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MULTIDIMENSIONAL CHROMATOGRAPHIC/MASS SPECTROMETRIC TECHNIQUES FOR THE TRACE DETERMINATION OF STEROIDS

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ABSTRACT

Research has centred on multidimensional chromatographic techniques which utilise the high specificity of immunoaffinity chromatography for extraction of analytes from complex biological matrices. On-line immunoaffinity chromatography-high performance liquid chromatography-mass spectrometry (IAC-HPLC-MS) systems (IAC and HPLC coupled *via* a loop interface) were developed for the confirmatory analysis of the corticosteroids dexamethasone and flumethasone with MS detection. Utilising an atmospheric pressure chemical ionisation (APCI) LC-MS interface, dexamethasone was confirmed in both spiked and post administration equine urine samples, with a detection limit of 0.1 $\mu g\ l^{-1}$. Detection by quadrupole ion trap mass spectrometry (ITMS) using a particle beam (PB) interface was performed for dexamethasone and flumethasone in post administration equine urine samples with high precision (6.9-7.4 %) with limits of detection in the range 3-4 $\mu g\ l^{-1}$. Studies were also conducted in this work into the antibody cross-reactivity and non-specific binding of corticosteroids on a HEMA bound anti-dexamethasone IAC column.

On-line IAC-HPLC and IAC-HPLC-GC have been developed and assessed for the determination of testosterone in equine urine. A novel approach to interfacing IAC with HPLC being achieved using a porous graphitic carbon (PGC) column.

The IAC-HPLC system developed was used for sample pre-treatment for combustion isotope ratio mass spectrometry analysis. The IAC-HPLC and IAC-HPLC-GC systems finally being coupled with mass spectrometry to enable confirmation of the endogenous steroid at 0.5 $\mu g\ l^{-1}$ and 1 $\mu g\ l^{-1}$ respectively in stripped equine urine.

ACKNOWLEDGMENTS

I would like to express my gratitude to my supervisor, Professor Colin S. Creaser, for his help and encouragement throughout this study. My thanks also goes to Dr. Ed. Houghton, Dr. Mark Seymour and Phil Teale at the Horseracing Forensic Laboratory (H.F.L.) for their support and advice. For the financial support of the project I thank the EPSRC and H.F.L., and for the experience gained in combustion isotope ratio mass spectrometry at the Open University, I am grateful to Dr. Paul Mason and Professor Colin Pillinger.

In my research time at Nottingham Trent I made a number of good friends who contributed ideas and encouragement. Special mention must go to the members of the group, Dr. James Stygall, who was helpful with the particle beam work, Brian, Tony, Derrick, Dan, Sarah and Debbie who all made life in the lab very enjoyable.

I thank my family for all the trouble they have gone to, to get me this far, and for their continuing love and support, and Jo, for all the love and patience she has shown to me. I love her very much.

This thesis is, to the best of my knowledge, original, except where due reference is made.

S. J. Feely, November 1997

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CHAPTER 1

INTRODUCTION

1.1. INTRODUCTION

The application of hyphenated or multidimensional chromatography procedures [1-7] to analyse complex samples is an area of growing interest in trace analysis. Multidimensional chromatography refers to the on-line coupling of methods which otherwise might be used in isolation. The combinations impart the potential for separations not possible by uni-dimensional techniques, and have provided the driving force for research in this area. The fact that these techniques occupy an expanding niche in chromatographic methodology is strong evidence that unidimensional (linear) systems (i.e. a single chromatographic column), for all their resolving power and high plate count, are often inadequate to solve separation problems without further separation steps [8].

The goal of trace analysis is to detect analytes at low levels in complex matrices. This may be achieved in multidimensional systems by a variety of methods including the use of pre-columns containing alkyl-bonded silicas [9,10], although certain samples require a much more selective purification such as enrichment provided by metal loaded and immunoaffinity columns [11,12] to achieve satisfactory results. Suitable enrichment and isolation of the analyte enables the compound(s) of interest to be observed above the inherent noise of the system and if an

identification detector is used, such as a mass spectrometer, the presence of the analyte may be confirmed [13]. Multidimensional analysis often achieves a greater sensitivity as well as a greater selectivity than can be obtained by a uni-dimensional system. Therefore, when two complimentary chromatographic techniques are coupled with mass spectrometry with its ability to produce diagnostic structural fingerprints of compounds, the combination provides an effective method for solving a number of the problems encountered in trace analysis [14,15].

1.2. BASIC THEORY OF CHROMATOGRAPHY

The term chromatography is used to describe a large number of greatly differing methods employed in analytical chemistry today. Chromatography is a chemical method of separation which relies on the distribution of solutes between two phases, one fixed (stationary phase) and one moving (mobile phase). It is a dynamic process which involves the solutes forming reversible interactions with the stationary phase. If these interactions are weak the component spends more time in the mobile phase and is transported quickly. On the other hand, if these interactions are strong the component spends less time in the mobile phase and is transported more slowly in comparison.

These interactions form the basis of chromatography and allow separations to be achieved between components which have varying affinities for the stationary and mobile phases. This competition between the mobile and stationary phases depends on the characteristics of the molecule and can be quantified as the partition coefficient $\mathbf{K}$, where $\mathbf{C_S}$ is the concentration of solute in the stationary phase and $\mathbf{C_M}$ is the concentration of solute in the mobile phase.

$$\mathbf{K = C_S / C_M} \qquad (1.2.1)$$

The capacity ratio or capacity factor ($\mathbf{k'}$) is a more practical value than $\mathbf{K}$ and is the ratio of the amount of solute in the stationary and mobile phases, and is given by

$$\mathbf{k' = K \frac{V_S}{V_M}} \qquad (1.2.2)$$

where $\mathbf{V_S}$ and $\mathbf{V_M}$ are the volumes of stationary and mobile phases, respectively.

The phase ratio β is given by

$$\beta = \frac{V_M}{V_S} \qquad (1.2.3)$$

so that

$$k' = \frac{K}{\beta} \qquad (1.2.4)$$

The fraction of the total time the solute spends in the stationary phase (F_S) is related to the capacity factor as follows

$$F_S = \frac{k'}{1+k'} \qquad (1.2.5)$$

and the fraction spent in the mobile phase is therefore conversely

$$F_M = \frac{1}{1+k'} \qquad (1.2.6)$$

The average velocity, $\bar{v}$, of the solute is given by the product of the average linear velocity of the mobile phase through the column, $\bar{u}$, and the fraction of time spent by the solute in the mobile phase F_M.

$$\bar{v} = \bar{u}F_M = \frac{\bar{u}}{1+k'} \qquad (1.2.7)$$

The time the solute spends in the column, the retention time, t_R, is therefore given as

$$t_R = \frac{L}{\bar{u}}(1+k') \qquad (1.2.8)$$

where L is the column length and $\bar{v} = L / t_R$. If there is no chromatographic retention, t_R becomes t_M, the retention time of a peak which was not retained:

$$t_M = \frac{L}{\bar{u}} \qquad (1.2.9)$$

The part of the retention time during which the solute is held back by the stationary phase is $t'_R = (t_R - t_M)$ and from equations (1.2.8) and (1.2.9) equation (1.2.10) is derived, which allows k' to be determined directly from the chromatogram (Figure 1.1):

$$k' = \frac{t'_R}{t_M} \qquad (1.2.10)$$

The concentration of solute as it emerges from the column corresponds to a Gaussian peak if equation 1.2.10 holds, which can be defined by the width at the base (w_b) or the width at half-height ($w_{0.5}$).

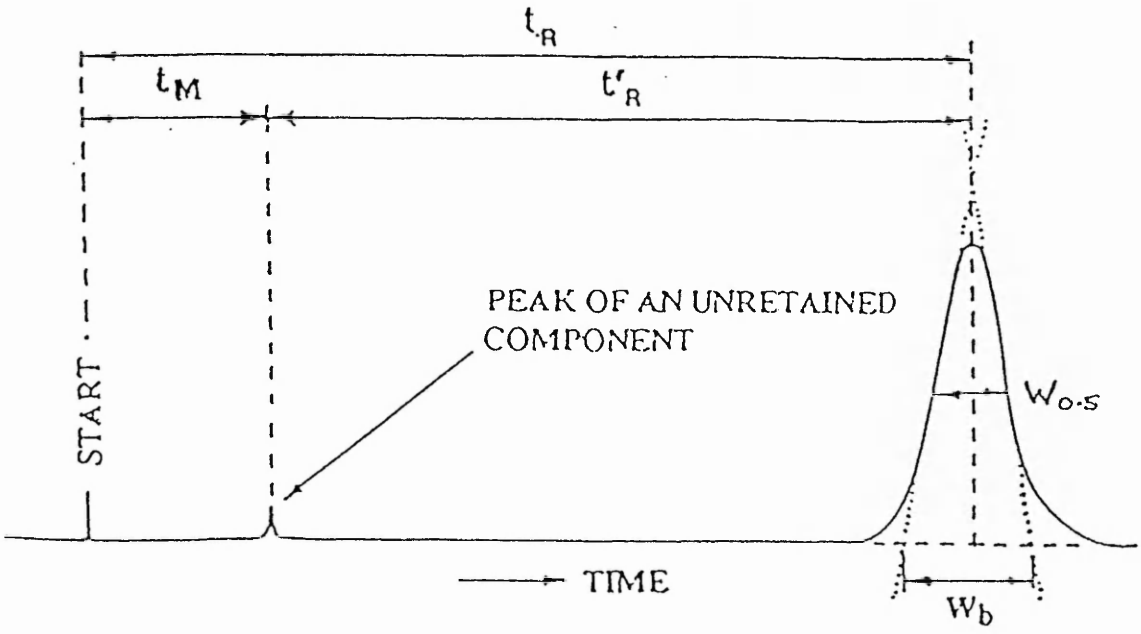


Figure 1.1. Chromatogram showing resolved peaks.

Clearly, the peak width, and hence the resolution, is related to the broadening of the solute band as it passes through the column. This is measured by the number of theoretical plates, **N**, by analogy with distillation theory, the number of equilibrations of the solute between the stationary and mobile phases in the column,

$$N = 16(t_R / w_b)^2 = 5.54\left(\frac{t_R}{w_{0.5}}\right)^2 \qquad (1.2.11)$$

Since **N** depends on column length, **H**, the height of one theoretical plate is equivalent to,

$$H = \frac{L}{N} \qquad (1.2.12)$$

The flow of mobile phase through the column and the diffusion of the solute in both phases influences the solute band width and, hence, efficiency and resolution. This dispersion is the result of four processes occurring as the solute band moves through the column, where **H** is the net effect of the band broadening. This relationship is described by the van Deemter equation, relating plate height to non-equilibrium diffusion processes and the average linear velocity [16, 17].

$$H = A + (B / \bar{u}) + C_S\bar{u} + C_M\bar{u} \qquad (1.2.13)$$

The total column dispersion is due to the combined effects of flow dispersion - **A** term, longitudinal diffusion - **B** term and mass transfer - **C** term. These contributions are shown in Figure 1.2. for gas chromatography (GC) and high performance liquid chromatography

(HPLC) separations. The effect of the multiple pathways through a packed column is given by

$$\mathbf{A = 2\lambda d_P} \qquad (1.2.14)$$

where λ is the packing uniformity, and $\mathbf{d_P}$ is the particle diameter of the stationary phase. In packed column chromatography (e.g. HPLC) this term is significant, however in open-tubular chromatography (e.g. capillary GC) this term is zero, since $\mathbf{d_P}$ is zero.

Longitudinal diffusion relates to the hindrance to the diffusion by the column packing. Termed the obstruction factor (γ) and solute diffusion in the mobile phase ($\mathbf{D_M}$).

$$\mathbf{B = 2\gamma D_M} \qquad (1.2.15)$$

The **C** terms express the effect of resistance to mass transfer in the mobile phase ($\mathbf{C_M}$) and the stationary phase ($\mathbf{C_S}$). $\mathbf{C_S}$ is dependant on the diffusion coefficient of the solute in the stationary phase ($\mathbf{D_S}$) and the effective film thickness of the stationary phase on the support particles ($\mathbf{d_f}$).

$$C_S = d_f^2 / D_S \qquad (1.2.16)$$

The C_M term describes the resistance to radial mass transfer caused by the particles of packing material. It is related to the particle diameter (d_p) and the diffusion coefficient of the solute in the mobile phase (D_M).

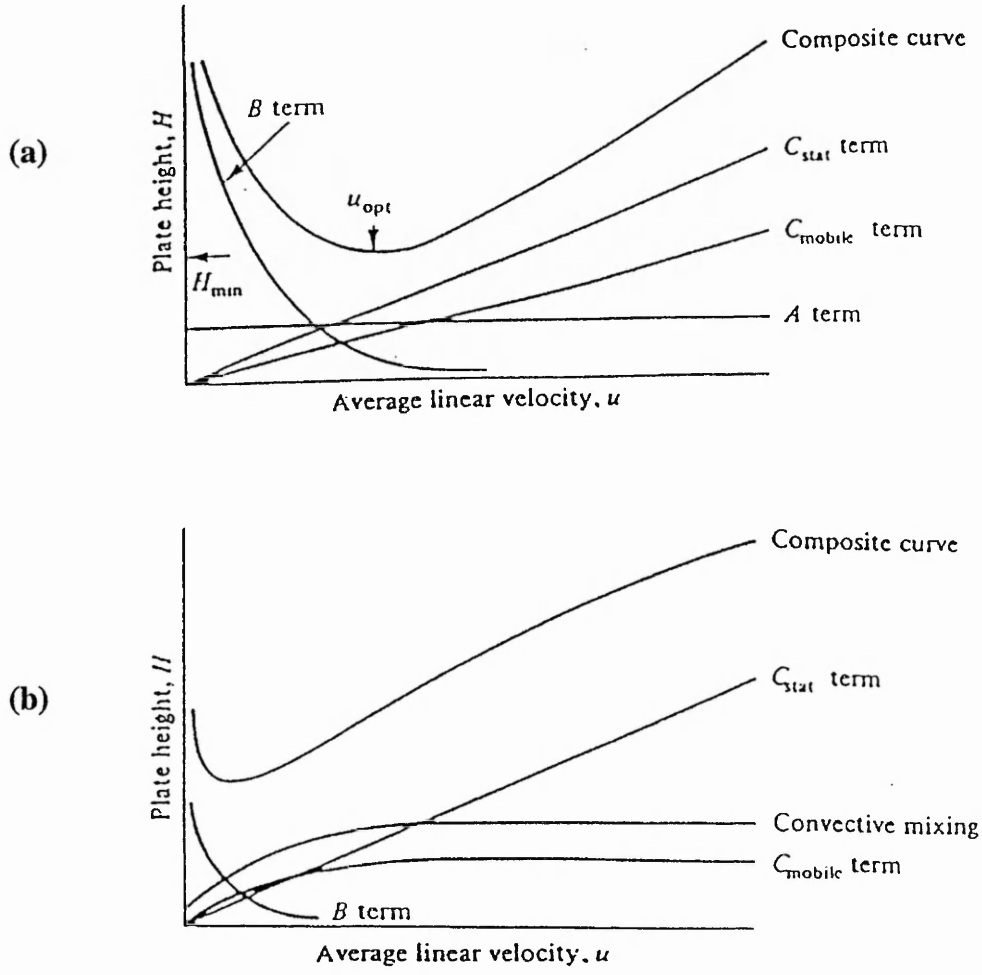


Figure 1.2. Plot of the van Deemter equation showing the contributions of the various terms for (a) GC and (b) HPLC separations.

$$C_M = d_p^2 / D_M \qquad (1.2.17)$$

The separation factor (α), depends on the relative retention, (k') for two solutes

$$\alpha = \frac{k_2'}{k_1'} \qquad (1.2.18)$$

α can be changed by varying the stationary and mobile phases.

The success of a chromatographic separation of two components, 1 and 2, is measured by the resolution, R_S, the ratio of peak separation to the average base width of the peaks (Figure 1.1.):

$$R_S = (t_{R1} - t_{R2}) / 0.5(w_{b1} + w_{b2}) \qquad (1.2.19)$$

R_S is related to N, and the average k' for the peaks in question by

$$R_S = \frac{N^{0.5}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'}{k'+1}\right) \qquad (1.2.20)$$

The term involving relative retention ($k'/k'+1$) is such that more plates are required to achieve the same resolution for early peaks than for later

eluting peaks. In practical terms, resolution is effectively complete for $\mathbf{R_S}$>1.5. However, resolution depends on the square root of the column length and doubling $\mathbf{L}$ only increases resolution by $\sqrt{2}$.

1.3. MULTIDIMENSIONAL CHROMATOGRAPHY THEORY

Peak capacity, $\mathbf{n_c}$ defines the ability of a chromatographic column, to separate a complex mixture into its components. This value describes the number of peaks which may be fitted side by side into the separation space of a chromatogram, in a given analytical resolution [1,18] and is the term commonly used to illustrate the advantages of a multidimensional chromatographic approach over a single chromatographic process.

$\mathbf{n_c}$ is a measure of the maximum number of peaks which may fit between retention times $\mathbf{t_{R1}}$ and $\mathbf{t_{R2}}$, at a minimum spacing $\mathbf{x_0}$, i.e. a minimum distance between two peaks which still allows two components to be identified as separate peaks where peak width remains constant for a single chromatographic process [19].

$$\mathbf{n_c} = \frac{(\mathbf{t_{R2}} - \mathbf{t_{R1}})}{\mathbf{x_0}} \qquad (1.3.1)$$

This equation holds over any given interval of the chromatogram, provided the component density is consistent. When this is the case for the whole of the chromatogram, then:

$$n_c = \frac{(t_{R\,max} - t_{R0})}{x_0} \qquad (1.3.2)$$

Where t_{R0} is the retention time for an unretained component and $t_{R\,max}$ is the maximum practical retention time. When in the isocratic or isothermal mode, peak widths gradually increase with increasing retention time, then n_c is given by:

$$n_c = \frac{N^{0.5}}{4R_S} \ln \frac{t_{R2}}{t_{R1}} \qquad (1.3.3)$$

(where N = number of theoretical plates) which for the whole chromatogram gives the following equation:

$$n_c = \frac{N^{0.5}}{4R_S} \ln \frac{t_{R\,max}}{t_{R0}} \qquad (1.3.4)$$

This can be written as,

$$n_c = \theta N^{0.5} \qquad (1.3.5)$$

Thus for a single chromatographic process the peak capacity, the number of components which can be separated in a mixture is proportional to the square root of the number of theoretical plates in the analytical column.

