 ml of ^{3}H-clenbuterol (8000 cpm) and 0 1 ml of antiserum were added, and the tubes were incubated at 37^{0}C for a further 15 minutes and overnight at 4^{o}C 0 5 ml of dextran-coated charcoal was added and the tubes were centrifuged for 10 min at 2000 rpm The supernatants were decanted into scintillation vials and 10 ml cocktail T was added The vials were counted for 5 minutes

5.3. RESULTS AND DISCUSSION

5 3 1 SELECTION OF THE AMOUNT OF PROPYL SULPHONIC ACID

The initial method development focused on determining a suitable amount (g) of PRS material for the MSPD/IE step The required amount of PRS was that which retained the β-agonists during the washing stages and allowed for their elution upon the addition of methanol containing ammonia Three amounts of PRS material (0 25, 0 5 and 1 0 g) were investigated in combination with 2 g of C18 EC bulk sorbent The C18 EC was chosen for this evaluation because, as it is the least polar of the reversed-phase sorbents under study, the ionic retention characteristics of the PRS material would be in greatest demand The other reversed-phase sorbents would contribute more to analyte interactions due to their higher polarity The amount of PRS sufficient for retention of β-agonists using C18 EC material was judged to be adequate for sorbents of higher polarity ^{3}H-clenbuterol and ^{3}H-salbutamol were used in this study to track β-agonist breakthrough in the washing stages and β-agonist recovery in the elution stage Profiles of the breakthrough and final recovery of the clenbuterol and salbutamol in the washing and elution solvents are shown in Figures 5 1 and 5 2, respectively All determinations were carried out in duplicate (Tables 5 1 and 5 2)

There is little breakthrough of clenbuterol in the acetic acid and methanol washing steps at any of the PRS amounts under investigation This effective retention may be attributed to the presence of two amino groups (one alkyl, one aromatic) on the clenbuterol molecule which interact with the cation exchange sorbent An elution solvent of 2% ammonia in methanol does not have the required strength to fully elute clenbuterol from the MSPD/IE blend, particularly when 1g of PRS sorbent is used Concentrations of ammonia in methanol of 4 and 6 % are effective in substantially eluting clenbuterol (i e ≥ 89 %) at the three amounts of PRS used and the highest recovery of clenbuterol is obtained with a 1 g quantity of PRS and an elution solvent of 6% ammonia in methanol

Higher sample breakthrough values are observed for salbutamol in the acetic acid washes particularly when using less than 1 g of PRS material The percentage breakthrough of salbutamol when using 0 25, 0 5 and 1 g PRS sorbent were 23, 19 and 9%, respectively The high breakthrough of salbutamol at lower amounts of PRS material may be due to an insufficient amount of the cation exchange sorbent being present to fully retain the β-agonist Salbutamol has two hydroxy groups on the aromatic ring and, therefore, is quite polar, this hydrophilic character coupled with the molecule having only one amino group for ionic interactions with the cation exchanger may explain the high sample breakthrough of salbutamol in the acetic acid washes at low amounts of PRS material Very little salbutamol breakthrough is observed in the methanol wash of the MSPD/IE columns The highest recovery of salbutamol is achieved using 1g PRS and an elution solvent of 4 to 6

Figure 5 1 Profile of breakthrough and recovery of ^{3}H-clenbuterol in the washing and elution solvents for each of the three amounts of PRS sorbent used in combination with 2 g C18 EC bulk sorbent

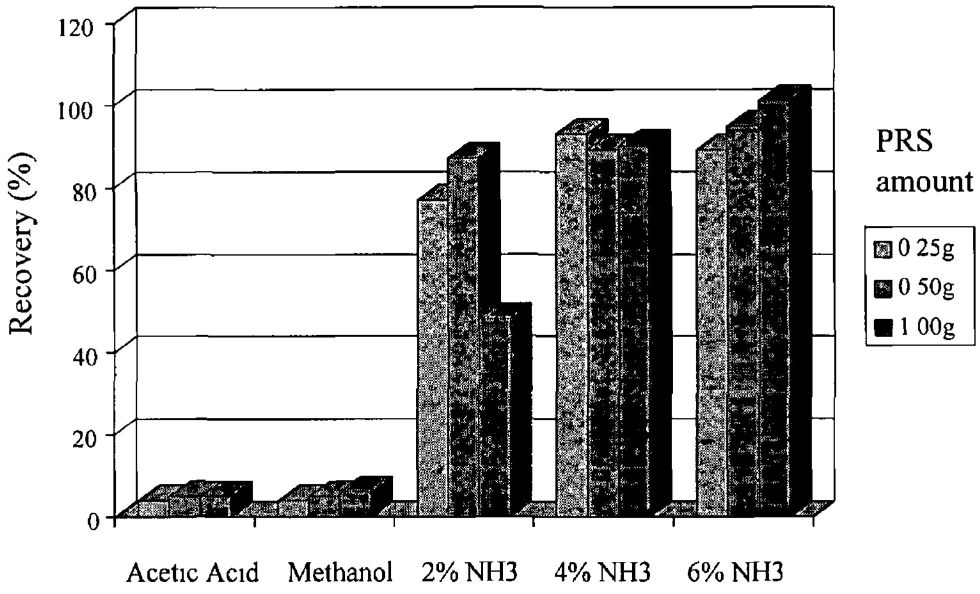


Table 5 1 Breakthrough and final recovery of ^{3}H-clenbuterol in the washing and elution solvents for each of the three amounts of PRS material used in combination with 2 g C18 EC bulk sorbent

	Recovery (%)				
PRS (g)	Acetic Acid	Methanol	2% NH_3 in MeOH	4% NH_3 in MeOH	6% NH_3 in MeOH
0 25	4	4	77	93	89
0 5	5	5	87	89	95
1 00	5	6	48	90	101

Figure 5.2 Profile of breakthrough and recovery of ^{3}H-salbutamol in the washing and elution solvents for each of the three amounts of PRS material used in combination with 2 g C18 EC material.

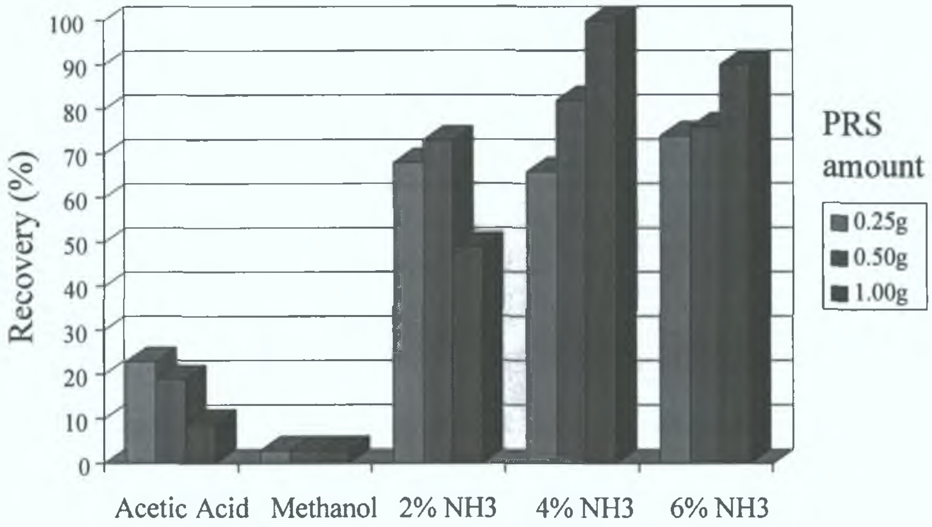


Table 5.2 Breakthrough and final recovery of ^{3}H-salbutamol in the washing and elution solvents for each of the three amounts of PRS material used in combination with 2 g C18 EC sorbent.

	Recovery (%)				
PRS (g)	Acetic Acid	Methanol	2% NH_3 in MeOH	4% NH_3 in MeOH	6% NH_3 in MeOH
0.25	23	3	68	66	74
0.50	19	3	73	82	76
1.00	9	3	49	100	90

% ammonia in methanol The low recoveries of salbutamol observed for the blends containing 0 25 g and 0 5 g PRS material are explained by the loss of salbutamol in the initial acetic acid washing steps Figures 5 1 and 5 2 show that 1g of PRS material used in combination with an elution solvent of 4 % ammonia or greater yields the highest recoveries for both β-agonists with minimal breakthrough in the washing steps

5 3 2 SELECTION OF REVERSED-PHASE SORBENT

The relative retention and clean-up characteristics of four reversed-phase sorbents (C18 EC, C8 EC, C18 and MF C18) were assessed, in combination with a 1 g amount of PRS sorbent, for the extraction of salbutamol and clenbuterol from fortified liver samples Liver samples fortified at 50 ng/g were used to assess the performance of the four sorbent types and the appropriate concentration of ammonia required for the elution of the analytes from the MSPD/IE blend Good quantitative measurement was obtained by preparing the salbutamol RIA standard curve in residue-free sample extract To allow for the variation in specificity of the antibody for the two β-agonists, individual standards (recovery standards) at the appropriate concentration for each analyte (200 pg) were prepared in residue-free sample extracts and assayed with the samples Profiles of the the recovery of clenbuterol and salbutamol from the four sorbent types using five concentrations of ammonia in methanol (ranging from 2 to 10 %) are shown in Figures 5 3 and 5 4, respectively All determinations were carried out in duplicate (Tables 5 3 and 5 4) Highest recovery of clenbuterol is obtained with MF C18 at ammonia concentrations of 4% or higher and with C18 EC and C8 EC sorbents at 6% or higher, while relatively poor recovery is obtained with the C18 sorbent at all concentrations of ammonia The most polar sorbent, non-endcapped C18, substantially retains clenbuterol on the MSPD/IE column on elution with 2% ammonia in methanol and, even at concentrations of 4%