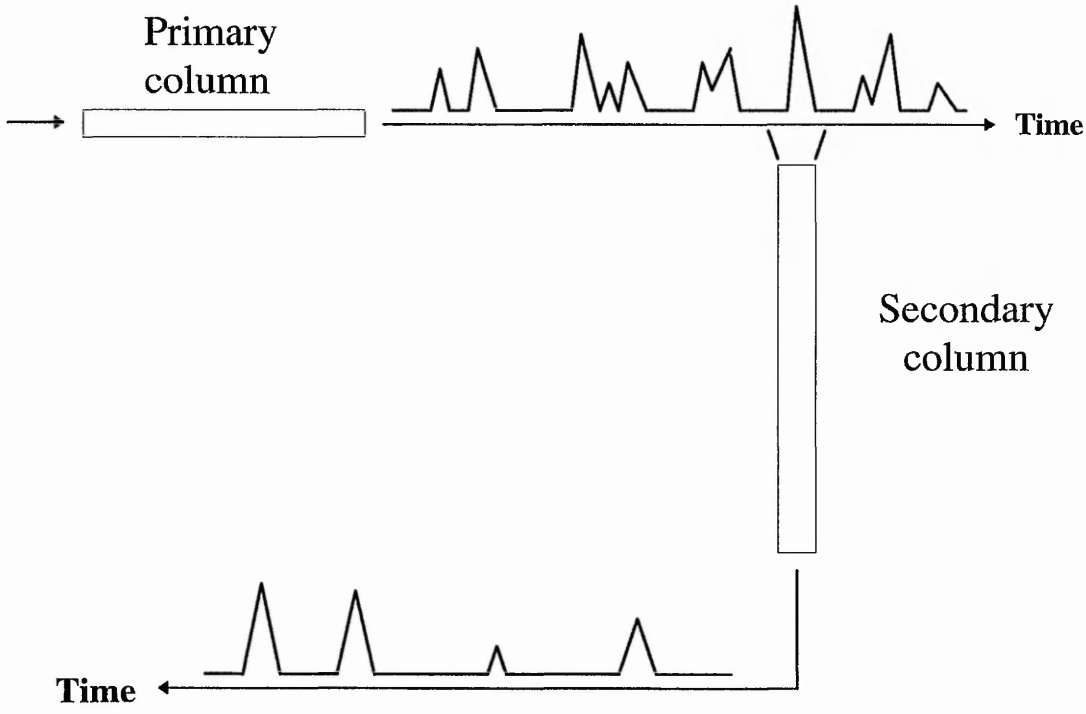


Figure 1.3. A coupled-column system in which fractions taken from the effluent of the primary column are routed to a secondary column.

In multidimensional chromatographic systems (Figure 1.3.), components are separated on a primary column, and then selected "fractions" of the effluent from this column are fed through a secondary column (then

tertiary etc.). In such approaches the migration mechanisms in the individual columns should be as different as possible, to maximise the effectiveness of the separation [19].

The basic theory is that components which co-elute from a column with a particular retention mechanism, due to their retention properties being too similar, may be separated by a different retention property, on a second column. Such multidimensional systems have the advantage over unidimensional separations that components which are not included in a fraction sent to the secondary column cannot merge together again and co-elute with those that are transferred [20]. The total peak capacity ($\mathbf{n}_{cc}$) of a coupled column system has been calculated as the sum of the individual peak capacities ($\mathbf{n}_i$) of the individual columns, which is given below:

$$\mathbf{n}_{cc} \approx \sum \mathbf{n}_i = \mathbf{n}_c \times \textbf{number of columns} \qquad (1.3.6)$$

Where $\mathbf{n}_c$ is the mean peak capacity. When the interactions which determine the separation on the first column are totally independent from those of the second, then the total peak capacity is equal to the product of the individual capacities [21], i.e.

$$n_{cc} = n_{c1} \times n_{c2} \times n_{c3} \ldots\ldots \text{etc.} \qquad (1.3.7)$$

Therefore, if each separation mode has approximately the same peak capacity, then for **j** separations (columns):

$$n_{cc} = n_c^j \qquad (1.3.8)$$

The above relationship is very difficult to achieve in practice with multi-dimensional chromatographic systems due to secondary effects which influence any chromatographic separation [22]. If there is complete redundancy throughout the separation, then

$$n_{cc} = \frac{(jN)^{0.5}}{4R_S} \ln j \frac{t_{R\,max}}{t_R} \qquad (1.3.9)$$

which approximates to

$$n_{cc} = j^{0.5} n_c \qquad (1.3.10)$$

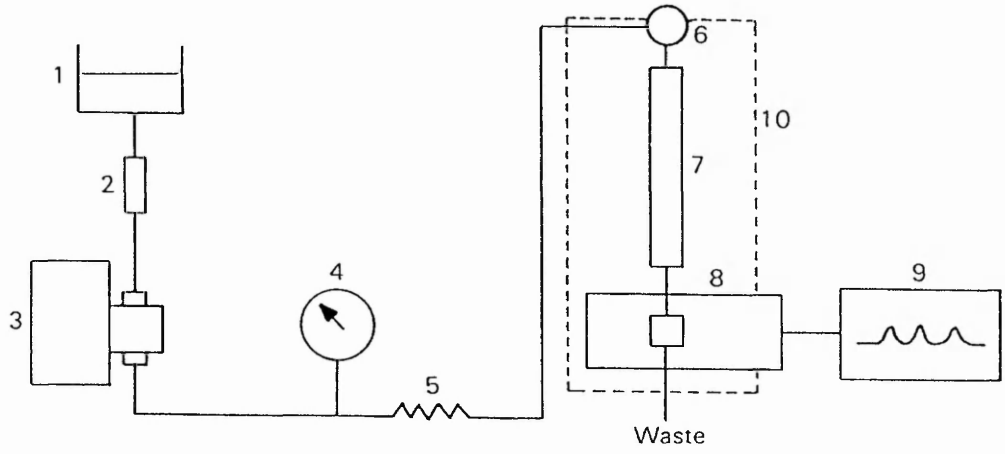
this basically describes the same relationship as equation 1.3.5 and illustrates that a coupled multidimensional chromatographic system consisting of **j** identical columns can separate $j^{0.5}$ more components in

the available separation space of the chromatogram. However, in practice the capacity of the system falls between the extremes implied by equations 1.3.8 and 1.3.10. In such systems though the analyst is frequently not interested in the separation of all the components of the starting mixture, but just a few or even one. The total quantity of peak information produced, is therefore much less than the peak capacity the system is capable of delivering, but this in no way diminishes the power that the multidimensional approach affords and, because of the ease with which a flowing coupled system lends itself to automation, it makes such approaches very attractive to the analyst involved in trace determination of substances in complex matrices.

1.4. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.

High performance liquid chromatography (HPLC) is a column chromatography which utilises a liquid as the mobile phase. Early in its development it was realised that massive increases in column efficiency would accompany decreases in the particle size of packings (Section 1.2) [23]. It was not however until the late 1960s that technology was sufficiently advanced to produce and use packings with particle diameters of 10 μm.

To achieve reasonable flow rates of mobile phase through such liquid chromatographic packings requires pumping pressures of several hundred atmospheres. As a consequence of this the instrumentation required is quite different from other types of chromatography. Figure 1.4. shows the essential components of a typical high performance liquid chromatograph.



1:eluent; 2:filter; 3:pump; 4:pressure gauge; 5:pulse damper; 6:injector; 7:column; 8:detector 9:recorder; 10:oven.

Figure 1.4. Schematic diagram of a high performance Liquid chromatograph.

1.4.1. The Mobile Phase

Unlike gas chromatography which will be discussed later there are many different mobile phases which may be used in HPLC. All of which have

a pronounced effect on the separations which can be achieved. The simplest way of performing a HPLC separation is by isocratic elution, in which a single solvent is used to transport analytes through the column. Often, however, a more effective separation may be achieved by a gradient elution, in which two solvents that differ greatly in polarity are employed. The ratio between volumes of the two solvents being varied in a pre-programmed way, sometimes continuously and sometimes in a series of steps.

1.4.2. Pumping Systems

The most widely used HPLC pump is the reciprocating positive displacement design pictured in Figure 1.5.. This pump consists of a piston in a low volume chamber that alternately draws-up mobile phase from a reservoir and then flushes the pump volume into the column often under high pressure.

To avoid backflow into the reservoir during displacement or filling, check valves are used, usually of the ball-and-seat design. There is no mobile phase delivered during the filling stage, therefore multiple out-of-phase pistons are used to minimise flow disruptions.

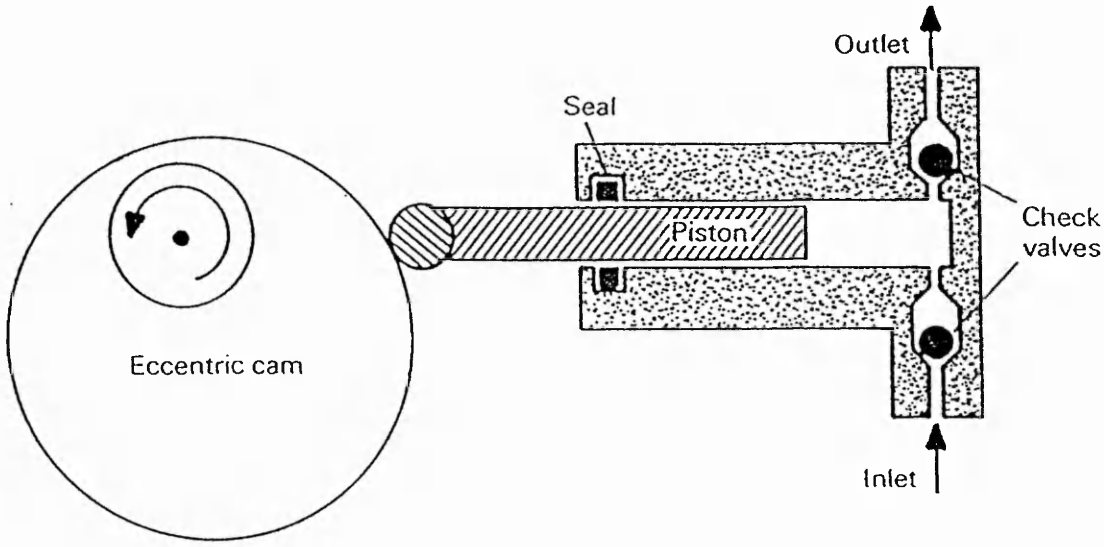


Figure 1.5. Reciprocating positive displacement pump design.

1.4.3. Sample Introduction

Analytes are injected in solution form into HPLC systems. The sample size introduced may vary greatly, from less than 1 µl with micro HPLC, to millilitres with the direct coupling of pre-treatment methods to analytical columns. It is typical however for a conventional 4.6 mm I.D. column to be injected with 5-100 µl of analyte solution. These volumes are determined by the size of the sample loop, which is fitted to an injection valve.

The valve has two positions: load and inject. In the load position the sample loop may be filled with analyte by a syringe. While in the inject position, the mobile phase can flush the contents of the loop onto the

column as shown in Figure 1.6.. The precision of injections with a typical sampling loop is a few tenths of a percent relative. Such valves can also link different flowing systems and columns together in multidimensional systems.

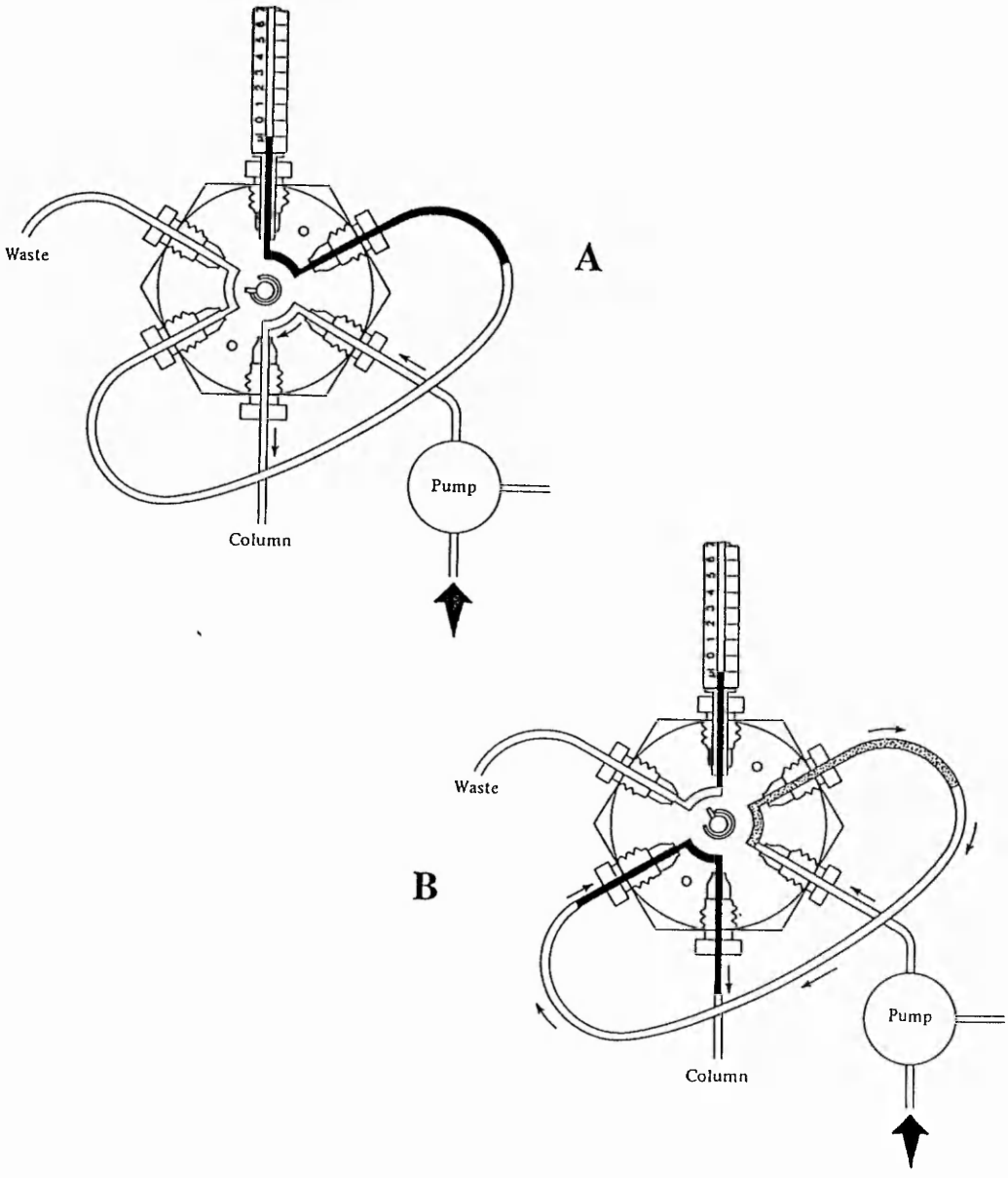


Figure 1.6. A rotary sample valve: (a) valve position for filling sample loop, and (b) for introduction of sample into column.

1.4.4. Columns

HPLC columns are mostly made of stainless steel tubing and range in length from 100-250 mm, with inside diameters of 1-4.6 mm. The particles packing the column being spherical and 3-10 μm in size. Such columns typically contain 20,000 plates in a 250 mm length and give fast separations with minimal solvent consumption.

Silica particles are the most common packing material. These may be coated with a thin organic film, which is either chemically or physically bonded to the surface. Other packings include alumina particles, porous-polymer particles, ion-exchanger resins and porous graphitic carbon particles which will be discussed in more detail later.

1.4.5. Bonded-phases

The term bonded-phase refers to the covalent binding of the stationary phase to the silica particles. Bonded-phases consist of an organic moiety covalently attached to silica with a siloxane type of bond (Si-O-Si-R). Reverse-phase packings are prepared by the attachment of hydrophobic groups such as octadecasilyl (ODS), octyl (C_8) and phenyl. Other side chains may be attached to give differing selectivities, such as cyanopropyl

and diol to give polar bonded-phases for normal phase separations. Bonded-phase packings are highly stable stationary phases, with minimal losses occurring following repeated use. The drawback of such packings being the limited sample capacity they offer.

1.4.6. Normal- and Reverse-phase Packings

Most low molecular weight, non-polar or polar compounds may be resolved either by reverse- or normal-phase chromatography. In reverse-phase chromatography the stationary phase is non-polar, usually a hydrocarbon, and the mobile phase is a relatively polar solvent such as water or methanol. Normal-phase chromatography refers to systems where there is a polar stationary phase (e.g. silica) and a non-polar mobile phase (i.e. hexane).

A large proportion of all HPLC separations are currently performed with reverse-phase, bonded octyl or octyldecyl siloxane packings (see Section 1.4.5.). In such separations the long-chain hydrocarbon groups align parallel to one another and perpendicular to the surface. The mobile phase used with these packings is usually aqueous solution containing various concentrations of solvents such as methanol.

1.4.7. Porous Graphitic Carbon

Porous graphitic carbon (PGC) has found a number of applications as an HPLC packing material because of its unique properties. PGC columns have enhanced separation power for diastereoisomers, geometric isomers and enantiomers, and exhibit improved retention possibilities for highly polar compounds. PGC packings may be used with both normal- and reverse-phase eluents. The particles of Hypercarb, the commercially available PGC packing, show a particle porosity of around 75 %.

At a molecular level PGC is very different from amorphous silica particles and is composed entirely of sheets of hexagonally arranged carbon atoms showing sp^2 hybridisation. The graphitic sheets are flat and the spacing of the carbon atoms within these sheets is very close to that in large polycyclic aromatic molecules. Indeed the individual graphite sheets may be regarded as gigantic aromatic molecules composed entirely of carbon. Within the sheets, carbon atom valency is fully satisfied and, unlike silica gels, there are no surface functional groups on the graphite.

The porous structure of PGC appears to be made up from layers of carbon atoms which are randomly twisted and interleaved in a complex way.

This is illustrated in the high resolution electron micrograph (Figure 1.7.) originally published by Knox, Kaur and Milward [24].