Figure 5 3 Profile of the recovery of clenbuterol from four reversed-phase sorbents, in combination with 1g PRS ion-exchange sorbent, on elution at different concentrations of ammonia in methanol

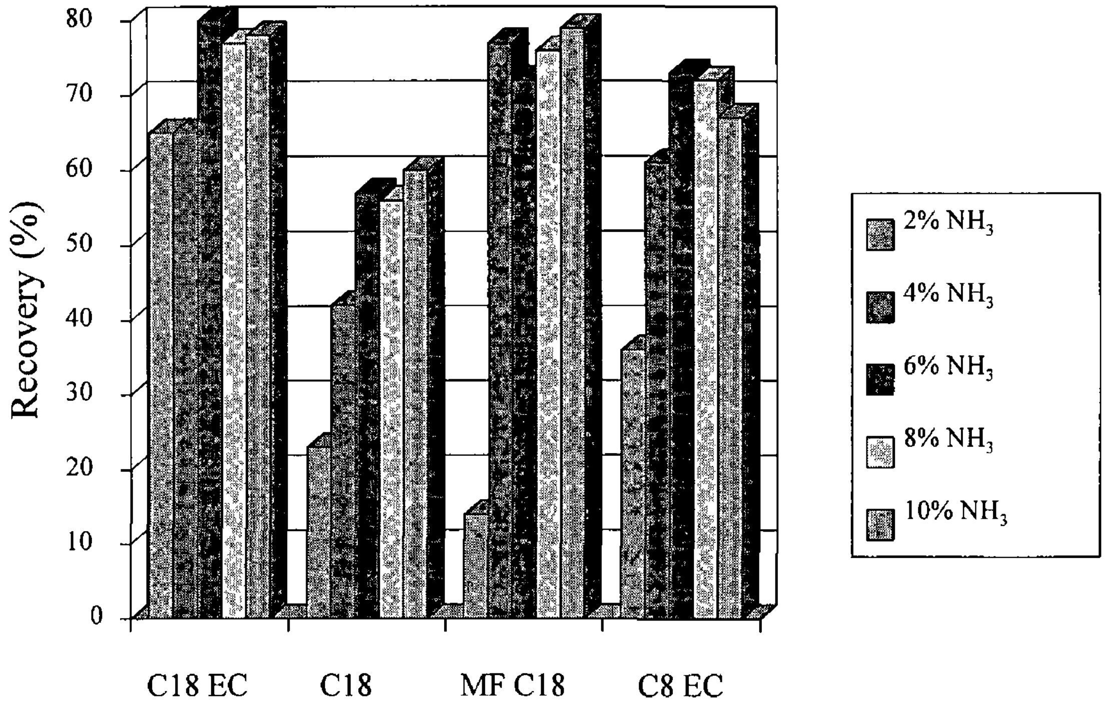


Table 5 3 Recovery of clenbuterol from four reversed-phase sorbents, in combination with 1g PRS ion exchange sorbent, on elution at different concentrations of ammonia in methanol

Ammonia in methanol (%)	Recovery (%)			
	C18 EC	C18	MF C18	C8 EC
2	65	23	14	36
4	65	42	77	61
6	80	57	72	73
8	77	56	76	72
10	78	60	79	67

Figure 5 4 Profile of the recovery of salbutamol from four reversed-phase sorbents, in combination with 1g PRS ion-exchange sorbent, on elution at different concentrations of ammonia in methanol

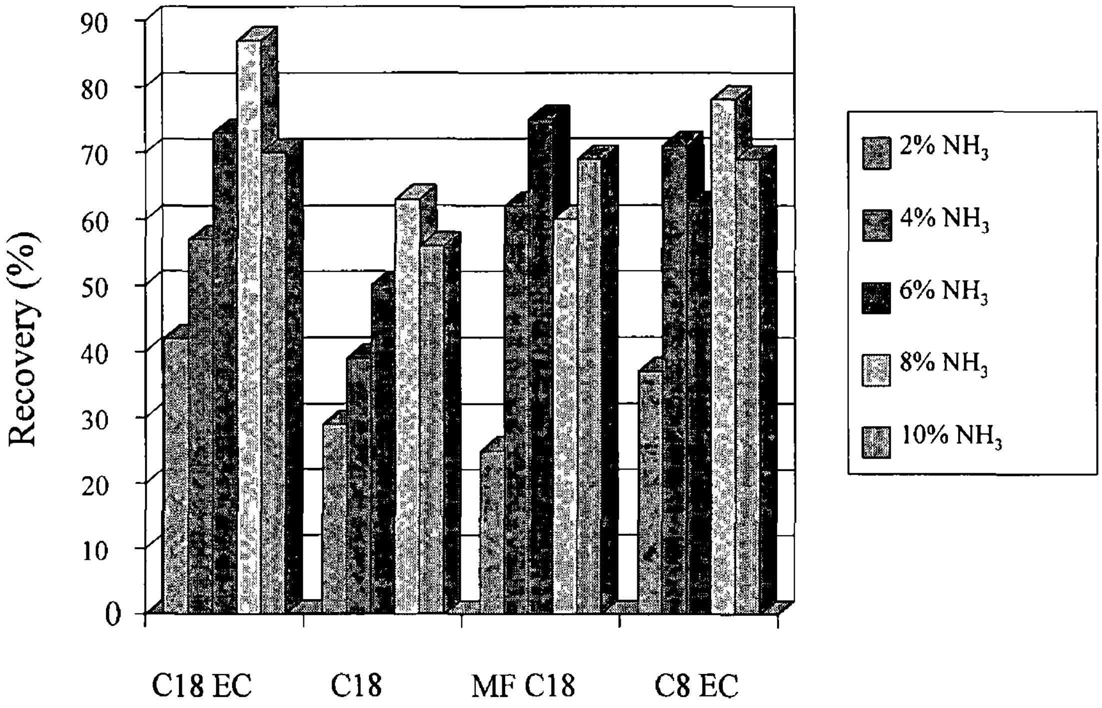


Table 5 4 Recovery of salbutamol from four reversed-phase sorbents, in combination with 1g PRS ion exchange sorbent, on elution at different concentrations of ammonia in methanol

Ammonia in methanol (%)	Recovery (%)			
	C18 EC	C18	MF C18	C8 EC
2	42	29	25	37
4	57	39	62	71
6	73	50	75	62
8	87	63	60	78
10	70	56	69	69

ammonia and above, the combined retention capacity of the reversed-phase and ion exchange sorbents are only partially overcome Monofunctional C18 sorbent shows strong discrimination between an eluent containing 2 % ammonia and eluents containing 4 % or higher ammonia, this discrimination may be useful in developing a clean-up step in the procedure The C18 EC sorbent, the least polar of all the bulk sorbents, yields a consistently high recovery of clenbuterol at all concentrations of ammonia in methanol and is, therefore, the preferred sorbent for use in a mixed-mode MSPD extraction of clenbuterol from liver

Highest recovery of salbutamol was obtained with MF C18, C18 EC and C8 EC sorbents at ammonia concentrations of 6 % (MF C18) and 8 % (C18 EC and C8 EC), while relatively poor recovery was obtained with the C18 sorbent at all concentrations of ammonia Again, as for clenbuterol, it is the C18 EC sorbent, the least polar of the four sorbents, which yields the highest recovery of salbutamol (87%)

The results indicate that the C18 EC sorbent is the most suitable sorbent for the simultaneous extraction of clenbuterol and salbutamol, and that an elution solvent of 8% NH_3 in methanol is an appropriate elution solvent for this combined extraction

5 3 3 REDUCTION OF MATRIX INTERFERENCE IN FINAL EXTRACTS

Determination of clenbuterol and salbutamol at fortification levels lower than 50 ng/g proved difficult due to the amount of matrix interference present in the sample extracts which affected the radioimmunoassay Where a small aliquot of sample extract can be used in the RIA (equivalent to 4 mg of tissue) interference is negligible, but use of larger aliquots of extract, which is necessary to achieve low ppb detection capability, requires much cleaner extracts The further clean-up was investigated by evaluating the possibility of using both organic and aqueous washing solutions in combination with the acetic acid and methanol MSPD/IE washing procedure The aqueous washes included a phosphate buffer wash (50 mM, pH 6 0) and a water wash, while the organic solvents investigated were hexane and acetonitrile Previous work with mixed-mode SPE has shown pH 6 0 to be an effective pH for the selective retention of the β-agonists and elution of interferences A phosphate buffer wash was evaluated both before and after the acetic acid wash and as a replacement for the acetic acid washing step Hexane and acetonitrile were evaluated as possible organic wash solvents All wash steps were applied as 8 ml volumes

Liver samples, assayed as being free of β-agonist residues, were blended with 2g C18 EC sorbent, combined with 1g PRS material and the various on-column washes were carried out The non-specific binding (NSB) values (a measure of the level of interference present in the final extract) of the resulting extracts (equivalent to 100 mg sample) were determined by radioimmunoassay The details of the six washing procedures and the non-specific binding value determined for each procedure are shown in Table 5 5 The acetic acid⇒methanol⇒8% NH_3 procedure, which gave a NSB value of 2625 cpm, is that used for assessment of the four reversed-phase sorbents (5 3 2 above) This high NSB value does not permit low ppb determinations The addition of hexane and acetonitrile washes, which gave NSB values of 2508 and 2777 cpm, respectively, do not contribute to any further clean-up Lower NSB values (928-1223 cpm) were achieved with the introduction of a phosphate buffer wash either before or after the acetic acid washing step, and also as an alternative to the acetic acid wash

Liver samples, fortified with clenbuterol and salbutamol at 2 ng/g, were cleaned-up using the acetic acid ⇒ phosphate buffer ⇒ methanol ⇒ 8% NH_3 washing procedure and yielded recoveries of 86% and 70% for clenbuterol and salbutamol, respectively The relatively high recovery of both β-agonists indicate the potential suitability of this washing procedure for the analysis of β-agonists at low ng/g levels