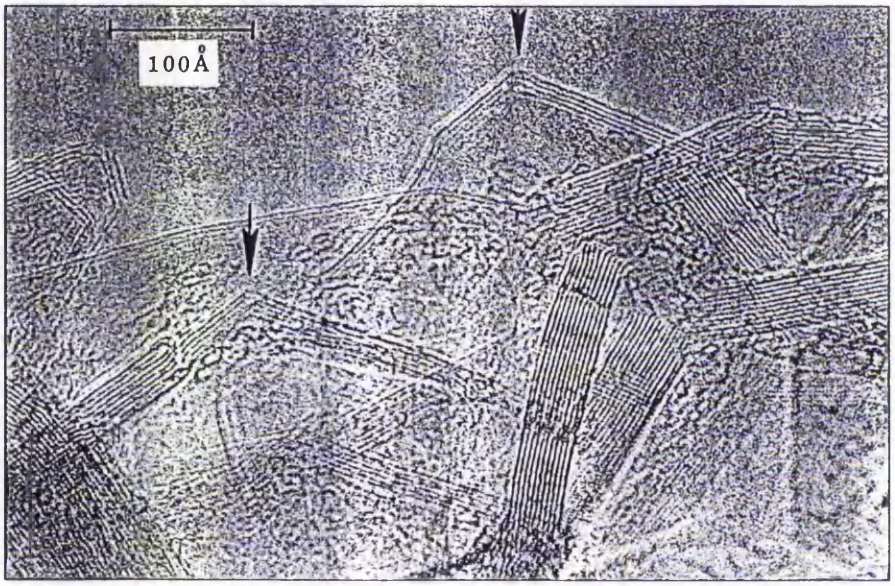


Figure 1.7. HREM section through PGC.

The spacing between the graphitic layers is typical for that of a three dimensional graphite, however unlike three dimensional graphite there is no ordering of the atoms between layers (Figure 1.8.) which gives rise to its mechanical strength. PGC characteristics can be clearly distinguished in both physical and chemical surface characteristics from the more conventional packings such as bonded silica. In Figure 1.9. the regular flat surface of hypercarb is compared to the random surface sites of silica. This flat graphite surface is the key to much of the enhanced regio- and stereo-selectivity shown by PGC packings.

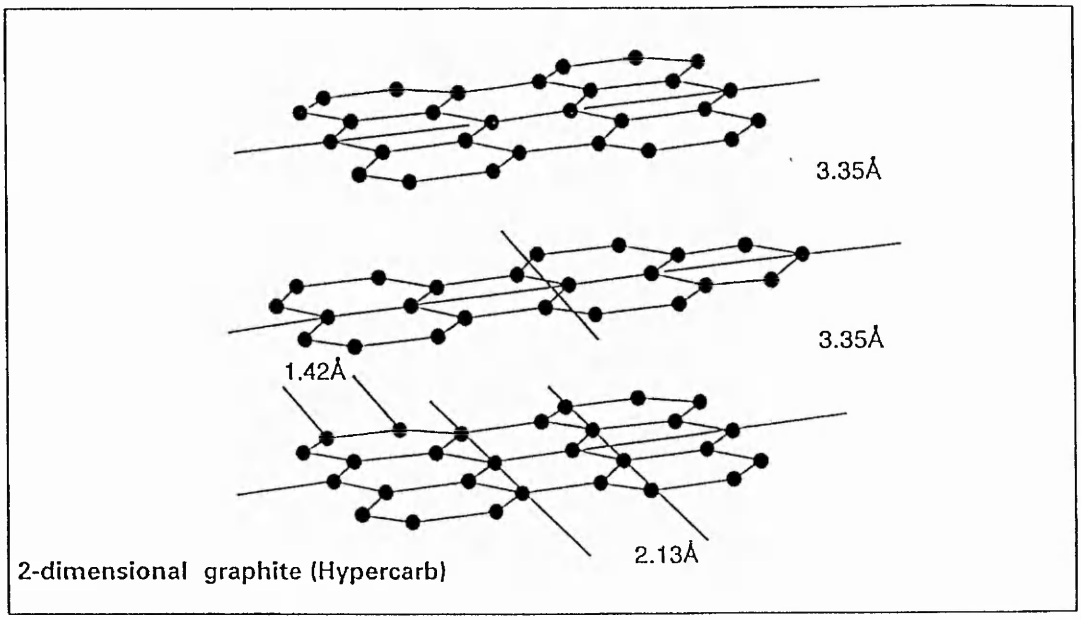


Figure 1.8. Crystal structure of hypercarb.

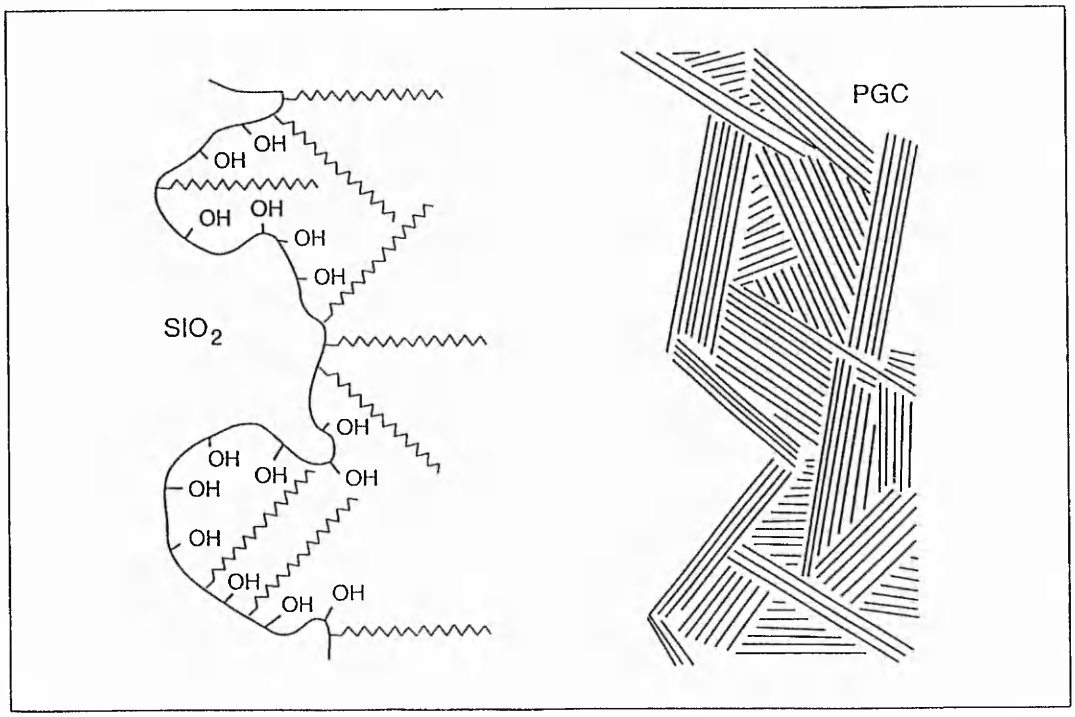


Figure 1.9. Comparison of surface bonded silica and hypercarb.

1.4.8. Detectors.

Two detection methods were used in the HPLC work; ultraviolet (UV) absorbance and mass spectrometry. UV detection will be described here. Mass spectrometry will be described in Section 1.7..

The UV detector (Figure 1.10.) is the most widely used detector in HPLC. Effluent from the column passes through a small sample cell, typically 8-10 µl in volume, fitted with quartz windows. Monochromatic UV radiation, selected by a grating or filter from a deuterium or xenon lamp source, passes through the windows and onto a detector which is either a photomultiplier or photodiode.

As the column effluent pass through the sample cell any analytes with chromophores which absorb light at the incident wavelength will cause a drop in detector response. This is converted to a peak on the output recorder or data acquisition system. UV detectors can either be fixed wavelength units (typically 254, 280 and 334 nm), variable wavelength, or diode array devices. The latter can operate fast enough to provide real time, full scan information. UV detectors are non-destructive, so they may be linked to other, complimentary techniques such as mass spectrometry.

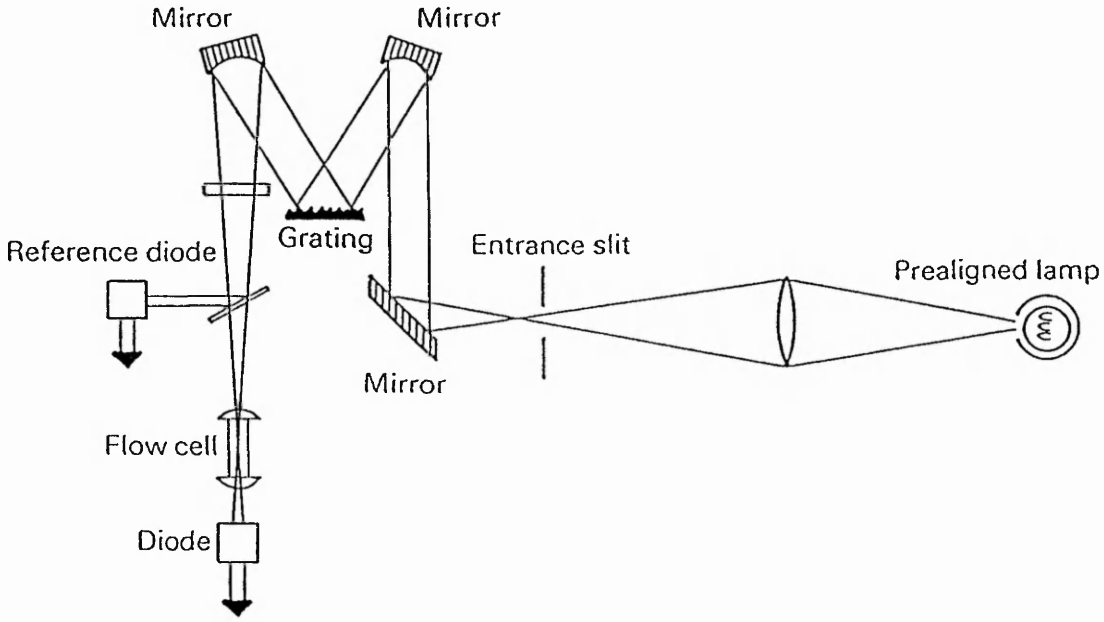


Figure 1.10. Schematic diagram of the optical path of a variable wavelength UV/Vis detector.

1.5. GAS CHROMATOGRAPHY

Gas chromatography was developed in 1952 as a means of separating volatile organic mixtures [25, 26]. Today capillary gas chromatography is capable of achieving greater resolution than HPLC, with around 50,000 theoretical plates being achieved commonly on a normal 30 m column.

The technique involves the vapour phase separation of analytes between a gas mobile phase (commonly H_2, He or N_2) and a liquid or solid stationary phase. In GC it is important that the compounds of interest are

volatile and thermally stable under the conditions selected for the analysis and this puts a limitation on the type of compounds which may be studied. However, capillary gas chromatography (CGC) has a vast range of well established applications in a number of fields in analytical chemistry. A typical gas chromatograph, represented schematically in Figure 1.11. consists of a sample introduction system, an oven (for controlling the temperature of the capillary column) and a detector which is linked to a data recording system.

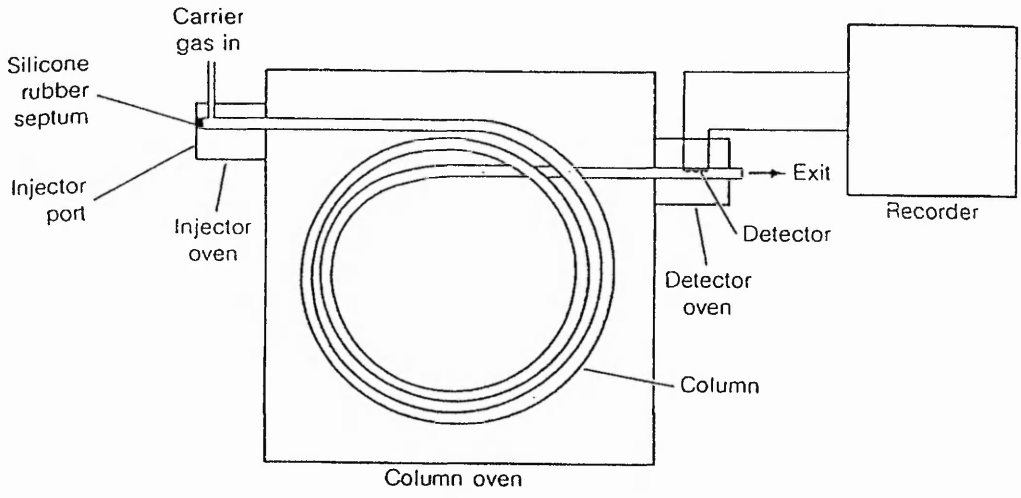


Figure 1.11. Schematic diagram of a typical gas chromatograph.

1.5.1. Sample Introduction

As with HPLC, the CGC sample is injected into the column through a specially designed inlet. This may either be a hot injection port which

introduces the sample to the column as a vapour, or a cold on-column injector which directly introduces the sample onto the column as a liquid.

The most common type is the heated split/splitless injector. In this inlet the sample is flash vaporised, and then introduced into the capillary column, with the entire sample entering the column (known as splitless injection), or with only a small portion of the sample entering the column (known as split injection). Typically, volumes of 0.5-5 μl of sample are injected through a septum by a syringe into a deactivated glass lined chamber upstream of the column. In both split and splitless operation, the injector is maintained at a temperature high enough to vaporise the sample, usually 150°C-300°C. In splitless injection, the column is at a temperature below the boiling point of the solvent to concentrate the analytes at the head of the column [27]. In the on-column injector sample is introduced directly on to the column using a syringe with a narrow bore needle. This method is often more effective than split/splitless injection for analytes of limited thermal stability [28].

1.5.2. Columns

Open tubular capillary columns are made from fused silica tubing which are coated externally with polyimide, which gives the column added

flexibility and robustness. The typical dimensions of these columns are 5-100 m in length with internal diameters of 100-530 μm. The stationary phase is internally coated or chemically bonded to the internal surface of the fused silica column to thickness' of 0.1-5.0 μm.

There have been a number of stationary phases developed for GC. Most of these consist of a polysiloxane backbone to which different functionalities are bonded to give different selectivities. Methyl, phenyl and cyanopropyl groups being examples of non-polar functionalities, whilst polar stationary phases are based on polyethylene glycols and polyesters.

Capillary GC (CGC) does not have a range of mobile phases like HPLC to help determine selectivity. It is however possible to alter the chromatographic conditions of a CGC separation during an analysis to improve performance, by increasing the column temperature. This enables volatile samples to be eluted at low temperatures and heavier less volatile compounds to be eluted at high temperatures, so achieving optimum separations, acceptable retention times and sharp peaks for all components in the mixture.

1.5.3. Detectors

A number of detectors are used in GC analysis, the most common being the flame ionisation detector (FID). The FID is a non-selective device which responds to nearly all organic compounds. In operation, analytes exiting the column pass through a small metal jet at the tip of which burns a hydrogen/air flame which is located under a tubular collector electrode. Analytes combusted in the flame, produce ions which are attracted to the collector and generate a current passing between the electrodes. This current is detected as a voltage drop, which is amplified and recorded.

The FID exhibits an excellent linear range of 10^6-10^7, and is very sensitive achieving sub-nanogram detection limits for certain compounds. Other common designs of detector include the electron capture, flame photometric and thermal conductivity detectors. In this research however the most important detector employed was the mass spectrometer (Section 1.7.).

1.6. IMMUNOAFFINITY CHROMATOGRAPHY THEORY

The first application of affinity chromatography was reported in 1910 and involved the selective adsorption of amylase onto insoluble starch. It was

not until 1967 though, with the development of a suitable support material [29], that affinity chromatography became a reliable tool in the analysts arsenal and began to expand into a number of different branches- immunoaffinity chromatography being one of these. A very selective and therefore powerful and useful technique for trace analysis, immunoaffinity chromatography (IAC) utilises the selectivity of antibodies as the stationary phase of a liquid chromatographic column.

1.6.1. Antibodies

Antibodies are glycoproteins synthesised and secreted by plasma cells in response to antigenic stimulation of certain cells. They are composed of four polypeptide chains, a pair of two light chains and two heavy chains (so named after their length and molecular weight), held together by interchain disulphide bonds.

Structurally all antibodies are immunoglobulins but functionally not all immunoglobulins are antibodies. When an immunoglobulin is known to react with a specific antigen it is called an antibody, but where the antigen specificity is not known it is just called an immunoglobulin.

1.6.2. Principles and Operation of Immunoaffinity Chromatography

The use of antibodies as an analytical tool for the determination of drugs, has evolved to the present day where analytical immunology is widely used for the determination of "known" substances. In general antibodies usually possess immense selectivity, targeting unique structural elements (epitopes) that consist of 3-12 amino acids in polypeptides or a few carbohydrate residues in oligosccharides. These relatively small epitopes on these large biological molecules are often greater in size than typical drugs and such low molecular weight substances do not generate a response from the immune systems of higher animals. Such compounds (haptens) require conjugation to immunogenic macromolecules to achieve an immunological response and production of appropriately selective antibodies.

Immunological recognition is based on the functional groups of the epitope of the antigen and the paratope of the antibody being complimentary in space and so aligned to form an antigen-hapten bond. In favourable cases, antibodies can be used to recognise a single substance in the presence of several thousand similar species, a useful function for a separation technique. This is the basis for the broad use of antibodies in forensic analysis.

An inherent problem with compound-specific antibodies is that they target epitopes, not the whole antigen. Multiple substances may have similar epitopes. When this is true, antibodies fail to discriminate by "cross-reacting" with structurally similar species. Such "false positives" are a negative feature of antibodies when the goal is to determine a single molecular species. In contrast, cross-reactivity is a positive attribute when the aim is to isolate families of molecules of similar structure. This biological recognition by the antibody reflects the degree of fit and the summation of the various molecular interactions (electrostatic, hydrophobic, hydrogen bonding and van der Waals) that exist when ligate is bound to ligand.

To generate a suitable immunoaffinity chromatography column an antibody which has been raised against a protein conjugate of the target compound to be separated, is covalently attached to a chromatographic bed material, the matrix (The matrix or chromatographic bed material should exhibit minimal non-specific binding [30]). The immobilised antibody should retain its specific binding affinity for the target, so giving an IAC stationary phase

The sample should be applied to the immunoaffinity column in loading buffer which maximises the affinity of the antibody for the analyte

(Usually aqueous buffer at a neutral pH). Interfering compounds are then washed away with loading buffer, leaving the analyte immobilised on the column. Analyte elution may be achieved by any agent which weakens the interaction between the antibody and the analyte. Use of an eluting mobile phase with a low pH or a high organic content which disrupts the antibody is usual. Elution conditions must not however be so extreme as to denature the stationary phase.

Immunoaffinity chromatography is therefore capable of:

- purifying substances from complex biological mixtures,
- separating native from denatured forms of the same substance and
- removing small amounts of biological material from large amounts of contaminating substances.