Table 5 5 Evaluation of various wash/elution procedures for MSPD/IE

Wash/elution procedure	Non-specific binding (cpm)
AA⇒MeOH⇒8% NH_3	2625
AA⇒PB⇒MeOH⇒8% NH_3	928
AA⇒HEX⇒MeOH⇒8% NH_3	2508
AA⇒ACN⇒MeOH⇒8% NH_3	2777
PB⇒MeOH⇒8% NH_3	1071
PB⇒AA⇒MeOH⇒8%NH_3	1008
PB⇒H_2O⇒MeOH⇒8% NH_3	1223

AA = Acetic acid, PB = phosphate buffer, HEX = hexane, ACN = acetonitrile, MeOH = methanol

5.5 CONCLUSION

As an extension of the work on use of mixed-mode SPE for extraction/clean-up of β-agonists from liver (chapter 4), the principles of that approach were combined with the MSPD methodology developed for β-agonists in the laboratory **[10-13]** The developed technique consists of a manual blending of liver with reversed-phase sorbent and further mixing of this blend with cation exchange sorbent, prior to its packing into a syringe barrel for a wash/elution sequence This technique gives high recovery of the different types of β-agonist and allows for the application of a range of wash steps without substantial loss in residue recovery However, the clean-up obtained was insufficient to allow for residue determination consistently at the low ppb level It would appear that the mechanism involved in retention of the β-agonists also causes retention of matrix co-extractives which act as interferents in the RIA determination Nonetheless, the developed method illustrates a possible new application of MSPD through the use of different sorbent chemistries to achieve a single-stage multi-residue procedure

5.6 REFERENCES

1 Barker, S A , Long, A R and Short, C R , J Chromatogr , **475**, 1989, 353

2 Schenk, F J , Barker, S A and Long, A R, J Assoc Off Anal Chem , **75**, 1992, 655

3 Iosifidou, E , Shearan, P and O'Keeffe, M , Analyst, **119**, 1994, 2227

4 Long, A R , Hsieh, L C , Malbrough, M S , Short, C R and Barker, S A , J Agric Food Chem , **38**, 1990, 430

5 Long, A R , Hsieh, L C , Bello, A C , Malbrough, M S , Short, C R and Barker, S A , J Agric Food Chem , **38**, 1990, 427

6 Long, A R , Hsieh, L C , Malbrough, M S , Short, C R and Barker, S A , J Agric Food Chem , **38**, 1990, 423

7 Long, A R , Soliman, M M and Barker, S A , J Assoc Off Anal Chem , **74**, 1991, 493

8 Long, A R , Malbrough, M S , Hsieh, L C , Short, C R and Barker, S A , J Assoc Off Anal Chem , **73**, 1990, 860

9 Long, A R , Hsieh, L C , Malbrough, M S , Short, C R and Barker, S A , J Assoc Off Anal Chem , **73**, 1990, 379

10 Boyd, D , Shearan, P , Hopkins, S , O'Keeffe, M and Smyth, M R , Anal Chim Acta **275**, 1993, 221

11 Boyd, D , Shearan, P , Hopkins, S , O'Keeffe, M and Smyth, M R , Anal Proc **30**, 1993, 156

12 Boyd, D , O'Keeffe, M and Smyth, M R , Analyst, **119**, 1994, 1467

13 Boyd, D , O'Keeffe, M and Smyth, M R , Anal Proc , **32**, 1995, in press

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

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

16 Gabiola, C , in "Proceedings of the Euroresidue II Conference, Veldhoven, The Netherlands, May 3-5, 1993", (eds N Haagsma, A Ruiter and P B Czedik-Eysenberg), CIP-Gegevens Koninklijke Bibliotheek, den Haag, ISBN 90-6159-016-7, 257

17 Montrade, M -P , Le Bizec, B and Andre, F , in "Proceedings of the FLAIR Workshops held in Ghent and Thessoloniki, 1992", (eds H A Kuiper and L A P Hoogenboom, RIKILT-DLO,Wageningen, The Netherlands), ISBN 90-5601-003-4, 143

APPENDIX A: PUBLICATIONS

1 *Applications of SPE to Extraction and Clean-up of Clenbuterol from Liver with Determination by Radioimmunassay*,

Sean Collins, Michael O'Keeffe and Malcolm R Smyth

"Proceedings of the FLAIR Workshops held in Liege and Nantes, 1993", (eds H A Kuiper and L A P Hoogenboom, RIKILT-DLO, Wageningen, The Netherlands), ISBN 90-5601-006-9, 97-100

2 *Multi-Residue Analysis for Beta-agonists in Urine and Liver Samples using Mixed Phase Columns with Determination by Radioimmunassay*

Sean Collins, Michael O'Keeffe and Malcolm R Smyth

Analyst, **119**, 1994, 2671-2674

3 *Rapid analysis of β-agonists in urine by thermospray tandem mass spectrometry*

J A Van Rhijn, M O'Keeffe, H H Heskamp and S Collins

Journal of Chromatography A, 1995, in press

APPLICATIONS OF SPE TO EXTRACTION AND CLEAN-UP OF CLENBUTEROL FROM LIVER WITH DETERMINATION BY RADIO-IMMUNOASSAY

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INTRODUCTION

The (re)-partitioning effect of β-adrenergic agonists in meat production has been well documented (1) The purpose of this study was to develop an efficient extraction procedure for clenbuterol from liver samples using Solid Phase Extraction (SPE), with determination by radioimmunoassay Following enzymatic digestion of liver in basic buffer and centrifugation, the supernatant is purified on a liquid/liquid extraction column (2 - 6) Further clean-up is carried out on a silica SPE cartridge and clenbuterol is determined in the final extract This method may be suitable as an automated procedure

EXPERIMENTAL

Extraction from liver samples

1 25 g of liver was weighed into a 60 ml centrifuge tube Tris buffer containing Protease enzyme (Type XXIV) and $CaCl_2$ was added Following homogenisation and digestion for one hour at 55°C with repeated vortexing, the digest was centrifuged and 20 ml of the supernatant was cleaned up on a liquid/liquid extraction column

Liquid/Liquid Extraction Column Clean-up

Clean-up of the primary digest on a Chem Elut column was used to link the extraction with a SPE clean-up and concentration step The recoveries of tritiated clenbuterol from 20 ml of primary extract under various conditions are shown in Table 1 Optimum recovery involved a pH adjustment of the primary extract to 12, followed by a twenty minute equilibration period with elution of clenbuterol by toluene (60 ml)

Table 1 Recoveries of tritiated clenbuterol from primary extract using Chem Elut columns under various conditions

Eluant	Hexane		Toluene		Tol DCM 3 1
pH	10	12	10	12	12
Recovery (%)	41	35	38	80	75

Tol = Toluene
DCM = Dichloromethane

Silica SPE extraction of clenbuterol from toluene

Tritiated clenbuterol was used to develop a method to retain clenbuterol from toluene on a silica cartridge (Sep-Pak) The average recovery of clenbuterol using elution with 9 ml ethanol was 88% The complete extraction and clean-up procedure (Figure 1) yielded extracts suitable for determination of clenbuterol by radioimmunoassay (RIA), using a kit supplied by Laboratoire d'Hormonologie, Marloie, Belgium

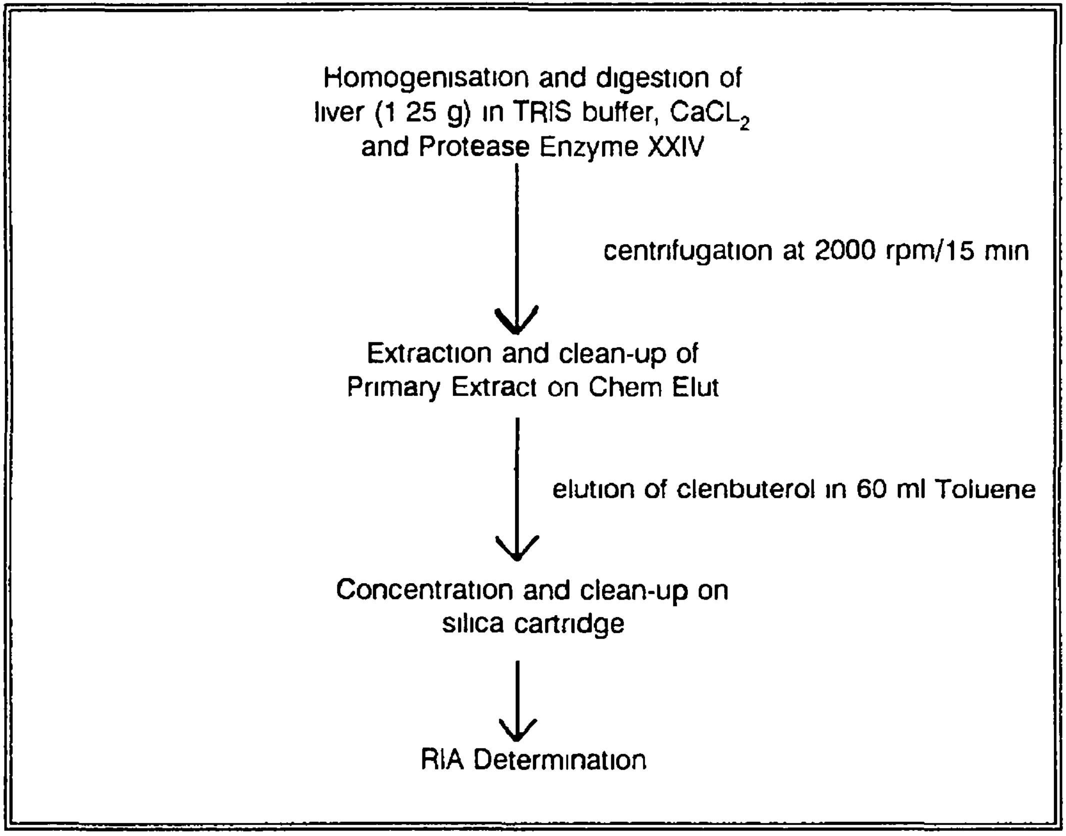

Figure 1 Flow Chart of Extraction and Clean-up procedure

RESULTS AND DISCUSSION

The inter-assay and intra-assay variations for the determination of clenbuterol in fortified liver are shown in Tables 2 and 3, respectively The inter-assay variation for determination of clenbuterol in an incurred sample is shown in Table 4 While recoveries with this method are relatively low, both the inter-assay and intra-assay variations have acceptable coefficients of variation The results in Table 4 indicate that the procedure can be successfully applied to incurred liver samples, the concentration of clenbuterol in the incurred sample, determined using a standard method, is 0 37 ± 0 08 ng/g