IAC is therefore an extremely powerful technique [31-35], ideal for coupling with HPLC or GC, to allow further analytical separation of the components in the purified IAC extract. The complimentary features of these techniques allow them to be combined to produce analytical systems with superior selectivity, speed and sensitivity. Enabling an increase in the throughput of samples; an important factor in routine monitoring laboratories.

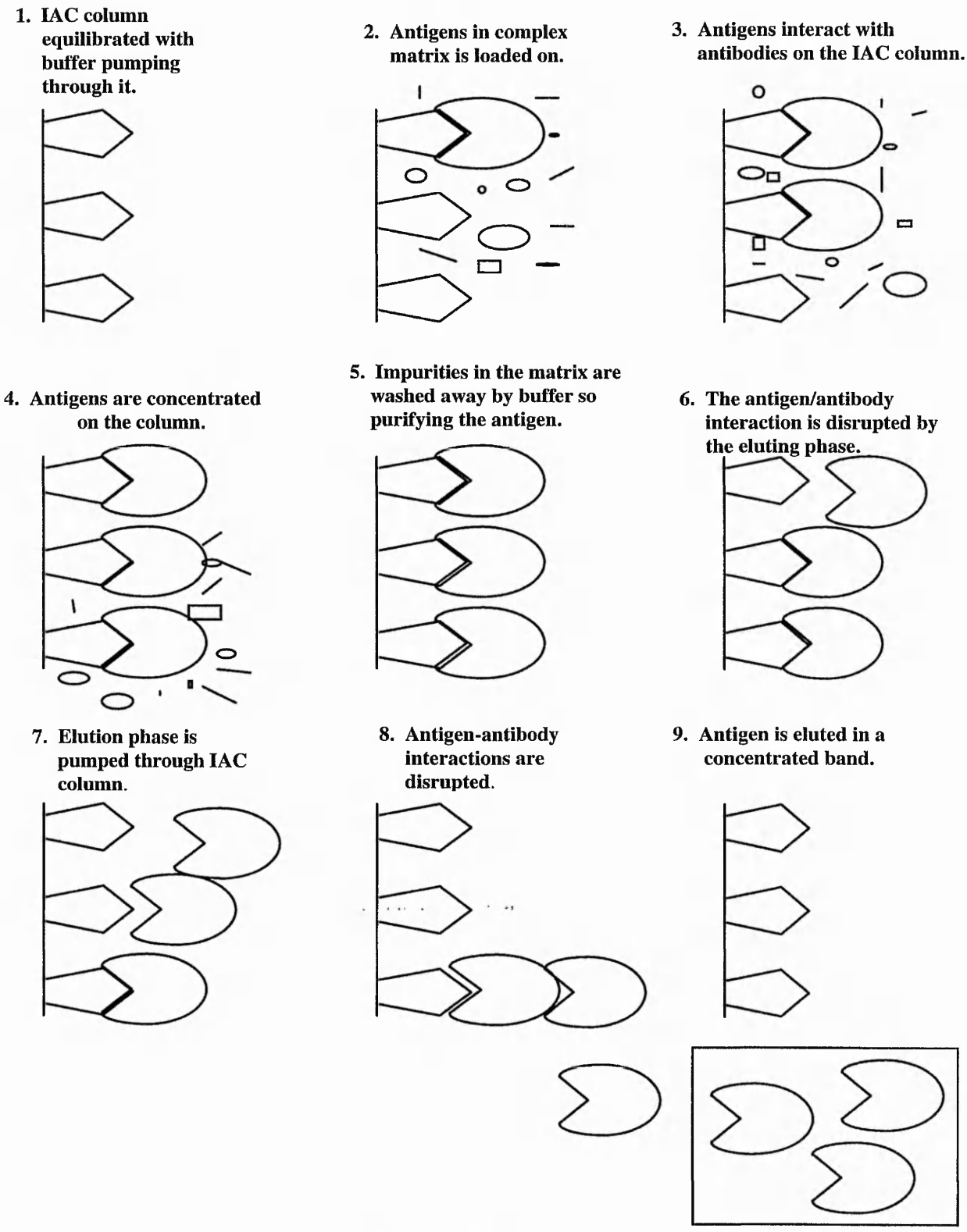


Figure 1.12. Schematic of the processes involved in IAC chromatography.

1.7. MASS SPECTROMETRY

Mass spectrometry (MS) was initially developed at the beginning of this century by J. J. Thompson [36]. Since those early investigations, the advancement of the technique has been driven by the fact that it is a spectrometric technique capable of high sensitivity and structural identification of compounds, yielding a unique 'fingerprint' spectrum in many cases. In MS, neutral molecules are ionised to form gaseous molecular and fragment ions. These are then separated on the basis of their mass to charge ratio (m/z), detected by an electron multiplier tube or other suitable analyser and their relative abundances recorded.

Both anions and cations maybe monitored, but positive ion mass spectrometry is the more established and usual approach. This instrumentation is very frequently coupled to chromatographic systems where it serves as the most versatile and powerful of chromatographic detectors.

1.7.1. Ionisation methods

To perform an MS analysis neutral molecules must be ionised [37]. Ionisation occurs directly after introduction of the sample into the ion

source chamber, which is upstream of the mass analyser. In this work electron ionisation, chemical ionisation and atmospheric pressure chemical ionisation were used.

1.7.1.1. Electron ionisation

Electron ionisation is one of the oldest [38], and still the most common ionisation method. Neutral compounds (M) are bombarded by high energy electrons from a tungsten or rhenium filament.

$$M + e^- \Leftrightarrow M^{+\bullet} + 2e^-$$

Any excess internal energy gained by the ionised molecule in this interaction, may be dissipated by the breaking of chemical bonds which can result in both fragment ions and neutral species. As an ionisation method, EI is inherently inefficient, with 0.1 % of a sample being converted to ions [39]. This is however compensated for by the tremendous sensitivity of the detectors employed in MS.

Although EI is very common, it has the disadvantage that it may result in such extensive fragmentation of some molecules, that the mass spectrum

obtained is comprised entirely of fragment ions [40]. Other methods have therefore been developed to avoid this problem and so compliment EI.

1.7.1.2. Chemical ionisation

Chemical ionisation (CI) is a 'softer' technique for the production of ions and is less likely to lead to fragmentation of the molecular ion. First described in 1966 by Munson and Field [41], the method has since gained wide acceptance. It requires the introduction of a reactant or reagent gas (e.g. ammonia, methane or iso-butane) into the source at a much higher pressure than that used for EI. Electron ionisation of reagent gas molecules lead to the formation of molecular radical cations. In the case of ammonia these newly generate ions at the higher pressure of the CI source collide with neutral ammonia to form ammonium reagent ions. The ammonium reagent gas ions react with electrophilic addition. Analyte molecules (M), instigating ionisation either by proton transfer or by addition.

$$M + NH_4^+ \quad \Leftrightarrow \quad [M + H]^+ + NH_3$$

$$M + NH_4^+ \quad \Leftrightarrow \quad [M + NH_4]^+$$

The result of the proton transfer reaction is the protonated quasi-molecular ion $[M + H]^+$. For this reaction to occur, the proton affinity of

the molecule must be greater than that of the reagent gas, reagent gases maybe varied to achieve the ideal results for a particular compound. This ion is generated with little excess internal energy and it is more likely that protonated molecules will be detected by CI than molecular ions will be detected by EI. APCI and ESI ionisation sources are described in section 1.7.5.1.

1.7.2. Mass Analysers

Once the ions have been formed in the ion source, they pass into the mass analyser where they are separated and focused according to their mass to charge ratio (m/z). There are a number of mass analysis devices such as magnetic sectors, time of flight and quadrupole instruments. Only quadrupole devices were used in this work and are described below.

1.7.2.1. Quadrupoles

The design for a quadrupole mass spectrometer was first patented in 1956 by Paul and Steinwedel [42, 43]. The quadrupole mass filter is made up of four parallel cylindrical, or hyperbolic rods fixed around a central axis. This is positioned close to a small aperture in the ion source casing from where the analyte ion may be easily introduced.

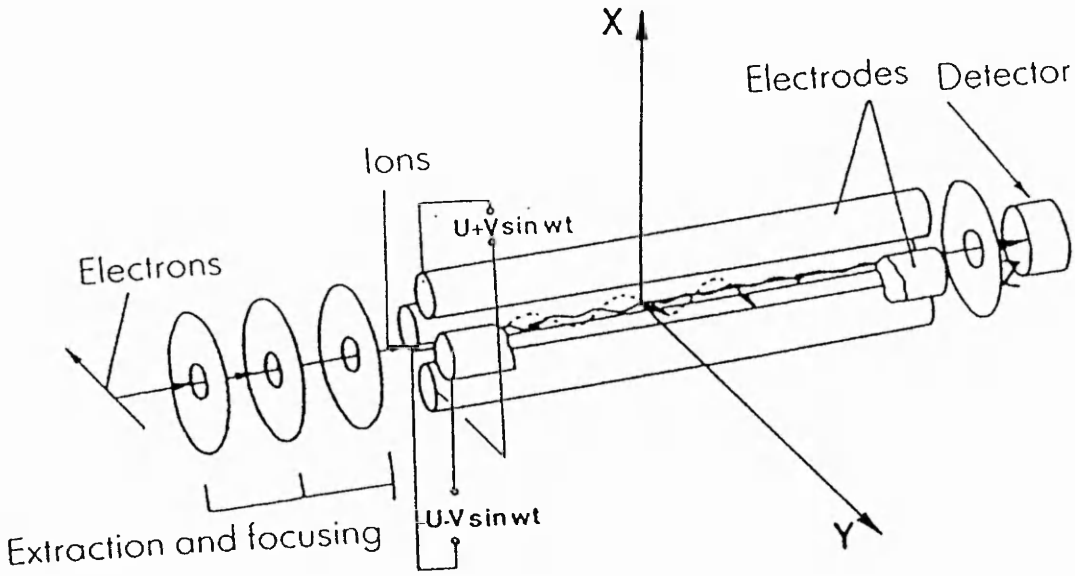


Figure 1.13. Schematic of the linear quadrupole.

The rods occupy the corners of a square (Figure 1.13.). Each rod is electrically paired with the rod diagonally opposite. One of the two pairs is kept at a positive ($+U_{dc}$) and the other at a negative ($-U_{dc}$), dc potential. Additionally, each pair has a superimposed radiofrequency potential (V), the signal to one pair being delayed by 180° relative to the other. By ramping the amplitude of the dc and rf potentials, ions can be accelerated into the analyser where they take on differing trajectories. A number of these are unstable, which leads to the ions colliding with the rods, but some are stable and the ions following these trajectories pass through the filter to the detector at the end. Adjustment of the dc and rf potentials enables individual m/z value ions to be transmitted, or a range of m/z values to be scanned.

1.7.2.2. Ion Traps

The quadrupole ion trap mass spectrometer (QITMS), is a mass filter device where the quadrupole field is applied in three dimensions. The ion trap, as it is commonly known, consists of a central circular 'ring electrode' and two 'end cap electrodes', all with hyperbolic surfaces, as is illustrated in Figure 1.14..

If rf and/or dc voltages are applied to these three components a rotational symmetric field is created in the space between them. In this field, ions exhibit a complex periodic motion and those which have stable trajectories are trapped. Increasing the amplitude of the rf voltage leads to the ejection of ions of increasing mass to charge ratio from the trap towards the detector allowing a mass spectrum to be obtained.

Sample is introduced as a gas into the analyser *via* the hole between the ring electrode and one of the end caps. Once within the trap ionisation may be performed by electrons gated into the trap through a hole in an end cap. The pressure inside the trap for EI is usually 10^{-3} torr. This relatively high pressure is a consequence of helium, which is introduced as a buffer gas to achieve good resolution and sensitivity [44].

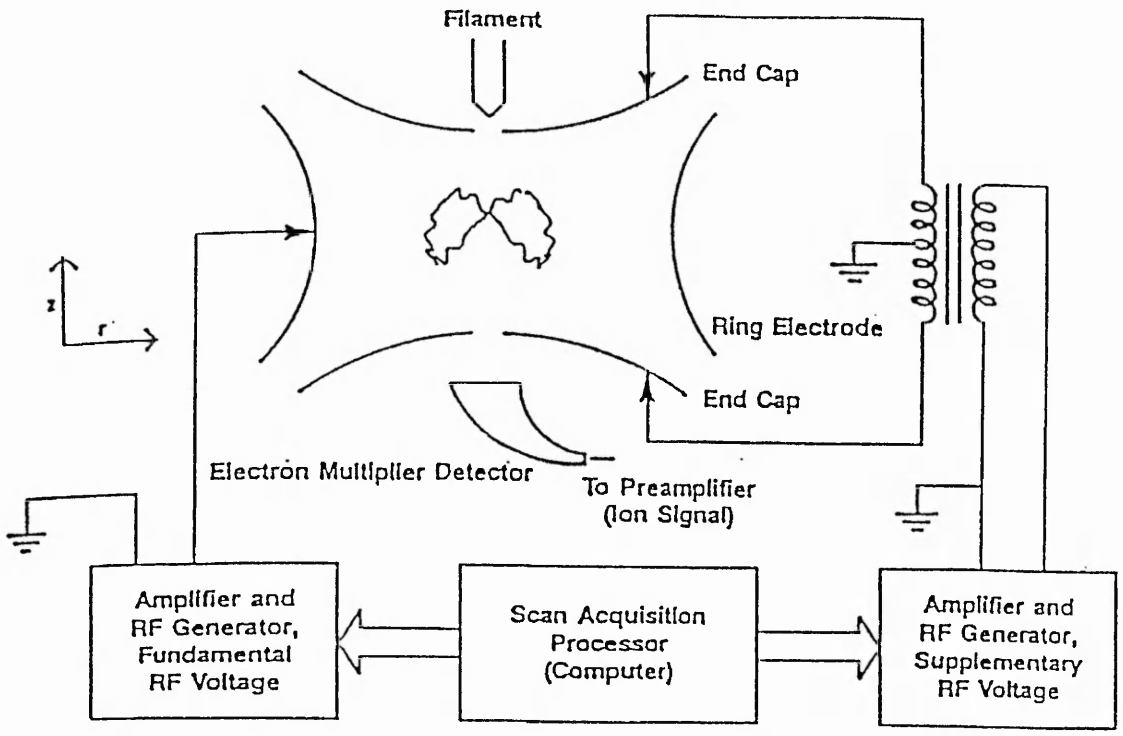


Figure 1.14. The configuration of the ion trap.

1.7.3. Detectors

No information could be gained from a mass spectrometer without a suitable detector. The ion current usually reaching a detector is 10^{-9}-10^{-18} A. This must be amplified and quantified for which task the most common design is the electron multiplier. An example of one design of this type of detector is the channeltron. It consists of a curved glass or ceramic tube coated internally with lead doped glass. When an ion hits the coating it causes a shower of electrons which impinge on a different section of coating further down the tube. Each electron creating yet more

electrons. This process occurs a number of times so achieving a cascade effect, the gain of which can be 10^6. The amplified signal 'hits' the collector plate where it is converted into a voltage and then processed under computer control to give a mass spectrum.

1.7.4. Tandem Mass Spectrometry

Tandem mass spectrometry involves taking mass-selected ions produced and separated in an initial mass spectrometry step, fragmenting them, usually by collisionally activated dissociation and subjecting the product ions to a second mass analysis. Following the ionisation (and fragmentation) of a molecule, a precursor ion(s) from the resulting spectrum is introduced or held in a chamber and allowed to undergo collisions with a suitable buffer gas usually helium, producing product ions. These product ions are then focused detected and recorded as before.

The technique of tandem mass spectrometry (or mass spectrometry-mass spectrometry, MS/MS) maybe used to obtain structural information about a precusor ion. Also, if complex mixtures are analysed, many different ions will be observed and the assignment of each component becomes difficult. In such circumstances, tandem mass spectrometry can allow

identification of a target component by mass-selection and fragmentation of a characteristic precusor ion.

MS/MS may be performed easily using a QITMS, and even be extended to multistage tandem experiments (MS^n). To achieve this, the sample is ionised, then all the ions are ejected from the trap except for those of the selected m/z ratio. This ion is then induced to collide with the helium buffer gas, leading to fragmentation and product ion formation. These may then be ejected and detected, or a product ion may be selectively retained in the trap and the process repeated.

1.7.5. Coupling chromatography to mass spectrometry

1.7.5.1. LC-MS

Mass spectrometers are typically operated at pressures of 10^{-3}-10^{-8} torr which eliminates scattering due to ion-molecule collisions. In HPLC the mobile phase eluting into the detector is of course a liquid. A liquid can give rise to a vapour volume in excess of 200 times that of the original liquid. This presents major problems for the pumping system of the mass spectrometer and to surmount these a number of interfaces have been designed and investigated with differing degrees of success. Combined

LC-MS was first reported over twenty five years ago [45] and is now a widely used technique. Several reviews of the subject having been published [46-54].

The first commercial LC-MS interface was the moving belt interface which was developed by Scott [55] and McFadden [56]. This was followed by the thermospray interface which was invented by Vestal in 1982 [57] and Contiuous-flow FAB, which was invented in the early 1980's by Ishii [58] and Caprioli [59]. The particle beam interface, sometimes known as the MAGIC (Monodisperse Areosol Generator for Interfacing Chromatography) LC/MS interface [60, 61] and the atmospheric pressure interfaces, atmospheric pressure chemical ionisation (APCI) and electrospray interfaces (ESI) are more recent developments. The particle beam and APCI/ESI interfaces were used in this work and are described below.

1.7.5.1.a. Particle Beam interface

The particle beam [62] is the only interface where transfer of neutral molecules from the column to the spectrometer is achieved solely by aerodynamic means. A typical design is shown schematically in Figure 1.15.. Eluent from the HPLC column enters the device together with a

flow of helium, thereby which is used to generate an aerosol of solvent droplets which move through the desolvation chamber.

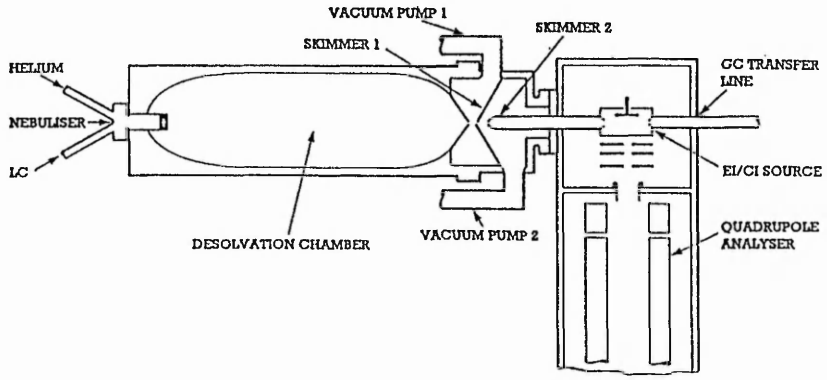


Figure 1.15. Schematic diagram of a particle beam interface.

The chamber is held at ambient temperature and pressure, causing the solvent to vaporise and produce a mixture of vapour, particles of analyte and helium. This mixture enters the first low pressure rotary pumped region through a narrow nozzle, a process which accelerates the gas/particle mixture, forming a supersonic jet containing a central, directed beam of particles. Solvent vapour and helium are "skimmed" and pumped away by a sequence of two nozzles facing the expansion jet, leaving the much heavier beam of analyte particles to travel directly into the ion source of the mass spectrometer where they strike a heated wall and are vaporised and ionised by EI or CI. The EI spectra can be

searched against libraries of known spectra, an advantage over other interfaces which this system shares with belt systems.

The particle beam interface can accommodate flows in the range, 0.1-1 ml min^{-1}. Originally designed for use on quadrupole mass spectrometers, it has been adapted for magnetic sector instruments and ion traps [63, 64]. The particle beam interface appears to have displaced the belt interface as the system of choice for compounds of medium molecular mass, largely because it has the advantage of mechanical simplicity and robustness. Detection limits lie most typically in the low nanogram range. The beam interface is generally though unsuitable for the study of high molecular mass or involatile, thermally labile materials.

1.7.5.1.b. APCI/electrospray

The atmospheric pressure chemical ionisation LC-MS interfaces (APCI/ESI) have achieved tremendous popularity since their introduction [65, 66]. The potential of this method, particularly for in the analysis of large molecules (polymers and small proteins) was recognised in 1988 [67]. The two techniques are dealt with together because as Figure 1.16. illustrates, the two designs are very similar.

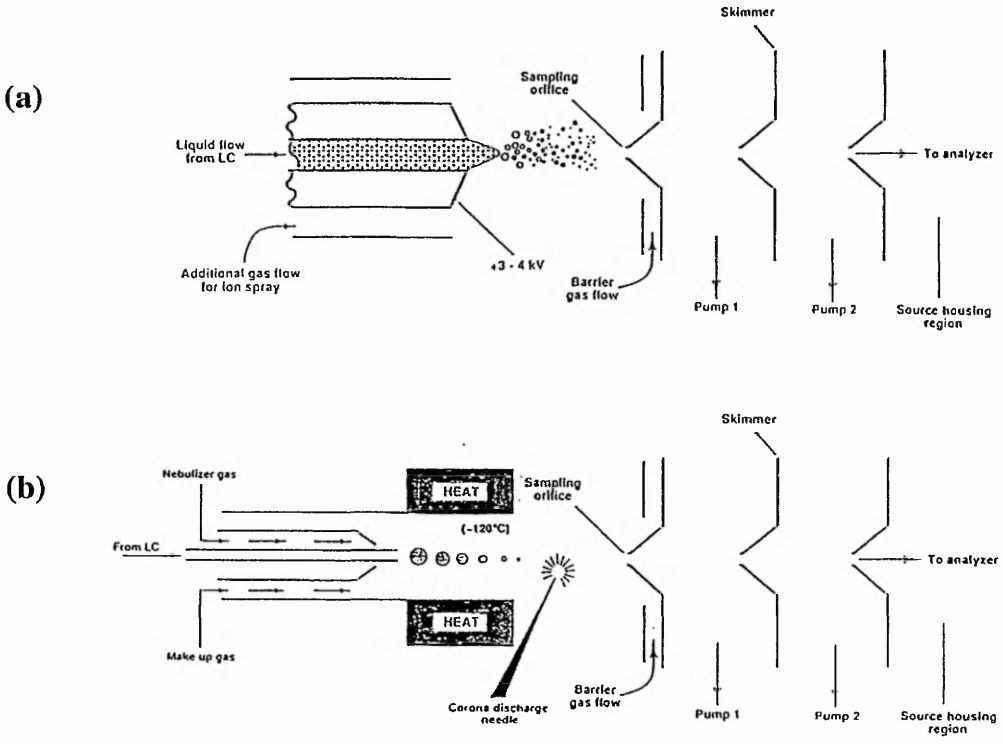


Figure 1.16. The (a) electrospray and (b) APCI interfaces.

The HPLC eluent is nebulised at atmospheric pressure, with ionisation occurring by application of a large (up to 6 kV) voltage either on the nebuliser tip itself (ESI) or on a corona discharge needle (APCI). In ESI, the ions are formed by ion evaporation from charged droplets, which break up into even smaller droplets (a well documented effect which is a result of coulombic repulsion), and APCI by gas phase ion-molecule reactions. A curtain of dry nitrogen flows behind and through the sampling orifice, preventing the entry of solvent vapour. The ions are then drawn through a series of electrostatic skimmers where pressure is sufficiently reduced for free ions to enter the analyser at high vacuum.

The nitrogen gas curtain fulfils a second role by breaking the hydrogen bonds in ion/solvent clusters which would otherwise complicate and confuse the mass spectrum achieved. APCI is generally used for small molecules and electrospray for large analytes such as peptides and proteins, which produce multiply-charged molecular ions capable of being measured by limited mass-range analysers. The sensitivity for both designs has been reported as being into the femtogram range.

1.7.5.2. GC-MS

The interfacing of a CGC column to MS does not cause as many problems as LC-MS, since the mobile phase is a gas, and the amounts introduced can be effectively controlled by the pumping system of the MS. The coupling of gas chromatography and mass spectrometry was therefore a very obvious step. The two methods compliment one another very well. The gas chromatograph being capable of resolving mixtures and the mass spectrometer of providing information to allow identification of unknown components. Both techniques operating in the gas phase.

GC-MS is excellent for separating, detecting and quantifying the components of complex organic mixtures. In CGC-MS the column is

either coupled directly through to the ion source, or *via* a deactivated fused silica transfer line. The set-up is extremely simple, the CGC column being fed through a heated sheath in to the ion source. The tip of the column rests just short of the electron beam that ionises the eluting molecules (Figure 1.17.).

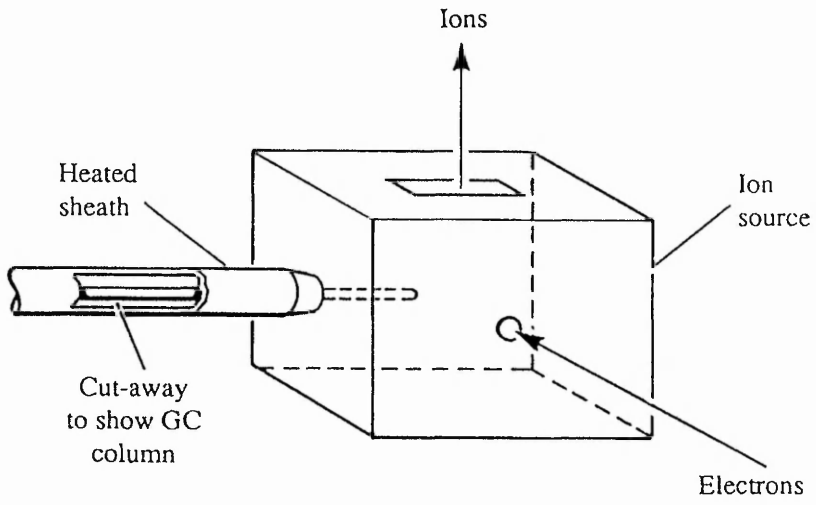


Figure 1.17. A schematic diagram of a mass spectrometer ion source, showing the direct coupling of a GC column. This entire volume is under vacuum.

1.8. OBJECTIVES

The purpose of this investigation was to evaluate the potential of IAC in on-line multidimensional chromatographic systems for the determination of steroids of interest to the Horseracing Forensic Laboratory, for which

existing methodology was both complex and time consuming. These procedures were to encompass the development of novel techniques, as well as the modification, improvement and refinement of existing methodology, with demonstration using real samples of complex matrices. Trace level detection limits in the $\mu g\ l^{-1}$ region were a pre-requisite for the methods that were to be developed with both conventional HPLC and GC detectors as well as with mass spectrometry. These studies were directed towards the determination of anabolic steroids and corticosteroids.

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CHAPTER 2

COUPLING OF AND HPLC FOR THE ANALYSIS OF POST ADMINISTRATION EQUINE SAMPLES FOR DEXAMETHASONE USING UV AND APCI-MS DETECTION

2.1. INTRODUCTION

Two categories of analysis are required for the successful detection of abuse of steroids: screen procedures and confirmatory analysis'. Screening procedures are required to analyse a large number of samples to pick out possible offenders. They need to be sensitive, fast and able to handle structurally related compounds. Methods used include thin layer chromatography, HPLC and immunoassays. Confirmatory analysis is required to confirm or reject positive results achieved in the screening procedure. Drugs and their metabolites must be unambiguously identified, and the method that can satisfy this requirement is usually gas chromatography - mass spectroscopy (GC-MS), with pre-treatment of the sample. The search is on however for more rapid and simple mass spectrometric methods to identify drug residues. An on-line multidimensional chromatographic approach being one area of research of tremendous interest.

2.1.1. Corticosteroids

The corticosteroids are characterized by an oxygen function on the C11 position. These compounds are produced naturally by the adrenal cortex (i.e. the outer part of the adrenal glands near the kidneys) under the

influence of the corticotrophic hormones from the pituitary gland. The introduction of a -CO.CH_2OH side chain at the C17 position, 4-ene-3-oxo configuration in the A-ring, and an oxygen function at the C11 position all affect the physiological activity. The presence or absence of a hydroxyl group at the C17 position also influences the activity.

There are two classes of corticosteroids, although they are not distinct from each other as their physiological effects often overlap. The first and the most important to equine medicine is the glucocorticoids, which effect both protein and carbohydrate metabolism, whilst having a powerful anti-inflammatory effect. The second group are the mineralocorticoids, which cause increases in sodium and water retention.

By modifying the natural corticosteroids, drug companies have been able to synthesise corticosteroids with increased potency whilst unwanted side effects, such as sodium and water retention, have been reduced (Table 2.1.).

When administered systematically, glucocorticoids can enhance a competing horse's performance. Blood glucose levels rise as protein, fat and carbohydrate metabolism increases and hence provides an extra reserve of energy for the horse and that has a stimulating effect [1].

Table 2.1. A comparison of the physiological effects of cortisol to other corticosteroids.

	Sodium retention	**Anti-inflammatory effect**
Natural Steroid		
Cortisol	1	1
Corticosterone	15	0.3
Synthetic Steroids		
Prednisolone	0.8	4
Dexamethasone	0	25

These steroids also have the capacity to prevent or suppress the local heat, redness, swelling and pain which are characteristics of inflammation, but do not remove the cause of the inflammation. An animal running on a glucocorticoid-injected injury could drastically worsen it, although this only becomes apparent when the anti-inflammatory effects of the drug have worn off.

The detection of the abuse of high-potency drugs such as synthetic corticosteroids in equine sport presents an analytical problem. As administration at low levels and extensive metabolism results in low

concentrations of drug/metabolites in biological fluids. Dexamethasone (Figure 2.1.) is one such synthetic corticosteriod, reputed to have been used as a pre-race shot in racehorses [2], it is a restricted substance under the Rules of Racing.

Dexamethasone (9α–fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione), is relatively potent and is one of the major synthetic corticosteroids used in veterinary practice [3]. Metabolic studies have shown the production of a number of phase I and phase II metabolites and rapid elimination of the parent drug [4-6]. Antibodies for dexamethasone are readily available and thus it was an ideal candidate for study by on-line IAC.

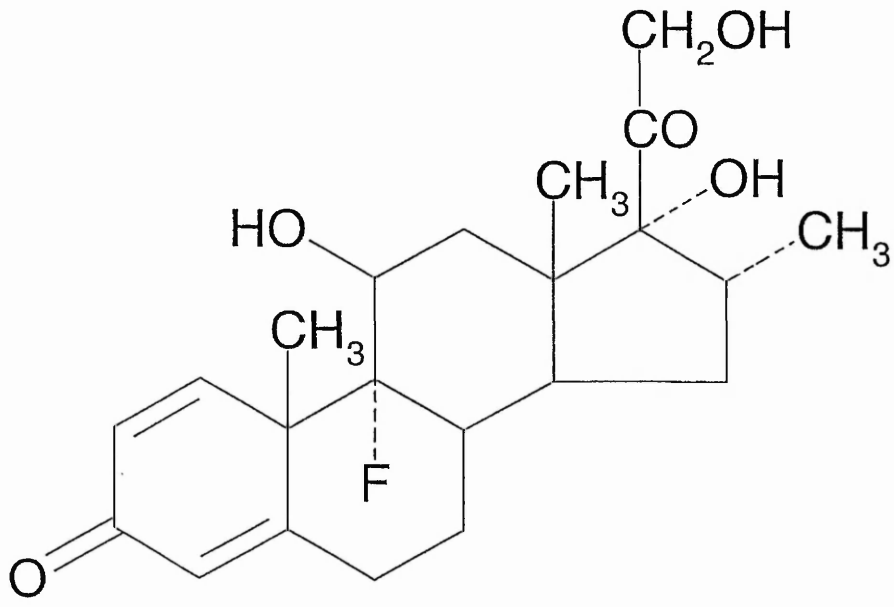

Figure 2.1. The structure of dexamethasone

Techniques reported for the determination of dexamethasone and associated synthetic corticosteroids are numerous [7-21]. Radioimmuno assay (RIA) is used widely for screening samples, but lacks the selectivity to allow the unambiguous identification of the analyte because of cross-reaction with other related corticosteroids.

2.1.2. IAC-HPLC Separations

The specificity that can be accomplished by the binding of analytes in a complex biological matrix to the antibodies of an immunoaffinity chromatography (IAC) stationary phase, provides a very efficient sample clean-up. This specificity has been utilised for on-line sample preparation of biological samples prior to HPLC with UV detection [22-25] and GC [26, 27].

Mass spectrometric detection in IAC-HPLC has also been reported, with atmospheric pressure chemical ionisation employed for the determination of propranolol and LSD in urine, using a short trapping column to interface IAC with HPLC [28]. Continuous-flow fast atom bombardment ionisation for the determination of diethylstilbestrol in the urine of rats and calves having also been demonstrated [29]. These reports (and others in a similar vein [17-19]) show the potential of IAC-HPLC-MS for the

determination of analytes at low levels in complex biological matrices. A tremendous asset when drug confirmation of potent steroids is required.

In this Chapter studies are reported of the development of a novel approach which provides the required selectivity and sensitivity for the detection of the synthetic corticosteroid dexamethasone in equine urine. On-line IAC-HPLC with mass spectrometric detection being the technique used to achieve a confirmatory analysis.

2.2. EXPERIMENTAL

2.2.1. Materials

Sepharose 4B CNBr - activated and Protein G stationary phases were obtained from Pharmacia (Uppsala, Sweden). Rabbit serum containing anti-dexamethasone antibody was provided by the Horseracing Forensic Laboratory (Newmarket, U.K.). Tritium labeled dexamethasone was obtained from Amersham International (Amersham, Buckinghamshire, U.K.). Dexamethasone, sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, sodium acetate and sodium azide were purchased from the Aldrich Chemical Co. Ltd (Dorset, U.K.). Propionic acid and methanol (Distol grade) were obtained from Fisons

(Loughborough, U.K.). Water was obtained from a MilliQ system (Millipore, Bedford, MA, U.S.A.). All eluents were filtered through 0.45 μm Millipore filters.

2.2.2. Standard and sample preparation.

A stock solutions of dexamethasone was prepared in methanol at a concentration of 1 mg ml^{-1}. Further dilutions were prepared in methanol to give concentrations in the range of 0.2-50 ng ml^{-1} for standard analysis' and spiking experiments. Spiked and post administration urine samples (20 ml) were adjusted to pH 7.0 and centrifuged at 1500 g for ten minutes prior to analysis. The supernatant was removed and 10 ml aliquots were introduced into the IAC-HPLC system.

2.2.3. Anti-dexamethasone IAC column

The crude rabbit serum samples were purified using a Protein G stationary phase (bed height 4 cm) packed in a C10 column (Pharmacia, Uppsala, Sweden). The appropriate fractions were collected, pooled and bound to CNBr - activated Sepharose 4B by the method described by the manufacturer [30]. Initially the Sepharose gel was swollen. This was then mixed with the dialysed IgG fraction and coupling buffer (0.1 M

$NaHCO_3$ + 0.5 M NaCl, pH 8.3). After the reaction was complete unreacted sites on the Sepharose were blocked using 0.1 M Tris/HCl, pH 8. The prepared anti-dexamethasone stationary phase was packed into a C10 column (Pharmacia, Uppsala, Sweden) to give a bed height of 5.4 cm. When not in use the IAC column was stored at 4°C in 20 mM phosphate buffer containing 0.5 M NaCl + 0.2 % sodium azide.

2.2.4. Instrumentation

2.2.4.1 Low pressure IAC system

Using a LKB low pressure chromatography system (Pharmacia), set-up as shown schematically in Figure 2.2. the tritium labeled experiments on the anti-dexamethasone IAC column were performed. Treated equine urine (Section 2.2.2) was spiked with [^{3}H]-dexamethasone at a suitable level. This was loaded on to the IAC column and was washed with loading buffer. The IAC column was then eluted with elution mobile phase and 1 ml fractions were collected, mixed with 3 mls of instagel scintillation fluid and were counted in a Beta V Liquid Scintillation Counter.

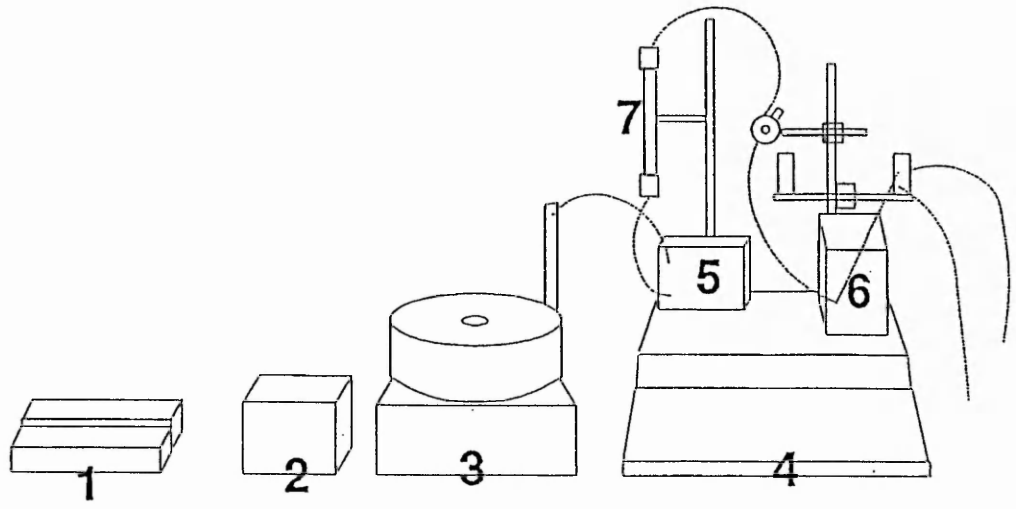

Figure 2.2. Schematic diagram of LKB low pressure chromatography system used to determine the elution of dexamethasone from the anti-dexamethasone IAC column. (1) chart recorder, (2) single path monitor-optical unit, (3) fraction collector (4) gradient programmer, (5) single path monitor-control unit (7) IAC column.