Table 2 Inter-assay variation for SPE/RIA determination of clenbuterol in liver

Clenbuterol added ng/g	n	Percentage Recovery	
		mean ± SD	CV (%)
0 2	5	71 3 ± 7 7	10 8
0 4	4	56 1 ± 6 6	11 7

Table 3 Intra assay variation for SPE/RIA determination of clenbuterol in liver

Clenbuterol added ng/g	n	Percentage Recovery	
		mean ± SD	CV (%)
0 2	6	75 3 ± 4 7	6 2
0 4	6	54 4 ± 9 0	16 5

Table 4 Inter-assay variation for SPE/RIA determination of clenbuterol in incurred liver

Sample	n	Clenbuterol determined (ng/g)	
		mean ± SD	CV (%)
Incurred liver	4	0 37 ± 0 08	21 5

Liquid/liquid extraction or liquid/liquid back extraction techniques are not involved, avoiding the problem of emulsion formation This, coupled with the lack of any solvent evaporation steps, allows for automation of the clean-up step once the primary extract has been generated The application of this procedure to other β-agonist residues, such as salbutamol and terbutaline, may result in poor recoveries due to the hydrophilic nature of these compounds which reduces their recovery from the liquid/liquid extraction column Clean-up steps involving more than one retention mechanism, such as mixed phase SPE, may give higher recoveries for multi-residues than the procedure outlined here

ACKNOWLEDGEMENT

This work was supported by a research grant from Teagasc and the Irish American Partnership fund

REFERENCES

1 A P Moloney and P Allen
Proceedings of the Workshops held in Ghent, May 22/24, 1992 and Thessaloniki, October 30/31, 1992 (Eds H A Kuiper and L A P Hoogenboom, RIKILT-DLO Wageningen, The Netherlands, ISBN 90-5601-003-4) FLAIR Concerted Action no 8, 89-101

2 L A Van-Ginkel, R W Stephany and H J Van-Rossum (1992) J Assoc Off Anal Chem, 75 (3), 554-560

3 D Courtheyn, C Desaever and R Verne (1991) J Chromatogr, 564, 537-549

4 H Hooijerink, R Schilt, W Haasnoot, and D Courtheyn (1991) J Pharm Biomed Anal, 9 (6), 485-492

5 J M Degroodt, B Wyhowski de Bukanski, H Beernaert and D Courtheyn (1989) Z Lebensm Unters Forsch, 189, 128-131

6 C Eddins, J Hamann, K Johnson (1985) J Chrom Sci, 23, 308-311

Multi-residue Analysis for Beta-agonists in Urine and Liver Samples Using Mixed Phase Columns With Determination by Radioimmunoassay*

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A method is described for the extraction of four β-agonists, clenbuterol, salbutamol, mabuterol and terbutaline from bovine urine and liver samples using radioimmunoassay (RIA) as the method of determination. Following enzymic digestion of the liver samples using protease enzyme, the digest is centrifuged and the harvested supernatant is saturated with sodium chloride and adjusted to pH 11.0. An ethyl acetate–propan-2-ol mixture is used to extract the β-agonists from the liver digest. The samples of urine and liver extracts are adjusted to pH 6.0 and applied to mixed phase (XtrackT) columns. The column is washed with water and methanol and the β-agonists are eluted with methanol containing 2% ammonia. After evaporation of the eluting solvent and reconstitution in ethanol the β-agonist residues are determined by RIA, with standard graphs prepared in residue-free sample extract. The procedure has been validated for clenbuterol, salbutamol, mabuterol and terbutaline. The mean recovery of the β-agonists from urine and liver is >75% and >85%, respectively. The detection limit is 0.13 ng ml^{-1} and 0.46 ng g^{-1} of clenbuterol in urine and liver, respectively. The high recoveries attained for both types of β-agonists are a result of an efficient liquid–liquid extraction step coupled with a selective mixed solid-phase extraction procedure.

***Keywords:** Beta-agonists, mixed solid phase extraction, residue analysis, radioimmunoassay*

Introduction

β-Agonists are used therapeutically in the treatment of broncho pulmonary disorders in humans and animals. It has been established that a number of β-agonists may exercise a repartitioning effect from adipose tissue towards muscle tissue, thus, increasing the carcass lean : fat ratio in animals. These metabolic effects have been described when β-agonists are administered at dosages of 5–10 times the recommended therapeutic dose.[1,2] A significant advantage of β-agonists for growth promotion is that they are orally active which allows them to be mixed with animal feed. An increase in the carcass mass of calves of about 10% occurred when clenbuterol was administered orally, without any increase in feed intake.[3] The use of compounds with hormonal or thyreostatic action was prohibited within the EU in 1985[4] except under veterinary prescription. Nevertheless, several cases of food poisoning were reported in Spain when bovine liver containing high levels (>100 ng g^{-1}) of clenbuterol were consumed.[5] Salbutamol had been implicated as an alternative repartitioning agent to clenbuterol,[6] while mabuterol had also been used to improve meat-production efficiency in livestock.[7]

Recent studies have shown that in edible tissues the highest concentrations of clenbuterol are found in the liver while an even greater accumulation occurs in the tissues of the eye.[8,9] Both classes of β-agonist, the more polar substituted phenol type (salbutamol, terbutaline) and the less polar substituted aniline type (clenbuterol, mabuterol), have been determined in a variety of biological matrices such as plasma, urine and liver. High-performance liquid chromatography (HPLC) coupled with ultraviolet, electrochemical or fluorescence detection has been used to determine clenbuterol[10–12] and salbutamol.[13–17] Immunoassays have been developed for clenbuterol[18] and salbutamol[19–21] and gas chromatography–mass spectrometry (GC–MS) has been used for the analysis of both clenbuterol[22] and salbutamol.[6]

Methods which allow for the simultaneous extraction and determination of several β-agonists from urine and liver have been developed using immunoaffinity chromatography (IAC),[23] matrix solid-phase dispersion (MSPD)[24] and mixed-mode solid phase extraction (SPE).[25–28] Variable recovery of β-agonists of different classes have been reported in previous work using mixed-mode solid phase extraction.

The present method uses a combination of liquid–liquid extraction and mixed mode solid-phase extraction for the extraction of four β-agonists [clenbuterol (**1**), salbutamol (**2**), mabuterol (**3**), terbutaline (**4**)] (Fig. 1) from urine and liver with determination by radioimmunoassay (RIA). The recovery of the more polar β-agonists from liver is enhanced through the use of a high ionic strength liquid–liquid extraction step while still maintaining the recovery of less polar β-agonists. The advantage of this method is that high recoveries are attained for all four β-agonists from urine and liver using a relatively simple procedure.

Experimental

Reagents and Equipment

Ethyl acetate ('pesticide' grade), methanol ('HiPerSolv') and ammonia solution (35%, 'AnalaR') were supplied by BDH (Poole, Dorset, UK). Propan-2-ol ('HPLC' grade) was supplied by Aldrich (Dorset, UK) and ethanol ('absolute GR') was obtained from Merck (Darmstadt, Germany). Other chemicals used were of AnalaR grade from BDH, or equivalent. *Suc d'Helix pomatia* [containing β-glucuronidase (100 000 U ml^{-1} and sulfatase (1 000 000 U ml^{-1})] was supplied by Sepracor (Villeneuve la Garenne, France). Protease enzyme, type XXIV (bacterial), was obtained from

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* Presented at The Second International Symposium on Hormone and Veterinary Drug Residue Analysis, Oud St. Jan, Bruges, Belgium, May 31–June 3, 1994.

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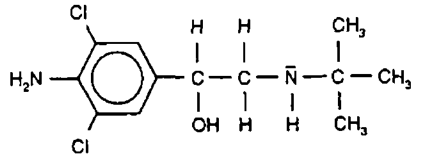
Sigma (St Louis, MO, USA) XtrackT mixed mode solid phase extraction columns (500 mg sorbent size) were supplied by Technicol (Stockport, UK) The immunoassay kit used for the determination of β-agonists was obtained from Laboratoire d'Hormonologie (Marloie, Belgium), with antiserum which had been raised against salbutamol and had principal cross-reactivities of 118% 100% 78% and 29% for **1** **2** **3** and **4**

Phosphate buffer (0 01 mol l^{-1} pH 7 0), containing 1 g l^{-1} gelatin and 0 1 g l^{-1} thimerosal was used for the RIA Dextran coated charcoal was prepared by adding 2 g of activated charcoal (Sigma) and 0 25 g of dextran T70 (Pharmacia Uppsala, Sweden) to 500 ml of doubly-distilled water The scintillation cocktail was Cocktail T (BDH) Clenbuterol hydrochloride salbutamol and terbutaline were obtained from Sigma Mabuterol was provided as a gift by Laboratoire d'Hormonologie

Extraction of Liver Samples

Liver (2 g) was weighed into a 60 ml centrifuge tube A 10 ml aliquot of TRIS buffer (0 2 mol l^{-1} pH 10) containing protease enzyme (0 33 mg ml^{-1}) and $CaCl_2$ (0 1 mol l^{-1}) was added Following homogenization and digestion for 1 h at 55 °C with periodic mixing, the digest was centrifuged for 20 min at 2000 rpm A 5 ml aliquot of the supernatant was collected, 1 8 g of sodium chloride was added and the pH was adjusted to pH 11 0 A volume of 5 ml of ethyl acetate–isopropanol (6 + 4) was added to the liver extract and the solution was mixed on a horizontal shaker for 15 min The sample was centrifuged (2000 rpm, 15 min) and the organic phase (4 ml) was collected This step was repeated and the combined organic phase (8 ml) was evaporated at 40 °C under a gentle stream of nitrogen



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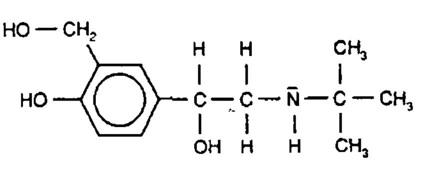

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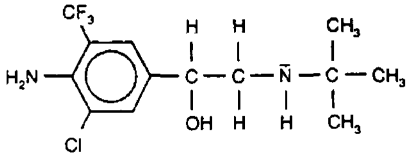

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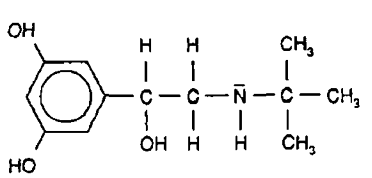

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Fig 1 Structures of clenbuterol (1) salbutamol (2) mabuterol (3) and terbutaline (4)

Mixed-mode Solid-phase Extraction

A 6 ml aliquot of phosphate buffer (0 1 mol l^{-1} pH 6 0) was added to the urine sample and the liver extract and the pH was adjusted to 6 0 The XtrackT column was conditioned by the addition of 5 ml of methanol, 5 ml of doubly-distilled water and 2 ml of phosphate buffer (pH 6 0, 0 1 mol l^{-1}) The sample was applied at a rate of 2 ml min^{-1} and the column was dried for 10 min by drawing air through the column The column was washed with 5 ml of water and 5 ml of methanol and was dried for 15 min The β-agonists were eluted from the column using 5 ml of methanol containing 2% ammonia The collected eluate was evaporated at 40 °C under a gentle stream of nitrogen and resuspended in 2 ml or 5 ml of ethanol for liver or urine respectively

Radioimmunoassay

Aliquots (0 2 ml) of liver and urine extracts were added to culture tubes 0 2 ml aliquots of control (residue-free) liver or urine extracts were added to culture tubes containing a clenbuterol standard at 0, 10, 40, 100, 250 and 1000 pg for the standard graph, and to culture tubes containing 500 and 200 pg (liver extract) and 1000 and 400 pg (urine extract) each of **1–4** The samples the clenbuterol standards and β-agonist recovery standards (in duplicate) were evaporated to dryness under a stream of nitrogen and redissolved in 0 5 ml of phosphate gelatin buffer (pH 7 0) After vortexing, the culture tubes were incubated at 37 °C for 10 min A 0 1 ml aliquot of [^{3}H]clenbuterol (7500 cpm) and 0 1 ml of antiserum were then added and the culture tubes were incubated at 37 °C for a further 15 min and overnight at 4 °C A 0 5 ml aliquot of dextran coated charcoal was added and the tubes were centrifuged for 10 min at 2000 rpm The supernatants were decanted into scintillation vials and 10 ml of Cocktail T were added The vials were counted for 5 min

Method Validation

Bovine urine and liver samples assayed as being free of β-agonist residues were used for fortification studies To urine samples (5 ml) 10 and 25 µl volumes of an ethanolic solution containing **1–4** at 1 µg ml^{-1} were added to give fortification levels of 2 and 5 ppb respectively To liver samples (2 g) 10 and 25 µl volumes of an ethanolic solution containing the four β agonists at 0 4 µg ml^{-1} were added to give fortification levels of 2 and 5 ppb, respectively The fortified liver samples were left to equilibrate for 10 min before extraction

Results and Discussion

The XtrackT columns contain two retention mechanisms based on octyl and benzene sulfonic acid groups bound to the silica particles The cation-exchange properties of the column coupled with its hydrophobic retention characteristics allow for retention of a range of β-agonists Columns of similar chemistry to these (Clean Screen DAU) were used previously for β-agonist determination in urine[25 26] and for salbutamol in liver[26] This paper contains data for the determination of a range of β-agonists in urine and liver The optimum pH for the retention of β-agonists was found to be pH 6 0 and the elution of these compounds occurred when methanol containing 2% ammonia was applied to the column An important characteristic of this column is that a pre elution wash with 100% methanol is possible which results in cleaner sample extracts a methanol wash on a conventional reversed phase (C_{18}) column would elute the analytes together with interferences

The application of liver extracts, following protein precipitation with HCl and centrifugation directly to the SPE column was not successful due to the amount of matrix interferences still present Studies using liver extracts fortified with [^{3}H]clenbuterol and [^{3}H]salbutamol yielded recoveries of <30% in the elution step from the SPE columns Two published liquid–liquid extraction procedures for β-agonists were evaluated, *tert* butanol + ethyl acetate (3 + 7)^{27} and ethyl acetate + isopropanol (6 + 4) ^{28} the latter solvent mixture was chosen because of the higher recoveries achieved The effect of the pH and ionic strength of the aqueous liver extract on the efficiency of the liquid–liquid extraction of salbutamol was investigated (Table 1) The saturation of the aqueous layer with sodium chloride prior to liquid–liquid extraction with ethyl acetate–propan-2-ol resulted in good recovery of salbutamol at all pH values Saturation of the aqueous layer also resulted in the formation of a well-defined interface during the extraction procedure

An increase in non specific binding (NSB) in the RIA is caused bv the sample extracts, particularly liver extracts mean NSB figures for the extract free buffer, urine extract in buffer and liver extract in buffer are 130 cpm (n = 5), 183 cpm (n = 8) and 672 cpm (n = 9), respectively Good quantitative measurement was obtained, therefore, by preparing the standard graph (clenbuterol) for both urine and liver assays in residue free sample extract To allow for the variation in specificity of the antibody for the four β agonists individual standards at an appropriate concentration for each analyte were prepared in residue-free sample extracts and assayed with the samples

The inter and intra assay variations of the method for urine and liver are shown in Tables 2–5 Adequate recovery of all four β agonists is achieved from both urine and liver at fortification levels of 2 and 5 ng g^{-1} (Tables 2 and 4) The cross reactivity of the antiserum for terbutaline (29%) deter mined the lowest fortification level at which the method could be validated for all four β agonists under identical experimental conditions The method is sensitive to lower levels of clenbuterol, salbutamol and mabuterol The intra assay variations for urine and liver demonstrate good repeatability of the method for urine and liver assays (Tables 3 and 5)

The results obtained from the analysis of residue free urine and liver samples were used to estimate the detection limit The detection limit was 0 13 ng ml^{-1} and 0 46 ng g^{-1} of clenbuterol in urine and liver respectively based on the mean response for the control sample extracts plus three times the standard deviation

Incurred liver samples with residue concentrations (deter mined by alternative methods) of 4 0 3 5 and 8 0 ng g^{-1} of clenbuterol, mabuterol and salbutamol, respectively, were assayed Good agreement was found for the concentrations of clenbuterol (4 3 ng g^{-1}) and mabuterol (4 7 ng g^{-1}) deter mined by this method but a considerably lower level of salbutamol (1 3 ng g^{-1}) was determined The lower level of salbutamol determined probably results from the occurrence

Table 1 Extraction of salbutamol (5 ppb) from an aqueous solution at different pH and sodium chloride levels

β Agonist	pH	Sodium chloride concentration in 5 ml/g	Recovery (%)
Salbutamol	2	0 0	38
		0 8	77
		1 8	91
	7	0 0	90
		0 8	70
		1 8	91
	10	0 0	74
		0 8	70
		1 8	88
	12	0 0	58
		0 8	64
		1 8	74

Table 2 Inter assay variation for recovery of clenbuterol salbutamol mabuterol and terbutaline in urine (n = 5)

β Agonist	Concen tration/ ng ml^{-1}	Recovery (%)		
		Range	Mean ± SD	Relative standard deviation
Clenbuterol	2	75–92	84 ± 6 0	7 2
	5	77–106	92 ± 10 4	11 3
Salbutamol	2	73–93	85 ± 8 3	9 7
	5	62–86	76 ± 10 8	14 3
Mabuterol	2	46–95	79 ± 20 6	26 2
	5	89–102	98 ± 5 3	5 5
Terbutaline	2	48–108	81 ± 27 2	33 4
	5	64–106	82 ± 18 0	21 0

Table 3 Intra assay variation for recovery of clenbuterol salbutamol mabuterol and terbutaline in urine (n = 4)

β Agonist	Concen tration/ ng ml^{-1}	Recovery (%)		
		Range	Mean ± SD	Relative standard deviation
Clenbuterol	2	73–80	77 ± 3 6	4 6
	5	86–89	87 ± 1 3	1 4
Salbutamol	2	55–68	65 ± 7 3	11 4
	5	70–86	77 ± 6 8	8 8
Mabuterol	2	84–91	90 ± 4 6	5 1
	5	87–92	91 ± 2 5	2 8
Terbutaline	2	48–78	69 ± 14 0	20 0
	5	85–100	94 ± 7 0	7 4