2.2.4.2. IAC-HPLC-UV

The IAC-HPLC-UV instrumentation (Figure 2.3.) consisted of a Waters model 6000A HPLC pump (Bedford, MA, U.S.A.) (pump 1) which delivered mobile phase to the IAC column *via* a six-port injection valve (Rheodyne 7010) containing a 10 ml stainless steel injection loop (12.7 m x 1 mm i.d.). The mobile phase flowed from the IAC column to V1, a six port switching valve (Rheodyne 7010) fitted with a 5 ml switching loop (6.4 m x 1 mm i.d.). This enabled eluted sample fractions to be transferred from the low pressure IAC column to the high pressure HPLC column. V1 was connected to two Waters 501 HPLC pumps (Bedford, MA, U.S.A.) (pumps 2 and 3). Pump 2 was used to flush the IAC band from the switching loop onto the HPLC column in water and pump 3 delivered the mobile phase to chromatograph the concentrated sample. The analytical separation was carried out on a 250 x 4.6 mm i.d. (5 μm) ODS column (Hichrom, Reading, Berks), and the effluent was transferred to a Waters Model 441 UV absorbance detector (λ = 254 nm).

2.2.4.3. IAC-HPLC-MS

The instrumentation for IAC-HPLC-MS is shown schematically in Figure 2.4.. It differed from the IAC-HPLC-UV set-up at valve V1. The sample

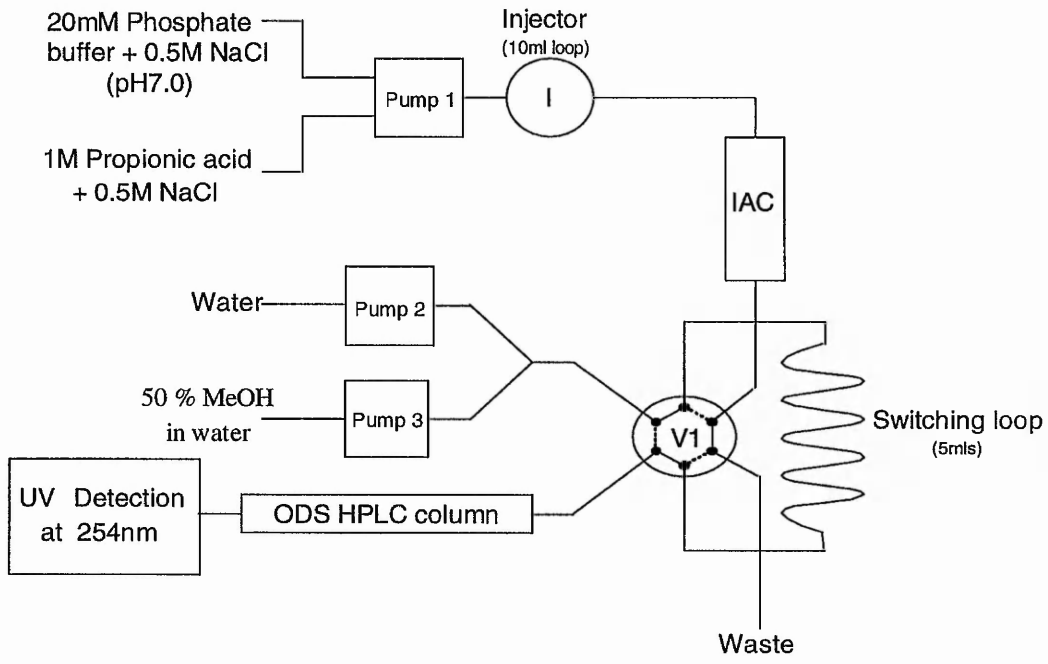

Figure 2.3. Schematic representation of the IAC-HPLC-UV system.

was flushed from the switching loop with mobile phase delivered by a Waters 501 HPLC pump (Bedford, MA, U.S.A.) (pump 2), to a T-piece fed by a second Waters 501 HPLC pump (Bedford, MA, U.S.A.) (pump 3). The sample passed through a 5 ml mixing loop (6.4 m x 1 mm i.d.) to V2, a six port valve (Rheodyne 7010) and onto the reverse phase analytical column. A Hewlett Packard 1050 LC delivery system (pump 4), delivered a methanol/water gradient to the column which was interfaced to a quadrupole mass spectrometer (VG Platform, Altringham, Cheshire) fitted with an atmospheric pressure chemical ionisation (APCI) interface.

2.2.5. Chromatographic Procedures

2.2.5.1. IAC-HPLC-UV

Mobile phase, 20 mM phosphate buffer + 0.5 M NaCl pH 7.0, was delivered to the IAC column at 1 ml min^{-1} and a 10 ml sample was injected. The IAC column was flushed with mobile phase for 15 minutes, then with the switching valve (V1) set to the load position, it was eluted with 1 M propionic + 0.5 M NaCl at 1 ml min^{-1}. At 30 minutes V1 was switched to the inject position and the chart recorder was started.

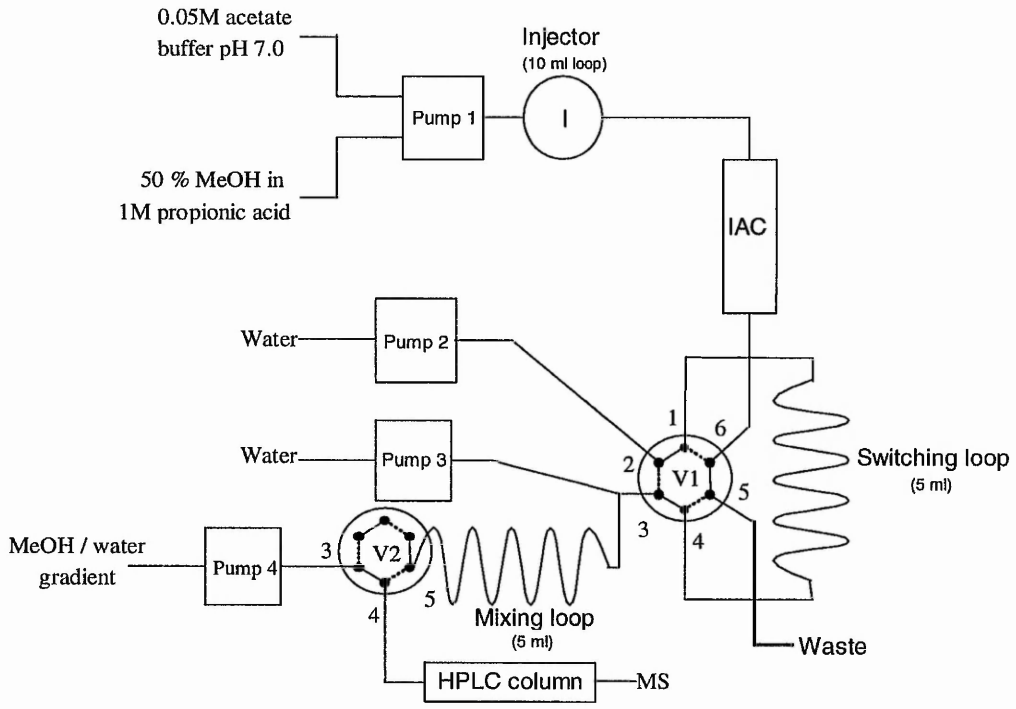

Figure 2.4. Schematic representation of the IAC-HPLC-MS system.

The contents of the switching loop were flushed onto the reverse phase analytical column with water at 1 ml min^{-1}. The sample was then eluted with 50 % methanol/water (v/v) at 1 ml min^{-1}.

2.2.5.2. IAC-HPLC-MS

Acetate buffer (0.05 M, pH 7.0) was delivered to the IAC column at 1 ml min^{-1} and the sample (10 ml) was injected. The switching valve (V1) was set to the load position and the IAC column was washed with buffer for 15 minutes, then eluting phase, 50 % MeOH in 1M propionic acid (v/v), was passed through the IAC column at 1 ml min^{-1}. After 30 minutes V1 was switched to the inject position and the eluted band was transferred to the reverse phase analytical column in water (1 ml min^{-1}) with an additional 2 ml min^{-1} of water (pump 3) *via* the 5 ml mixing loop. At 55 minutes the analytical column was switched on line with pump 4 (Hewlett Packard 1050) which eluted the sample with a 0 to 100 % water/methanol gradient (15 minutes) followed by 100 % methanol at 1 ml min^{-1} for 5 minutes. Mass spectral detection was started 55 minutes after injection. Dexamethasone was determined by selected ion monitoring of m/z 393 and 333 with a dwell time of 100 ms.

2.3. RESULTS AND DISCUSSION

2.3.1. On-line IAC-HPLC with UV detection

Sepharose, a beaded agarose gel, consists of linear chains of agarobiose units in which the ionic charge of the repeating 1,3-linked β-D-galactopyranose and 2,4-linked 3,6-anhydro-α-galacto-pyranose moieties are removed by reduction with sodium borohydride under alkaline conditions. As there are no natural covalent bonds between the linear polysaccharides, these are introduced by treatment with epi-chlorohydrin, improving the mechanical and chemical properties of the gel, thus permitting higher flow rates without compression of the gel bed and leading to improved stability at higher temperatures.

The Sepharose used as the support for the anti-dexamethasone antibodies in the IAC column is, however, unable to withstand the high pressures encountered in conventional HPLC. The problem was therefore to find an interface which would generate only a minimal back pressure on the IAC column, but which would enable a high percentage transfer of purified analyte from the IAC column to the HPLC system. The literature contains a number of examples of methods which incorporate small reverse-phase trapping columns in on-line IAC-HPLC systems [22-25],

but this route proved difficult with poor trapping of the target compound being achieved using the Pharmacia Sepharose column and the appropriate elution protocol.

An alternative approach investigated in this project was to use a switching loop to transfer eluted sample from the IAC column to the HPLC column. From studies of the anti-dexamethasone IAC column performed using tritium labeled dexamethasone (results stated later in this Chapter) it had been determined that the analyte was eluted in either a 3 ml or 5 ml band. It was therefore probable that, with correct flow rates of mobile phases and timing of column switching, a wide bore 5 ml loop would be capable of allowing the coupling of IAC on a Sepharose column with HPLC (Figure 2.5.).

This method would not generate a high back pressure on the IAC column and should allow the eluted analyte to be flushed on to the HPLC column. In initial investigations of the interface, 1 M propionic acid + 0.5 M NaCl was used as the eluting phase. It was expected that this mobile phase would allow easy focusing of the lipophilic corticosteroid at the head of the reverse phase analytical HPLC column (as depicted in Figure 2.6.). If an eluting phase containing organic modifier was used it was envisaged that a diluting stage would have to be incorporated into the system.

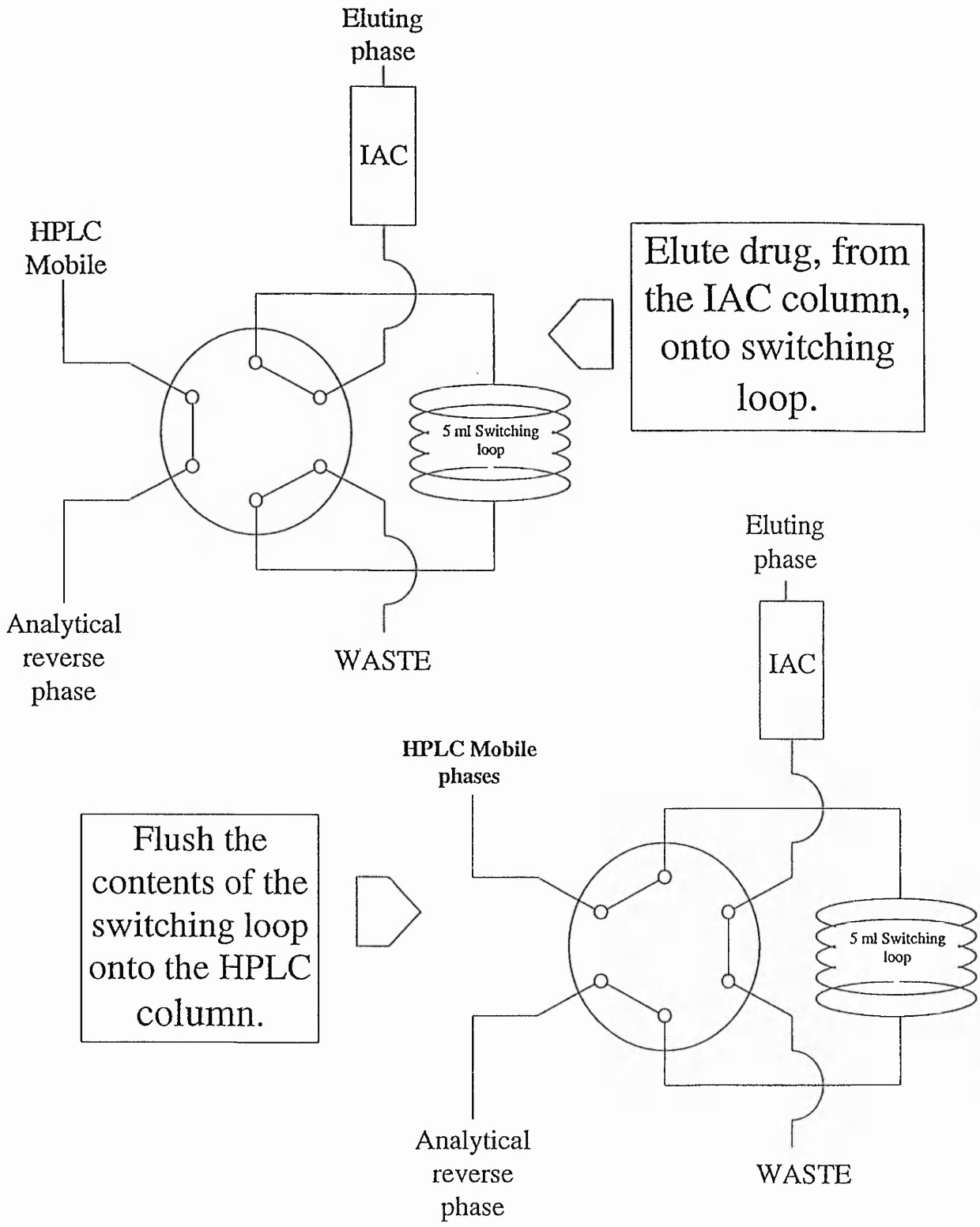

Figure 2.5. Schematic of the loop interface between the IAC and HPLC columns.

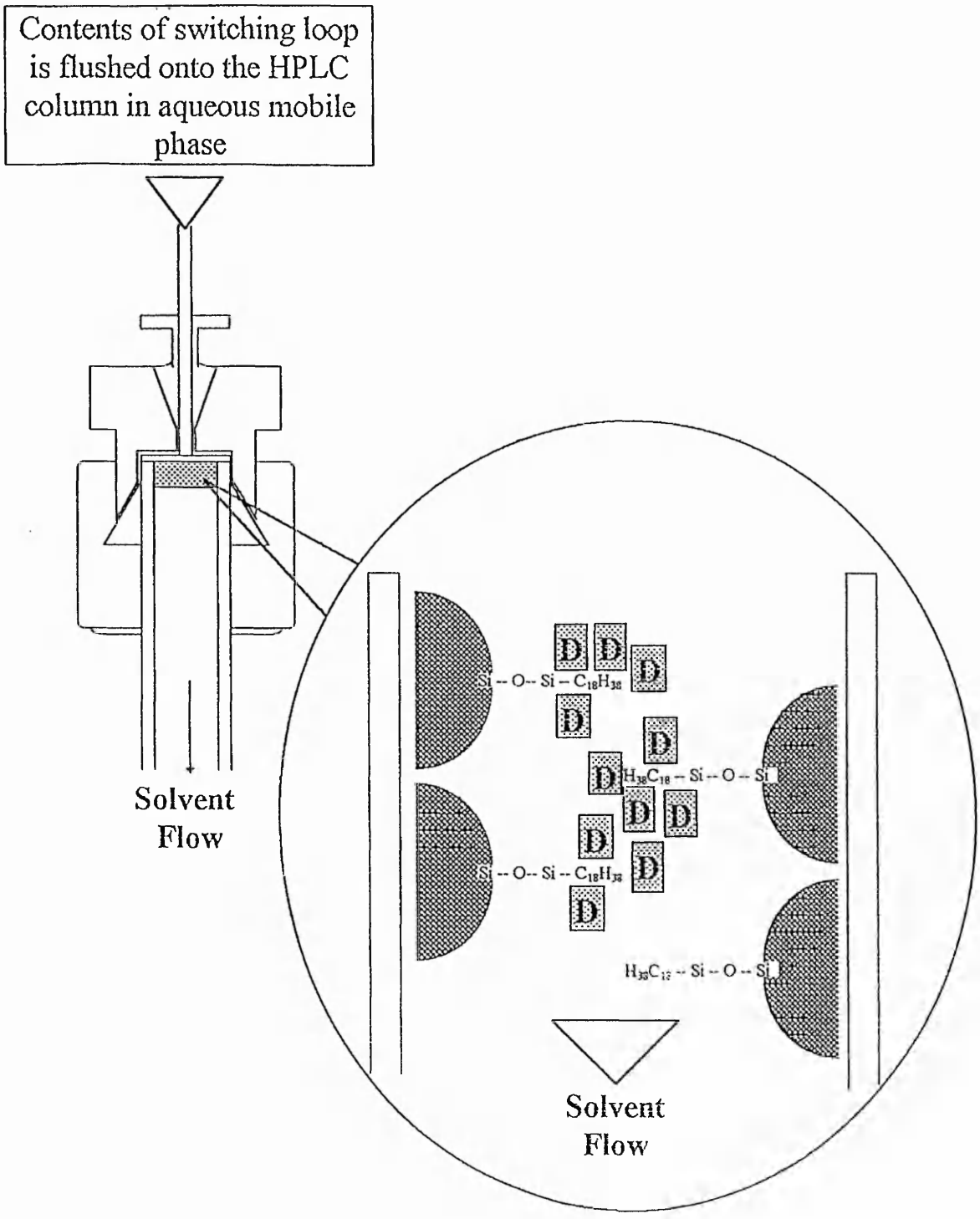

Figure 2.6. Interaction between dexamethasone and the C18 groups on the HPLC column.