Table 4 Inter assay variation for recovery of clenbuterol salbutamol mabuterol and terbutaline in liver (n = 5)

β Agonist	Concen tration/ ng g^{-1}	Recovery (%)		
		Range	Mean ± SD	Relative standard deviation
Clenbuterol	2	82–116	92 ± 14 1	15 0
	5	70–108	93 ± 14 3	15 4
Salbutamol	2	80–124	108 ± 17 7	15 4
	5	99–120	109 ± 8 0	7 3
Mabuterol	2	85–116	100 ± 11 2	11 2
	5	82–108	94 ± 10 4	11 1
Terbutaline	2	78–119	100 ± 15 0	15 0
	5	80–103	96 ± 9 2	9 6

n = 4

Table 5 Intra assay variation for recovery of clenbuterol salbutamol mabuterol and terbutaline in liver (n = 4)

β Agonist	Concen tration/ ng g^{-1}	Recovery (%)		
		Range	Mean ± SD	Relative standard deviation
Clenbuterol	2	87–99	92 ± 5 9	6 4
	5	81–99	90 ± 8 2	9 0
Salbutamol	2	77–117	93 ± 17 0	18 0
	5	89–94	93 ± 2 5	2 7
Mabuterol	2	80–93	86 ± 5 6	6 5
	5	83–97	89 ± 6 0	6 7
Terbutaline	2	88–123	110 ± 17 0	15 3
	5	62–81	73 ± 8 2	11 2

Table 6 Comparison of the recovery of terbutaline from urine using XtrackT columns of different size and at different rates of sample application ($n = 4$)

Terbutaline concentration/ ng ml^{-1}	Sample application rate/ ml min^{-1}	XtrackT particle size/ µm	Recovery (%) Mean ± SD	Recovery (%) Relative standard deviation
2	0.5	125–210	82 ± 2.7	3.2
	0.5	40–50	69 ± 14.0	20.0
	2.0	40–50	21† ± 14.4	68.5
5	0.5	125–210	93 ± 7.6	8.1
	0.5	40–50	94 ± 7.0	7.4
	2.0	40–50	41† ± 20.5	50.2

† Within each fortification level mean recovery values with different superscripts are significantly different ($p < 0.01$)

of salbutamol in liver largely as a conjugated residue[28] An enzymic deconjugation step was introduced into the method, 0.66 ml suc d'Helix Pomatia (diluted 1 + 10) was added to the 5 ml of liver digest supernatant which was adjusted to pH 5.0 and incubated at 37 °C overnight With this modification a salbutamol residue concentration of 9.0 ng g^{-1} was determined in the sample

As a result of the hydrophilic character of terbutaline and its biphenol like structure (Fig 1), application of terbutaline containing urine samples to the SPE columns at a rate in excess of 0.5 ml min^{-1} resulted in a significantly reduced recovery (determined by analysis of variance) of the compound (Table 6) The effect of the rate of application of the samples on recovery was not as pronounced for clenbuterol salbutamol or mabuterol XtrackT columns containing the same material, but having different particle size, were also investigated for their effectiveness in retaining terbutaline from urine samples applied at a rate of 0.5 ml min^{-1} It is apparent that a larger particle size of the mixed solid-phase extraction column results in higher recoveries of terbutaline and lower variability (Table 6), possibly owing to the increased surface area of the column

Conclusion

The described method is suitable for the extraction of clenbuterol salbutamol mabuterol and terbutaline from bovine urine and liver with determination by RIA Owing to the limited cross reactivity of the antibody used in the immunoassay step only four β-agonists could be determined however if a detection technique such as GC–MS was used a wider range of β agonists could be determined An enzymic deconjugation step is required to ensure that β agonists which occur in a conjugated form (such as salbutamol) are extracted and determined This method may be used as a screening procedure for β agonists giving high recovery of clenbuterol salbutamol mabuterol and terbutaline from urine and liver samples

The work reported in this paper was undertaken as part of co-ordinated research under the EU FLAIR programme (Concerted Action No 8) S C gratefully acknowledges the financial assistance from the Irish American Partnership Scholars Programme

References

1 *Beta Agonists and Their Effects on Animal Growth and Carcass Quality* ed Hanrahan J P Elsevier London 1987
2 Hanrahan J P Quirke J F Bomann W Allen P McEwan J C Fitzsimons J M Kotman J and Roche J F in *Recent Advances in Animal Nutrition* ed Haresign W and Cole D J A Butterworths London 1986 pp 126–138
3 Lafontan M Berlan M and Prud hon M *Reprod Nutr Dev* 1988 **28** 61
4 Commission of the European Communities *Off J Eur Commun* 1985 **L191** 27
5 Navarro-Martinez J F *Lancet* 1990 **336** 1311
6 Furst P Furst C and Groebel W *Dtsch Lebensm Rundsch* 1989 **85** 341
7 Van Ginkel L A Van Rossom H J and Stephany R W in *Proceedings of the EC Workshop RIVM* Bilthoven ed Van Ginkel L A Van Rossom H J and Stephany R W 1991 p 72
8 Degand G Bernes Duyckaerts A and Maghuin Rogister G J *Agric Food Chem* 1992 **40** 70
9 Heinrich H Meyer D and Rinke L M *J Anim Sci* 1991 **69** 4538
10 Haasnoot W Ploum M E Paulinsen R J A Schilt R and Huf F A *J Chromatogr* 1990 **519** 323
11 Eddins C Hamann J and Johnson K *J Chromatogr Sci* 1985 **23** 308
12 Hooijerink H Schilt R Haasnoot W and Courtheijn D *J Pharm Biomed Anal* 1991 **9** 485
13 Bland R E Tanner R J N Chern W H Lang J R and Powell J R *J Pharm Biomed Anal* 1990 **8** 591
14 Emm T Lesko L J Leslie J and Perkal M B *J Chromatogr* 1988 **427** 188
15 Hutchings M J Paull J D and Morgan D J *J Chromatogr* 1983 **227** 423
16 Tan Y K and Soldin S J *J Chromatogr* 1984 **311** 311
17 Ramos F Castilho M C Noronha da Silveira M I Prates J A M and Dias Correia J H R *Anal Chim Acta* 1993 **275** 179
18 Bucknall S D MacKenzie A L Sauer M J Everest D J Newman R and Jackman R *Anal Chim Acta* 1993 **275** 227
19 Loo J C K Beaulieu N Jordan N Brien R and Mcgilveray I J *Res Commun Chem Pathol Pharmacol* 1987 **55** 283
20 Adam A Ong H Sondag D Rapaille A Marteau S Bellemare M Raymand Ph Giroux D Loo J K and Beaulieu N *J Immunoassay* 1990 **11** 329
21 Howells L Sauer M Sayer R and Clark D *Anal Chim Acta* 1993 **275** 275
22 Blanchflower J W Hewitt S A Canavan A Elliott C T and Kennedy D G *Biol Mass Spectrom* 1993 **22** 326
23 Van Ginkel L A Stephany R W and Van Rossum H J *J AOAC Int* 1992 **75** 554
24 Boyd D O Keeffe M and Smyth M R *Analyst* 1994 **119** 1467
25 Montrade M P Le Bizec B Monteau F Siliart B and Andre F *Anal Chim Acta* 1993 **275** 253
26 Dumasia M C and Houghton E *J Chromatogr Biomed Appl* 1991 **564** 503
27 Leyssens L Driessen C Jacobs A Czech J and Raus J *J Chromatogr Biomed Appl* 1991 **564** 515
28 Montrade M P Riverain S Le Bizec B and Andre F in *Proceedings of the FLAIR Concerted Action No 8 Workshop* ed Kuiper H and Hoogenboom L A P Thessaloniki Greece October 1992 Rikilt DLO Wageningen The Netherlands p 143

Paper 4/03382E
Received June 6 1994
Accepted August 22 1994

ELSEVIER

Journal of Chromatography A, 000 (1995) 000–000

JOURNAL OF
CHROMATOGRAPHY A

Rapid analysis of β-agonists in urine by thermospray tandem mass spectrometry

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Abstract

A method is described for the analysis of β-agonists in urine of cattle. The method uses solid-phase extraction (SPE), followed by analysis of the resulting extract by flow injection thermospray tandem mass spectrometry (TSP-MS–MS). Sample preparation is performed using a mixed-bed SPE procedure using a sorbent having both hydrophobic and ionic properties. MS–MS analysis, following thermospray ionization, is performed in single-reaction monitoring parent mode. In that way isotope dilution can be used for quantitation of clenbuterol. Data are presented on precision and accuracy for clenbuterol and related compounds. Furthermore, data acquisition was performed in full-scan neutral loss mode to indicate the suitability of flow injection analysis (FIA)–TSP-MS–MS for exploratory analysis. Detection of β-agonists in this mode is based on the presence of the N-*tert*-butyl-β-ethanolamino moiety and, in that respect, detection of known as well as unknown compounds having this moiety will take place. This feature is exemplified by the analysis of samples containing several compounds.

1. Introduction

In the EC the use of β-agonists as growth promoting agents in the fattening of animals for human consumption is banned. Nevertheless, these compounds are frequently found in the urine of cattle and analysis for the purpose of regulatory control is carried out in most, if not all, countries. Besides regulatory control, there is a growing interest in methods suitable for "real-time" analysis to perform process control. Within the FLAIR (Food-Linked Agro-Industrial Research) Concerted Action nr. 8 this subject was studied extensively and part of the presented work was carried out within the framework of this EC project.

Furthermore, an additional analytical problem is the use of slightly modified compounds for growth promotion. These compounds are usually not detected whenever a target-compound approach is applied.

For regulatory control, the analysis is usually carried out using GC–MS in the multiple ion detection (MID) mode [1,2]. Although a very effective technique, it has three major drawbacks: the compounds are targeted, so related compounds will not be detected, derivatization is usually necessary and this may impart unwanted selectivity and variability and, furthermore, the procedure typically takes two days to carry out.