Initial investigations of IAC sample clean-up using anti-dexamethasone anti-bodies in a sepharose column coupled directly to HPLC *via* a loop interface were carried out with UV detection. The dexamethasone was eluted from the IAC column with aqueous propionic acid. Experiments with tritium labeled dexamethasone (Figure 2.7.) established that the analytes eluted from the IAC column in a 5 ml band and by correct timing of valve switching this band could first be flushed onto the switching loop and then onto the reverse phase analytical column. The dexamethasone was focused at the head of the column due to its strong interaction with the ODS stationary phase in the presence of aqueous mobile phase. The focused band was then chromatographed isocratically with methanol/water mobile phase.

The total recovery of dexamethasone for the IAC-HPLC system was 70 % and this procedure allowed the detection of dexamethasone spiked in urine at a level of 30 $\mu g\ l^{-1}$ (Figure 2.8.). The limit of detection was determined not by the minimum detectable quantity of dexamethasone, but by the interfering peaks observed in the chromatogram which originated from the equine urine matrix. Non-selective adsorption by the IAC Sepharose stationary phase was the most likely reason for the retention of matrix components by the IAC column.

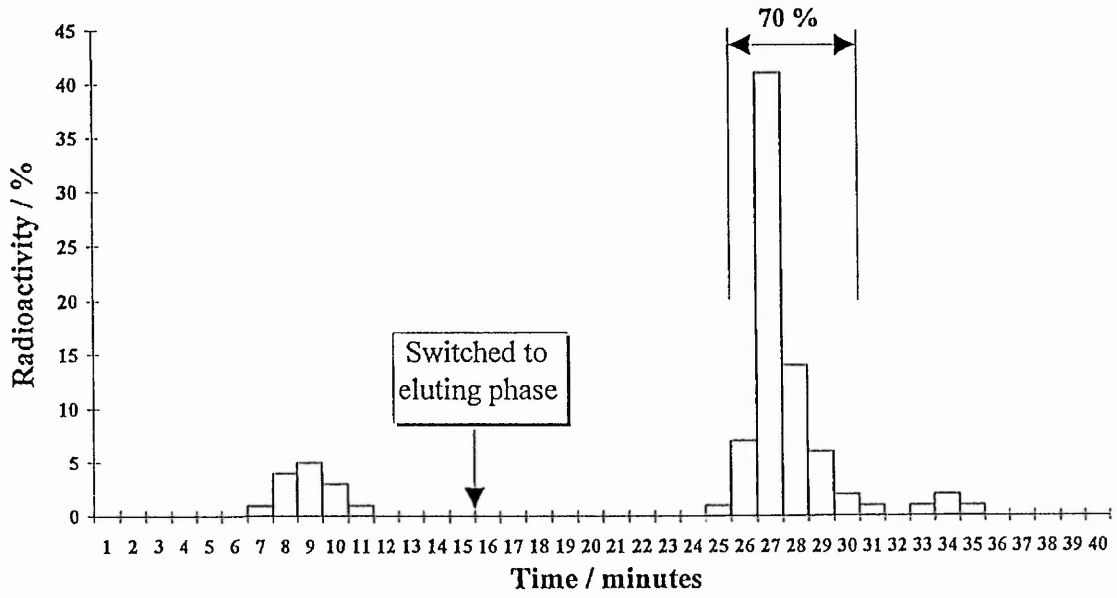

Figure 2.7. The elution profile of tritium labelled dexamethasone from the IAC column using 1 M propionic acid + 0.5 M NaCl as eluting phase.

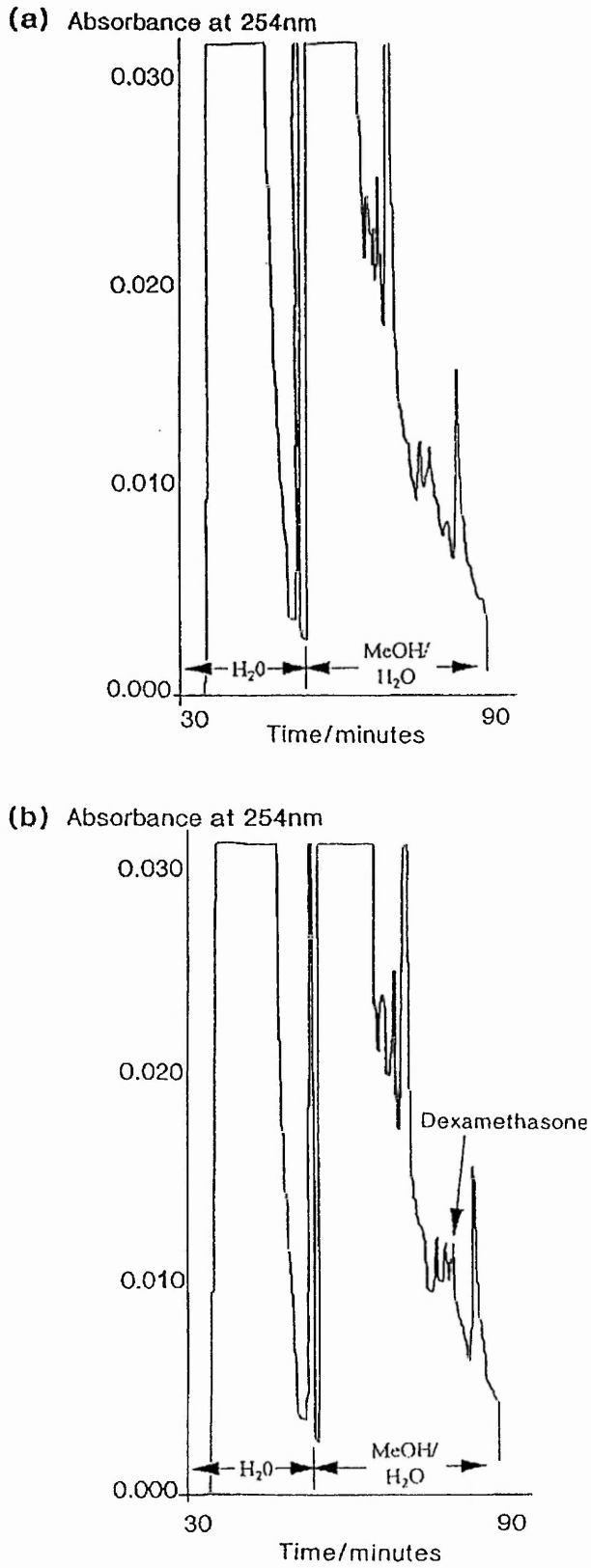

Figure 2.8. Chromatograms from IAC-HPLC-UV analysis of (a) blank equine urine and (b) 30 μg l^{-1} dexamethasone spiked in equine urine.

The IAC-HPLC-UV system established the potential of the coupling of the anti-dexamethasone IAC column with the HPLC column *via* a loop interface, but did not exhibit the required selectivity for the analysis of post administration equine urine samples for dexamethasone, due to the potency (and therefore low dose) of this synthetic corticosteroid.

2.3.2. On-line IAC-HPLC with mass spectrometry detection.

The combination of IAC-HPLC with mass spectrometry was investigated using an atmospheric pressure chemical ionization (APCI) interface. The mobile phase used to elute dexamethasone from the IAC column was changed to 1 M propionic acid in methanol/water for IAC-HPLC-MS, because of the incompatibility between the sodium chloride, used in the IAC eluting phase for the IAC-HPLC-UV system, and the APCI interface of the MS detector. The elution of dexamethasone from the IAC column by the methanol/aqueous propionic acid mobile phase was determined using tritium labeled dexamethasone as before and the dexamethasone was observed to elute in a 3 ml band between 11 and 14 minutes after the IAC column was switched to the eluting phase. However, the methanol in the IAC eluting phase presented a problem, since it prevented focusing of the dexamethasone at the head of the ODS column. To overcome this difficulty a dilution stage was introduced which involved the addition of

water to the IAC elutent in a 5 ml mixing loop ahead of the reverse phase analytical column (Figure 2.4.). This gave the necessary focusing at the head of the HPLC column and the dexamethasone was then chromatographed using a water/methanol gradient over 15 minutes.

Under APCI conditions dexamethasone showed a protonated molecular ion, $[M + H]^+$ at m/z 393 and a prominent fragment ion, corresponding to loss of the C-17 side chain, at m/z 333 (Figure 2.9). Selected ion monitoring (SIM) of the m/z 393 and 333 ions, and the chromatographic retention time were used to confirm the presence of dexamethasone following IAC-HPLC clean-up. The IAC-HPLC-MS system gave a recovery of 75 % for dexamethasone and had a limit of detection calculated at 0.1 $\mu g\ l^{-1}$ (signal : noise = 3:1). A good linear response, with a correlation coefficient of R = 0.995 was obtained for spiked urine samples in the range of 0.2-50 $\mu g\ l^{-1}$. Replicate injections of a spiked sample urine (1 $\mu g\ l^{-1}$) showed a coefficient of variation of 6.8 %.

The on-line combination of IAC-HPLC-MS eliminated the need for extraction and derivatisation procedures [31, 32] which are necessary when using IAC followed by off-line GC-MS. This methodology is also simpler in terms of sample manipulation and, with less manual intervention,

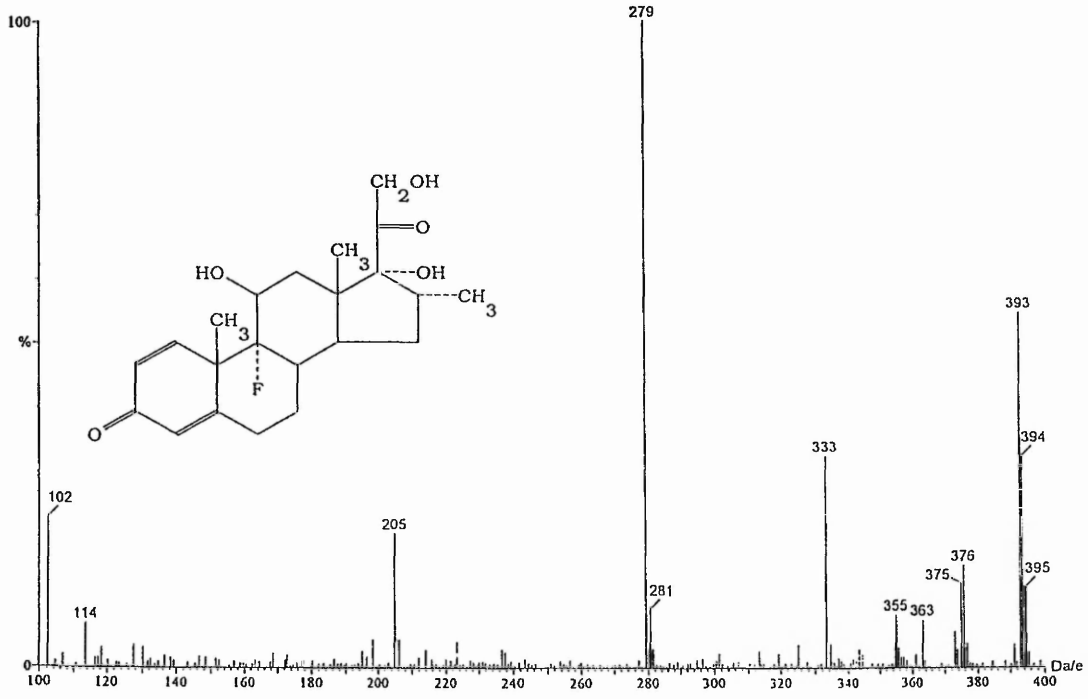

Figure 2.9. APCI mass spectrum of dexamethasone.

there are fewer stages where potential losses or contaminations might occur.

2.3.3. Comparison of on-line IAC-HPLC-MS with off-line IAC with GC-MS analysis.

Comparing the total analysis times of the two systems, the fully automated procedure is clearly superior (Figure 2.10.). This is due to the shorter analysis time for a single sample and the fact that the IAC column can be re-equilibrated whilst the LC-MS analysis is being carried out, and visa versa. Note, however, that the benefit in terms of analysis time per sample decreases as sample number increases, because the off-line extraction and derivatisation stages are both batch processes.

The on-line methodology is also simpler in terms of sample manipulation and, with less manual intervention, there are fewer stages where potential losses or contamination might occur.

It should be noted, however, that in terms of mass spectrometer time, which is relatively expensive, the on-line method in fact ties up an instrument for longer (305 min for 5 samples, c.f. 75 min for off-line GC-MS).

Table 2.2. Comparison of the analysis times for off-line IAC GC-MS and on-line IAC-HPLC-MS.

Number of samples	Total analysis time (min)		Difference in analysis time per sample (min)
	On-line	Off-line	
1	85	275	190
5	305	580	55
20	1130	1630	25

This drawback could be overcome using what might be termed a "near-line" system, in which the IAC stage of the procedure is decoupled from the LC-MS. Fractions containing the eluted analyte are collected for all samples. These are then analysed directly by LC-MS, using a large volume autosampler and a trace enrichment system. This would however re-introduce manual interventions. Such a set-up would require less instrument time (approximately 105 min for 5 samples), but would give a

longer total analysis time than the on-line system (approximately 380 min for 5 samples), and would require more monitoring time by the analyst.

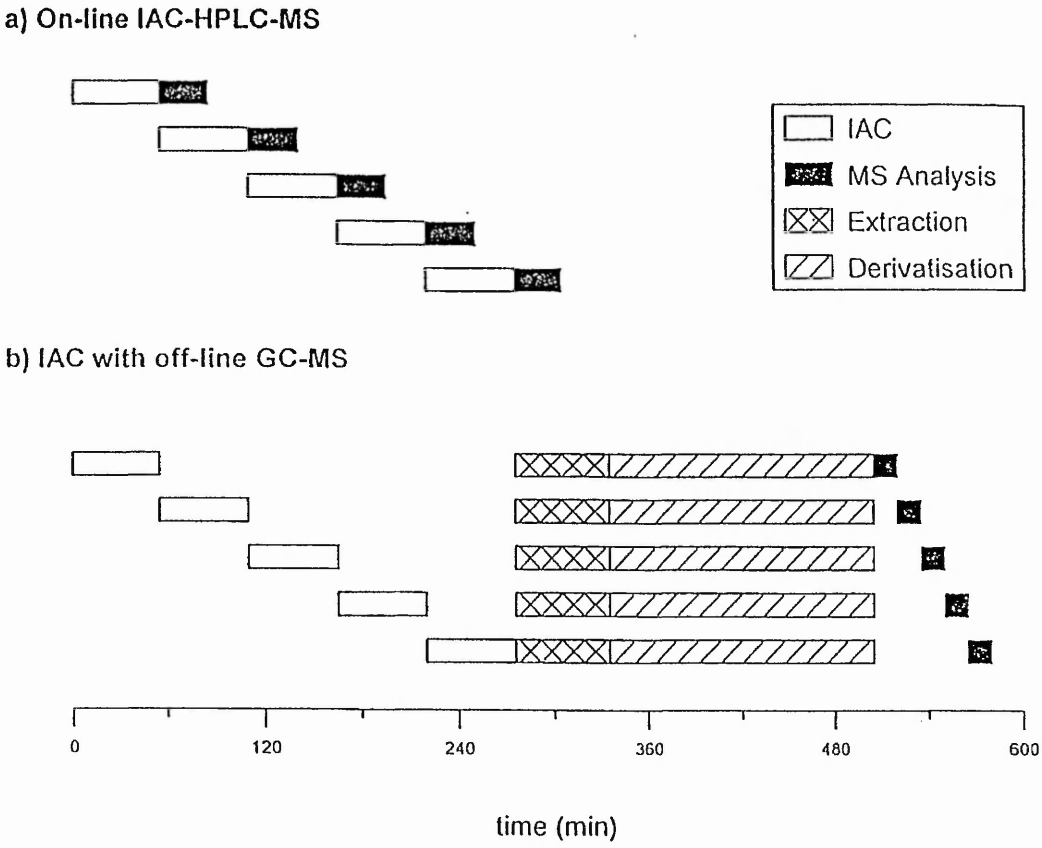

Figure 2.10. Comparison of analysis times for five samples.

Another approach which would maintain the advantages of the on-line technique and decrease the instrument time used would be to decrease the duration of the limiting step (the IAC pre-treatment in the hyphenated system) of the analysis so that the MS analysis time was limiting.

The IAC-HPLC-MS system described has been used to analyse both equine samples spiked with dexamethasone and post administration samples. Figure 2.11.a gives the selected ion chromatogram (m/z 393 + 333) from a sample spiked with dexamethasone (retention time: 14.7 minutes) at 5 $\mu g\, l^{-1}$. Figure 2.11.b shows the chromatogram from a sample collected 8 hours after a single intra-muscular injection (20 $\mu g\, kg^{-1}$) of a Dextran preparation of dexamethasone to a thoroughbred horse (retention time 14.8 minutes). The concentration of dexamethasone in this sample was measured as 4 $\mu g\, l^{-1}$.

2.4. CONCLUSION

On-line IAC-HPLC coupled with APCI-MS is shown to be an effective method for the confirmatory analysis of dexamethasone in equine urine at concentrations down to 0.1 $\mu g\, l^{-1}$. The technique showed a reasonable % RSD as well as good linearity. With the method being shown to be faster for 5 samples than the alternative off-line IAC GC-MS approach. The use of APCI-MS with its increased detector selectivity for dexamethasone, compared with UV detection, allowed the technique to be applied to the determination of post administration samples.