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0021-9673/95/$09.50
SSDI 0021-9673(95)00531-5

Whenever the results of analysis should be available on a short term, as for "real-time" analysis, GC–MS methods generally are not very suitable In that case application of LC–MS–MS techniques may offer better performance

We present here a flow injection (FIA)–TSP-MS–MS method that may be used in either of two ways (a) a method for semi-quantitative target-compound analysis that is suitable for very rapid analysis of residues of β-agonists and (b) a method for rapid qualitative group-specific analysis of nanogram amounts of known as well as unknown β-agonists

2 Experimental

2 1 *Materials and instrumentation*

All reagents used were of analytical grade The absence of β-agonists in the blank urine used for spiking experiments was demonstrated using a method based on GC–MS with a limit of detection of 0 2 ng/ml [1]

Clenbuterol-d_6, the analogue of clenbuterol where six protons at the *tert*-butyl moiety are substituted with six deuterons, was used as an internal standard, allowing quantitation by isotope dilution

Sample preparation was performed using XtrackT columns XRDAF515 (World Wide Monitoring, Bristol, PA, USA) containing 500 mg of a sorbent having ionic and hydrophobic properties The clean-up procedure was adapted from the procedure published by Montrade et al [3] In brief, samples are hydrolyzed using glucuronidase/arylsulfatase from *Helix pomatia* at pH 4 8, using acetate buffer for pH adjustment Following hydrolysis, the pH is adjusted to 6 0 using 0 1 *M* phosphate buffer and the extract is applied to a column conditioned with 3 ml each of methanol, water and 0 1 *M* phosphate buffer The column is washed with 0 1 *M* phosphate buffer and the analytes are eluted with methanol containing 3% concentrated ammonium hydroxide

The analysis is carried out using a Finnigan MAT TSQ70 (San Jose, CA, USA) mass spectrometer equipped with a thermospray II interface The interface is operated at 200°C block temperature and 90°C vaporizer temperature The repeller was at 80 V, no discharge ionization was used Argon was used as collision gas at a pressure reading of 1 2 mTorr and a collision offset of −13 eV was applied The carrier eluent consisted of 30% methanol in water with an overall concentration of 0 05 *M* ammonium acetate The solvent was pumped at a flow-rate of 1 0 ml/min by a Gilson 305 pump (Villiers-le-Bel, France) equipped with an additional pulse damper Aliquots of 50 μl were injected directly into the carrier eluent by means of a Gilson 231-401 autosampler

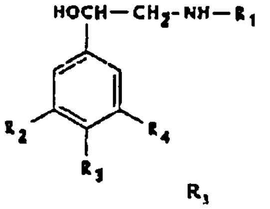
2 2 *Data acquisition*

To perform semi-quantitative analysis, a procedure for single reaction monitoring (SRM) in parent mode was used, monitoring the loss of water (M_r 18) and methylpropene (M_r 56) from the molecular ions For cimaterol and clenbuterol-d_6 corresponding losses were monitored (Table 1) By applying parent mode data acquisition, the data system will record the masses of the precursor ions rather than the masses of the product ions In this way the use of clenbuterol-d_6 as an internal standard becomes possible regardless of the fact that the mass of the product ion monitored is the same as for unlabelled clenbuterol

Urine samples were spiked with five β-agonists (cimaterol, clenbuterol, terbutaline, mabuterol and salbutamol) at 1, 2 and 5 ng/ml, respectively Aliquots of 5 ml of urine each from each spiking level were analyzed in triplicate Following clean-up, to each extract 15 ng of clenbuterol-d_6, corresponding to 3 ng/ml in the urine, was added as an internal standard The extracts were evaporated to dryness and redissolved in 0 50 ml of carrier eluent Subsequently the extracts were analyzed using FIA–TSP-MS–MS in SRM parent mode Aliquots of 50 μl, corresponding to 0 5 ml of urine, were injected in duplicate

To perform group-specific qualitative analysis, data acquisition is performed in full-scan neutral

Table 1
Molecular structure and SRM data for the investigated analytes



Name	R_1	R_2	R_3	R_4	SRM
Clenbuterol (CLEN)	$C(CH_3)_3$	Cl	NH_2	Cl	277→203
Clenbuterol-d_6 (CLEN-d_6)	$C(CD_3)_2CH_3$	Cl	NH_2	Cl	283→203
Mabuterol (MAB)	$C(CH_3)_3$	Cl	NH_2	CF_3	311→237
Terbutaline (TER)	$C(CH_3)_3$	OH	H	OH	226→152
Salbutamol (SAL)	$C(CH_3)_3$	CH_2OH	OH	H	240→166
Cimaterol (CIM)	$CH(CH_3)_2$	CN	NH_2	H	220→160

loss mode The scanning range of the first quadrupole is from 200 to 370 u at one scan per second Consequently the scan range of the second quadrupole is from 126 to 296 u

3. Results and discussion

3 1 *Semi-quantitative determination*

Fig 1 shows a typical result for the analysis of urine samples spiked at 1 ng/ml Table 2 presents the corresponding quantitative data of the three spiking levels tested From Fig 1 it is clear that detection of all analytes except salbutamol is readily performed at 1 ng/ml level with sufficient signal-to-noise ratio For salbutamol, signal-to-noise ratio is low and, although this compound is detected, the limit of detection (LOD) is equal to 1 ng/ml For the other compounds, based on signal-to-noise ratio, a LOD of at least 0 5 ng/ml in the urine is achievable Reagent blanks do not indicate the presence of any of the analytes Analysis of the blank urine extract, however, seems to indicate the presence of cimaterol and clenbuterol For cimaterol this is most likely caused by interfering compounds, because all urine blanks analyzed during this study show a comparable blank offset These interferences were not characterized any further Consequently, for cimaterol the clean-up has to be improved or, alternatively, chromatographic separation of the analyte and the interference may be applied For clenbuterol, other urine blanks did not show an offset, so for this compound it may have been incidental contamination However, especially for clenbuterol the signal is rather small and quantitation yields a blank offset of approximately 0 3 ng/ml

Quantitation is carried out by using clenbuterol-d_6 as an internal standard For clenbuterol the quantitative results are therefore acceptable, especially when taking into account that clenbuterol-d_6 is only added after clean-up so recovery losses are not corrected For the other compounds, quantitative results should be

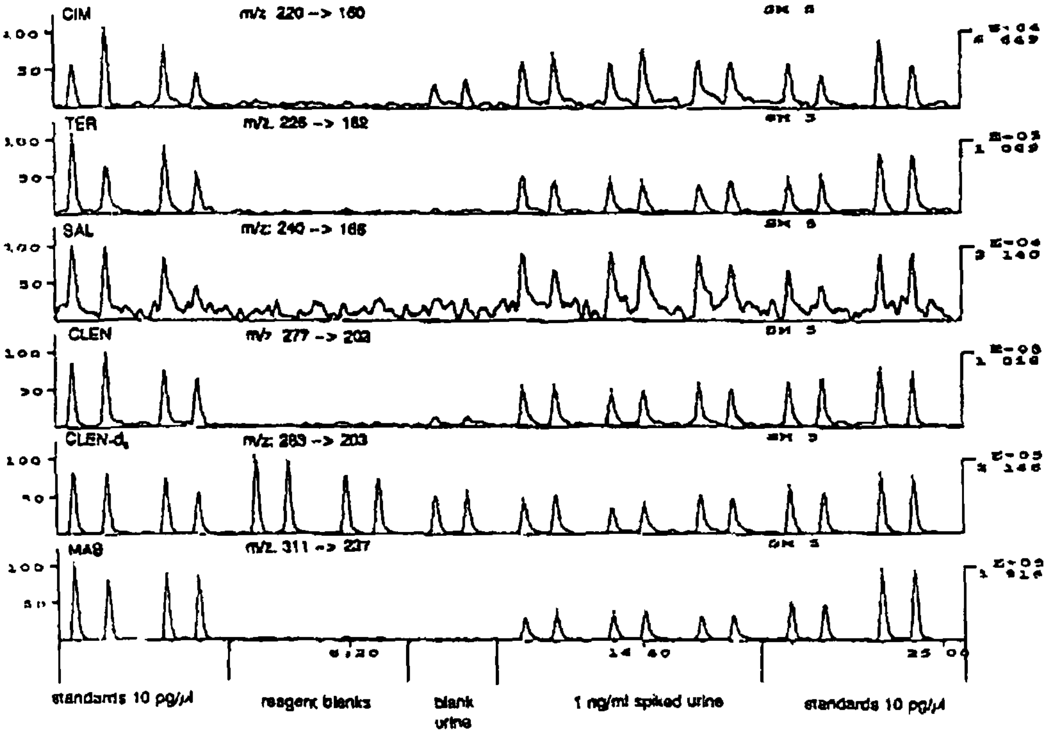


Fig 1 SRM profiles for the FIA–TSP MS–MS analysis of urine samples spiked at 1 ng/ml with five β-agonists

> [illegible] caption

improved Repeatability is again acceptable for clenbuterol and perhaps for mabuterol and terbutaline, but also here improvements have to be made However, as the use of these compounds in the fattening of animals for human consumption is banned within the EC, indication of the presence itself is more important than accurate quantitative results Improvement of quantitation may be achieved by using isotope-labelled standards added before clean-up Particularly for salbutamol the use of salbutamol-d_6 as an internal standard could improve quantitation The results of analysis justify the statement that the presented method yields at least semi-quantitative information, except for salbutamol

Together with the clean-up, not including hydrolysis, the entire procedure, including calibration, can be performed within two hours for a

Table 2
Results for the analysis of clenbuterol (CLEN) salbutamol (SAL), cimaterol (CIM), terbutaline (TER) and mabuterol (MAB) in spiked urine samples purified on XtrackT SPE columns

Spike level	CLEN		SAL		CIM		TER		MAB	
(ng/ml)	Mean (ng/ml)	CV (%)	Mean (ng/ml)	CV (%)	Mean (ng/ml)	CV (%)	Mean (ng/ml)	CV (%)	Mean (ng/ml)	CV (%)
1 0	1 1	15	1 6	28	1 5	25	1 0	21	0 7	21
2 0	2 4	18	4 3	31	2 7	19	1 9	9	1 5	18
5 0	4 4	12	12 2	19	5 9	7	5 6	14	2 9	8

set of 20 samples In that respect the method is far more rapid than any GC–MS method now available

3 2 *Qualitative determination*

Tandem mass spectrometry may also be used for exploratory analysis to detect the possible presence of unknown compounds Using neutral loss scanning, the loss of 74 u can be monitored, corresponding to the loss of water followed by the loss of methylpropene from the molecular ion It is assumed that for all N-*tert*-butyl substituted β-ethanolamines, this fragmentation will occur when using alike experimental parameters For all known compounds this has been confirmed in our laboratory

Fig 2 shows the analysis of a 50-μl aliquot corresponding to 0 5 ml of urine, of a urine extract spiked prior to clean-up, at 3 ng/ml, with several β-agonists The presence of the peak at 4 4 min indicates the presence of one or more compounds that comply with the scanning requirement the loss of 74 u

The inset in Fig 2 shows the CID spectrum averaged over the peak at 4 4 min This spectrum indicates the presence of salbutamol $[M+H]^+$ at m/z 240 and $[M+H-H_2O]^+$ at m/z 222, terbutaline $[M+H]^+$ at m/z 226, clenbuterol $[M+H]^+$ at m/z 277/279 and mabuterol $[M+H]^+$ at m/z 311/313 It is obvious that these compounds are readily detected at this concentration level The presence of two fragment ions for salbutamol may be the reason for the low sensitivity in the semi-quantitative analysis, because there the loss of 74 u from only one ion (m/z 240) is monitored

To exemplify the detection of possible unknowns, a rather artificial experiment was carried out Recently the abuse of the brominated analog of clenbuterol was discovered [4] At that time, in our laboratory, GC–MS was used for the analysis One of these samples was analyzed using the presented method Experimental conditions were the same as in Fig 2 Again an aliquot equivalent to 0 5 ml of urine was injected while scanning the mass spectrometer for the loss of 74 u Fig 3 indicates the presence of com-

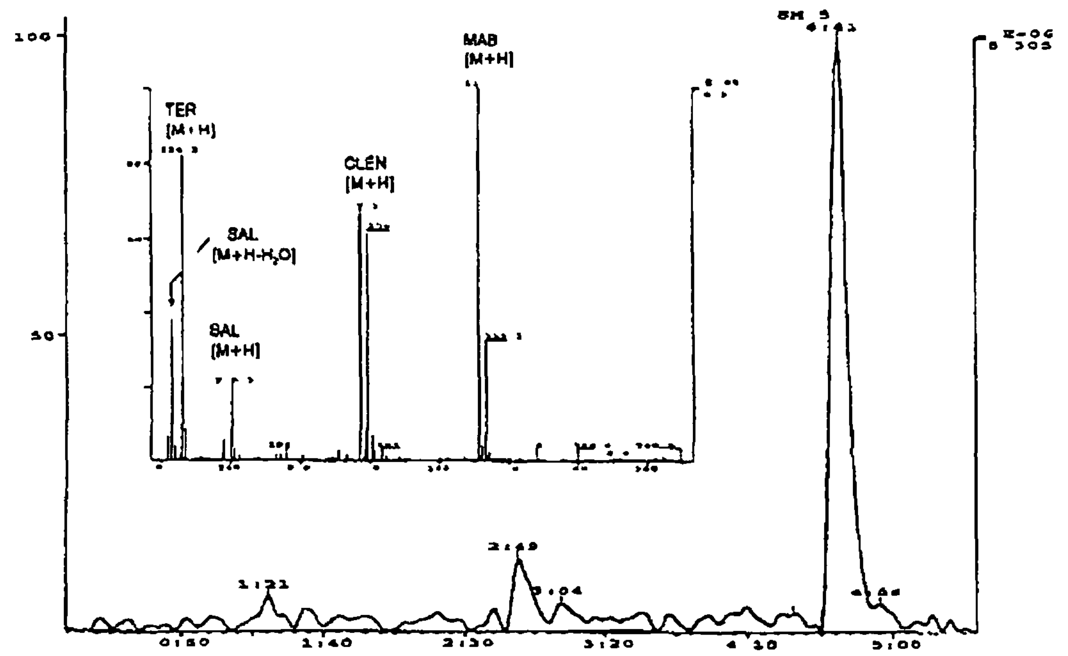


Fig 2 Injection of an extract of a urine sample spiked at 3 ng/ml indicating the simultaneous detection of all N-*tert* butyl substituted compounds present The mass spectrometer was operated in full scan neutral loss (74 u) mode

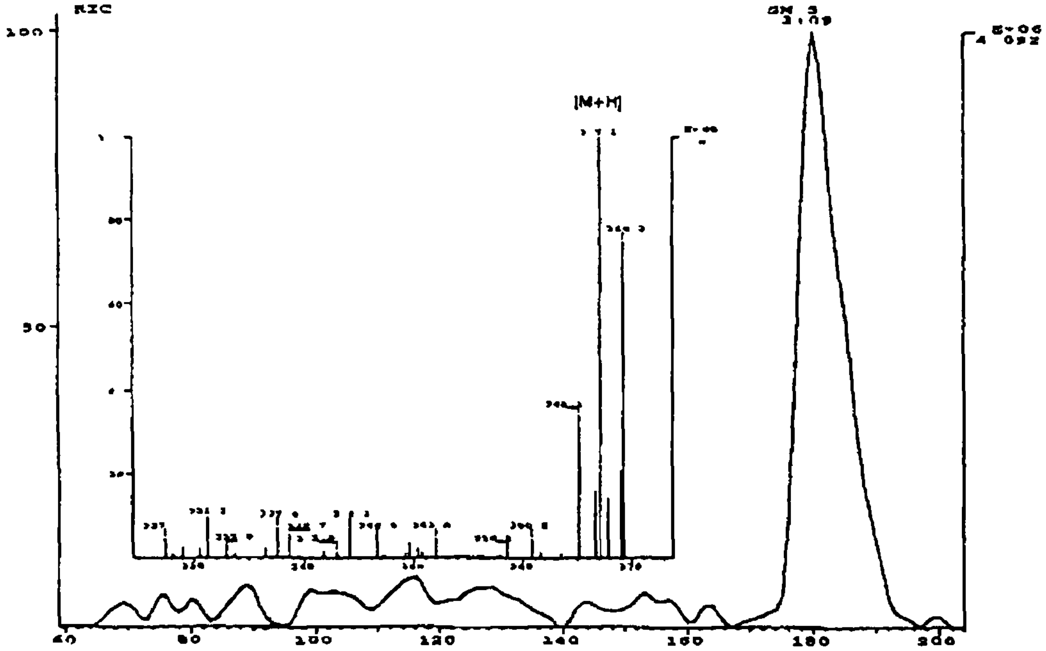


Fig 3 Injection of an extract of a urine sample exemplifying the detection of a possible unknown compound The CID spectrum reveals the presence of bromobuterol The amount was estimated at 0 7 ng/ml in the urine

pound(s) losing 74 u, and the averaged CID spectrum reveals the presence of a compound containing two bromine atoms with a monoisotopic molecular mass of 364, $[M+H]^+ = 365$ This compound was previously identified as bromobuterol, the brominated analog of clenbuterol Using GC–MS, the amount was estimated at 0 7 ng/ml assuming a response per mass unit equal to clenbuterol

From the above examples the possibilities of tandem MS for group-specific detection, in this case specific for N-*tert*-butyl substituted β-ethanolamines, are apparent even for low concentration levels like 1 ng/ml, although this strongly depends on the compound It should be emphasized, however, that the loss of 74 u may not be an exclusive feature of N-*tert*-butyl substituted β-ethanolamines alone, so interference may occur Only the analysis of a large number of samples from as diverse origin as possible will indicate whether or not the specificity is sufficient Currently this item is under investigation

4 Conclusion

The presented method may be used for rapid analysis of several β-agonists in urine in a semi-quantitative fashion at sub-ng/ml levels Analysis is rapid but specificity should be studied more extensively Operating the mass spectrometer in full-scan neutral loss mode, the possibilities of tandem mass spectrometry for group-specific detection of structure-related compounds are indicated Detection of an unknown as well as known compounds at ng/ml levels is demonstrated

Acknowledgements

This work was in part performed within the framework of the FLAIR Concerted Action nr 8 and presented at the FLAIR Workshop in Porto, April 1994 The authors wish to thank the managing committee for their support Other participants are acknowledged for their contributions to stimulating discussions on the subject

References

[1] J A van Rhijn, W A Traag and H H Heskamp J Chromatogr , 619 (1993) 243
[2] L A van Ginkel R W Stephany and H J van Rossum J Ass Off Anal Chem , 75 (1992) 554
[3] M P Montrade, B Le Bizec I Monteau B Siliart and F Andre Anal Chim Acta 275 (1993) 253
[4] L Leyssens J van der Greef H Penxten, J Czech J P Noben P Adriaensens, J Gelan and J Raus in N Haagsma A Ruiter and P B Czedik-Eysenberg (Editors) Residues of Veterinary Drugs in Food-Proceedings of the EuroResidue II Conference, Veldhoven, Netherlands 3–5 May 1993, Vol 2, p 444