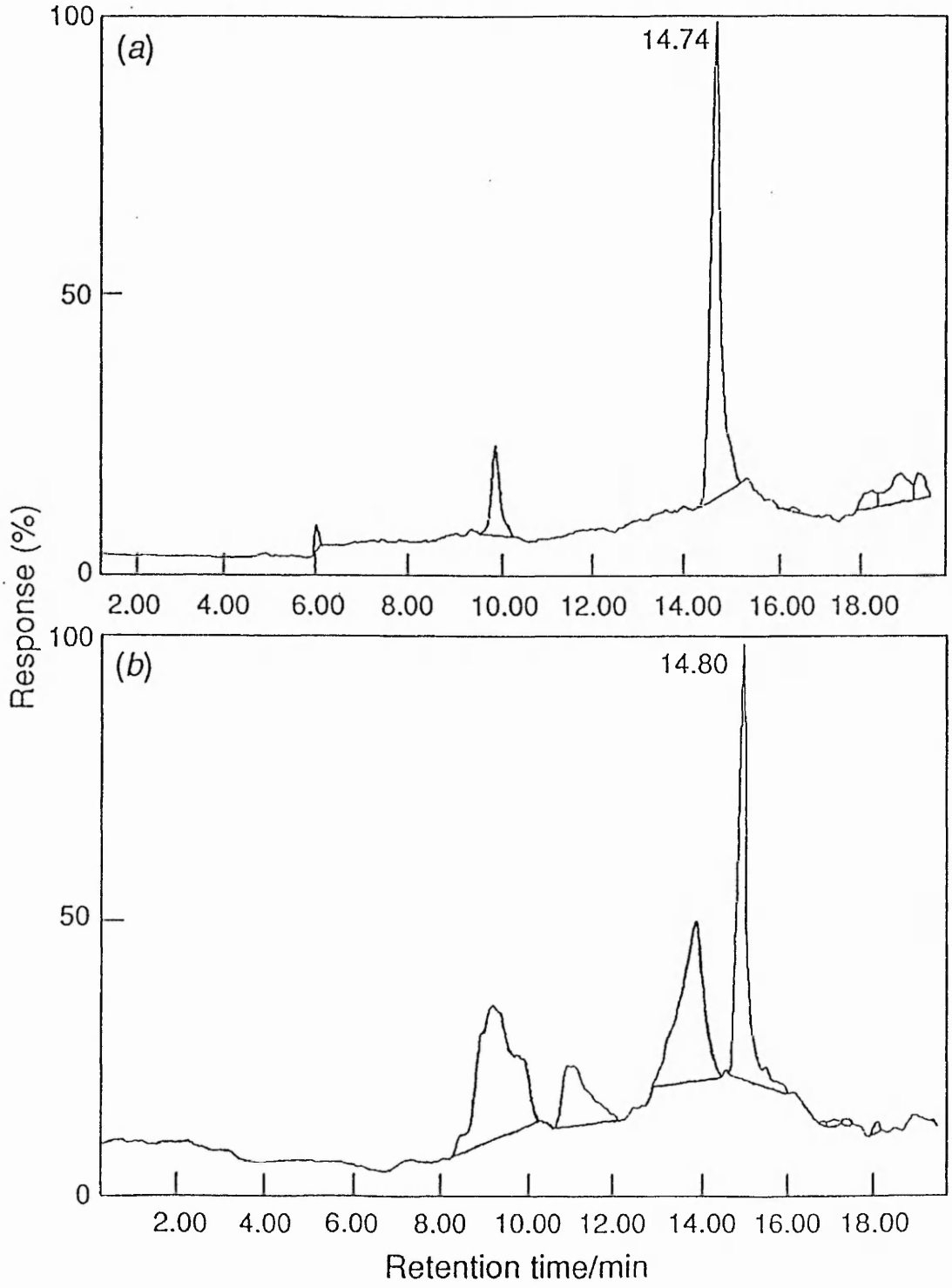

Figure 2.11. Selected ion chromatogram (m/z 393 + 333) of (a) a 5 μg l^{-1} dexamethasone spiked urine sample, (b) an 8 hour post administration urine sample.

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CHAPTER 3

IMMUNOAFFINITY CHROMATOGRAPHY ON-LINE WITH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-PARTICLE BEAM-MASS SPECTROMETRY FOR THE DETERMINATION OF CORTICOSTEROIDS

3.1. INTRODUCTION

The potential of immunoaffinity chromatography-high performance liquid chromatography-mass spectrometry (IAC-HPLC-MS) has been demonstrated for the determination of analytes at low levels in complex biological matrices [1-2] (Chapter 2). The powerful clean-up afforded by the supported antibodies in the IAC column, selectively enriching the analyte of interest, compliments the diagnostic power of MS in these procedures.

The soft gel supports, such as Sepharose, are commonly used in IAC columns. These display minimal non-specific binding, but there are restrictions on their use in multi-dimensional chromatographic systems because they are sensitive to high pressures and flow rates. The development of more robust antibody support materials would therefore be of great benefit in a multi-dimensional system incorporating IAC. Such a support is HEMA (poly-hydroxyethyl methacrylate) a commercially available rigid polymer which has been used in IAC columns for the extraction of LSD [3].

The synthetic corticosteroids (as stated in Chapter 2) are used by veterinarian practitioners for their anti-inflammatory effect but are

prohibited substances in equine sports due to their effects on performance [4]. The determination of this class of steroids presents an analytical challenge because their high potency and extensive metabolism (both phase I, involving oxidation, reduction and hydrolysis reactions, and phase II, constituting the formation of conjugates) [5-7] results in low concentrations of drug in the biological fluids of the offender.

There are many reports of methods for the determination of the corticosteroids [8-22]. Some of these may be classified as screening methods, which often have limited selectivity between compounds of the same class. The others are confirmatory methods which usually involve extensive off-line clean-up prior to analyte determination. However, work performed by Stanley *et al* [18-20] has highlighted the advantages of an immunoaffinity chromatographic multidimensional approach to confirming the presence of drug residues in the equine. These approaches are quicker and have less sample manipulation than certain GC-MS [23, 24] methods and give tremendous sensitivity.

A particle beam (PB) interface was used in this IAC-HPLC-MS study. The PB being a mechanically simple interface for LC-MS, which allows desolvation and transport of solute molecules to the MS, where they may undergo electron ionisation or chemical ionisation [25]. The

nebulisation/desolvation processes of the PB is critical, since the amount of analyte reaching the mass spectrometer ion source is inversely proportional to the heat of vaporisation and heat capacity of the mobile phase [26-28]. Normal phase HPLC is therefore usually the method of choice for the PB/MS [20], although reversed-phase HPLC has been used successfully with EI as the ionisation mode [29]. The sensitivity of the particle beam interface for combined IAC-HPLC under reversed-phase conditions was enhanced in this work by the use of quadrupole ion trap mass spectrometry (QITMS) with mass selective ion accumulation of analyte ions during the ionisation step.

In this chapter, the development of an on-line IAC-HPLC-MS method using HEMA as a support for anti-dexamethasone antibodies in an IAC column, is described. Coupling of reversed-phase HPLC with quadrupole ion trap mass spectrometry (QITMS) using a particle beam (PB) interface was evaluated for the analysis of the corticosteroids dexamethasone and flumethasone in post administration equine urine.

3.2. EXPERIMENTAL

3.2.1. Materials

Dexamethasone, cortisol, prednisolone, deoxymethasone, betamethasone, flumethasone, sodium hydrogen orthophosphate, sodium dihydrogen orthophosphate, sodium azide and sodium acetate were purchased from the Aldrich Chemical Co. Ltd. (Dorset, UK). Tritium labeled dexamethasone was obtained from Amersham International (Amersham, Buckinghamshire, U.K.). Propionic acid and methanol (Distol grade) were obtained from Fisons (Loughborough, UK). Water was obtained from a MilliQ system (Millipore, Bedford, MA, USA). All eluents were filtered through 0.45μm filters (Millipore, Bedford, MA, USA). HEMA 1000VS-L was purchased from Presearch Limited (Herts, UK). Rabbit serum containing anti-dexamethasone antibody was provided by the Horseracing Forensic Laboratory (Newmarket, UK).

3.2.2 Standard and sample preparation

Stock solutions of the corticosteroids were prepared in methanol at a concentration of 1 mg ml^{-1}. Further dilutions were prepared in methanol to give concentrations in the range of 0.2-50 ng ml^{-1} for standard analysis'

and spiking experiments. Spiked and post administration urine samples (20 ml) were adjusted to pH 7.0 and centrifuged at 1500 g for ten minutes prior to analysis. The supernatant was removed and introduced into the IAC-HPLC system.

3.2.3 Anti-dexamethasone IAC column

The crude rabbit serum samples were purified using a Protein G stationary phase (bed height 4 cm) packed in a C10 column (Pharmacia, Uppsala, Sweden). The appropriate fractions were collected and pooled. The antibodies were bound to HEMA and the HEMA was blocked using the method described by the manufacturer [30]. The HEMA was first swollen with water and then washed with 0.1 M NaOH/0.5 M NaCl, pH 8.3 coupling buffer. The anti-dexamethasone IgG solution (5 ml) was added to the HEMA (1 g) and coupling buffer (5 ml) and the resulting slurry was mixed overnight. The gel was washed and blocked with 0.1 M Tris base (pH 9.0). The HEMA anti-dexamethasone stationary phase was packed into a stainless steel HPLC column (50 mm x 4.6 mm). The IAC column was stored at 4°C in 30 mM phosphate buffer containing 0.5 M NaCl + 0.2 % sodium azide when not in use.

3.2.4. Instrumentation

3.2.4.1 Low pressure IAC system

Using a LKB low pressure chromatography system (Pharmacia), set-up as shown schematically in Chapter 2. the tritium labeled experiments on the HEMA anti-dexamethasone IAC column were performed. Treated equine urine (Section 3.2.2) was spiked with [^{3}H]-dexamethasone at a suitable level. This was loaded on to the IAC column and was washed with loading buffer. The IAC column was then eluted with elution mobile phase and 1 ml fractions were collected, mixed with 3 ml of instagel scintillation fluid and were counted in a Beta V Liquid Scintillation Counter.

3.2.4.2. IAC-HPLC-UV

The IAC-HPLC-UV instrumentation (Figure 3.1.) consisted of a Waters module 6000A HPLC pump (Bedford, MA, USA) (pump 1) which delivered mobile phase to the IAC column *via* a six-port injection valve (Rheodyne 7010) containing a 20 µl stainless steel injection loop. The mobile phase flowed from the IAC column to V1, a six port switching valve (Rheodyne 7010) fitted with a 5 ml switching loop (6.4 m x 1 mm

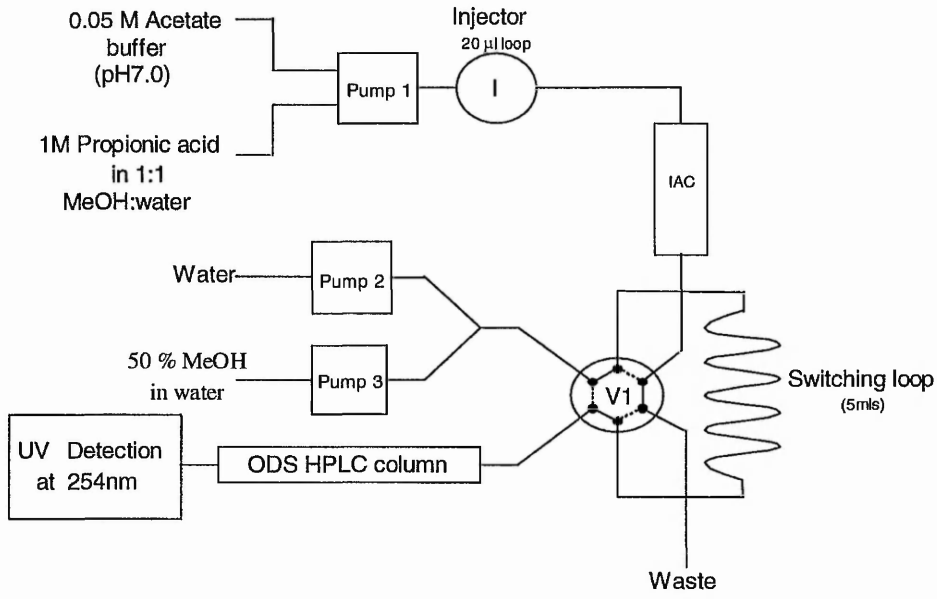

Figure 3.1. Schematic diagram of IAC-HPLC-UV system.

i.d.), which allowed eluted sample factions to be transferred from the IAC column to the HPLC column. Valve V1 was connected to two Waters 501 HPLC pumps (Bedford, MA, USA) (pumps 2 and 3). Pump 2 was used to flush the IAC band from the switching loop onto the HPLC column in water and pump 3 delivered the mobile phase to the HPLC column. The analytical separation was carried out on a 125 mm x 4.9 mm i.d. column packed with 5µm ODS (Hichrom, Reading, Berks), and the effluent was monitored by a Waters Model 441 UV absorbance detector ($\lambda = 254$ nm).

3.2.4.3. IAC-HPLC-PB/QITMS

The IAC-HPLC-PB/QITMS instrumentation (Figure 3.2) used the same IAC-HPLC column switching configuration as that employed for UV detection with the 20 µl injection loop being replaced by a 10 ml stainless steel loop (12.7 m x 1 mm i.d.). The effluent was transferred from the HPLC column to the particle beam interface. The interface was of modular design [31] and consisted of a heated stainless steel nebuliser, desolvation chamber and two-stage momentum separator, which was differentially pumped to remove solvent. The desolvation chamber was maintained at 50°C with a helium nebulizer pressure of 50 psi. The interface was

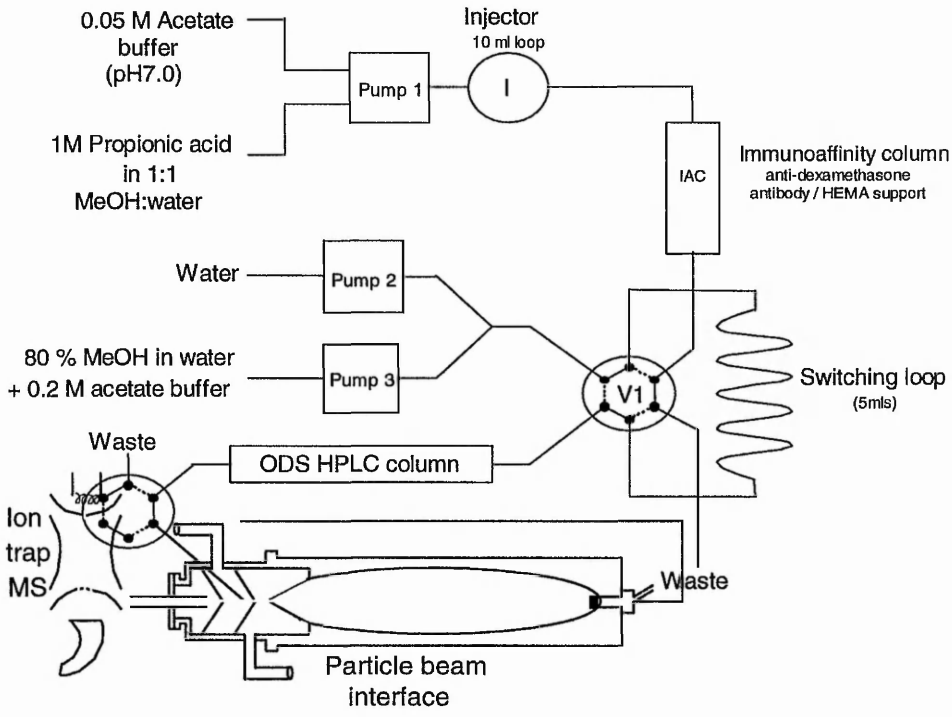

Figure 3.2. Schematic diagram of IAC-HPLC-PB/QITMS system.

coupled to the quadrupole ion trap mass spectrometer (Finnigan MAT ITMS, San Jose, Ca) *via* a stainless steel transfer line. The QITMS was operated in electron ionization (EI) mode and a filtered noise field (Teledyne Scientific Instruments, Sunny Vale, CA) was used to accumulate analyte ions mass selectively during the ionization period (400 ms).

3.2.5. Chromatographic procedures

3.2.5.1 IAC-HPLC-UV

The injected sample (20 µl) containing 0.2-0.8 µg of each of the corticosteroids was delivered to the IAC column in acetate buffer (0.05M, pH 7.0) mobile phase at a flow rate of 1 ml min^{-1}. The IAC column was flushed with mobile phase, with the switching valve (V1) set to the load position, then eluted with 50 % MeOH in 1 M propionic acid (v/v) at 1 ml min^{-1}. At 12 minutes V1 was switched to the inject position and the recorder was started. The contents of the switching loop were flushed onto the reversed-phase analytical column with water at 3 ml min^{-1}. The sample was then eluted with 50 % methanol/water (v/v) at 2 ml min^{-1}.

3.2.5.2. IAC-HPLC-PB/QITMS

Mobile phase (0.05 M acetate buffer, pH 7.0) was delivered to the IAC column at 2 ml min^{-1} and 2 x 10 ml aliquots of urine were injected. The IAC column was flushed with mobile phase for 15 minutes and then, with the switching valve (V1) set to the load position, the IAC column was eluted with 50 % methanol in 1 M propionic acid (v/v) at a flow rate of 1 ml min^{-1}. At 22 minutes V1 was switched to the inject position and the contents of the switching loop were flushed onto the reversed phase analytical column with water at 3 ml min^{-1}.

The sample was eluted with 80 % methanol/0.2 M ammonium acetate (v/v) at 2 ml min^{-1} for 3 minutes with the effluent going to waste. The flow rate was then reduced to 0.3 ml min^{-1} and the eluate switched to the particle beam interface.

3.3. RESULTS AND DISCUSSION

3.3.1. Evaluation of IAC column

IAC sample clean-up for a range of coricosteroids using anti-dexamethasone anti-bodies bound to a HEMA support, which was

coupled directly to a HPLC column *via* a loop interface were carried out initially with UV detection. Experiments with tritium labeled dexamethasone established that the analytes eluted from the IAC column in a 2 ml band and by correct timing of valve switching this band could first be flushed onto the switching loop and then onto the reverse phase analytical column (as described in Chapter 2). The corticosteroids (Figure 3.3) were eluted from the IAC column with 1 M propionic acid in methanol/water, under similar conditions as those used with the IAC Sepharose column. The packing of the HEMA supported antibodies into a normal 4.6 mm i.d. HPLC column gave a distinct operational advantage over the Pharmacia low pressure columns which were used with the Sepharose supported antibodies.

HEMA proved to be a robust support material for on-line IAC-HPLC, with the higher back pressures and flow rates possible through the IAC column in the multi-dimensional system allowing more rapid sample throughput. Soft gel supports such as Sepharose, which was used in the earlier studies with anti-dexamethasone antibodies, as described in Chapter 2, failed under similar flow and pressure conditions. Flow rates of 5 ml min^{-1} being employed on the HEMA column for loading and washing of injected sample, with no obvious deleterious effect to the

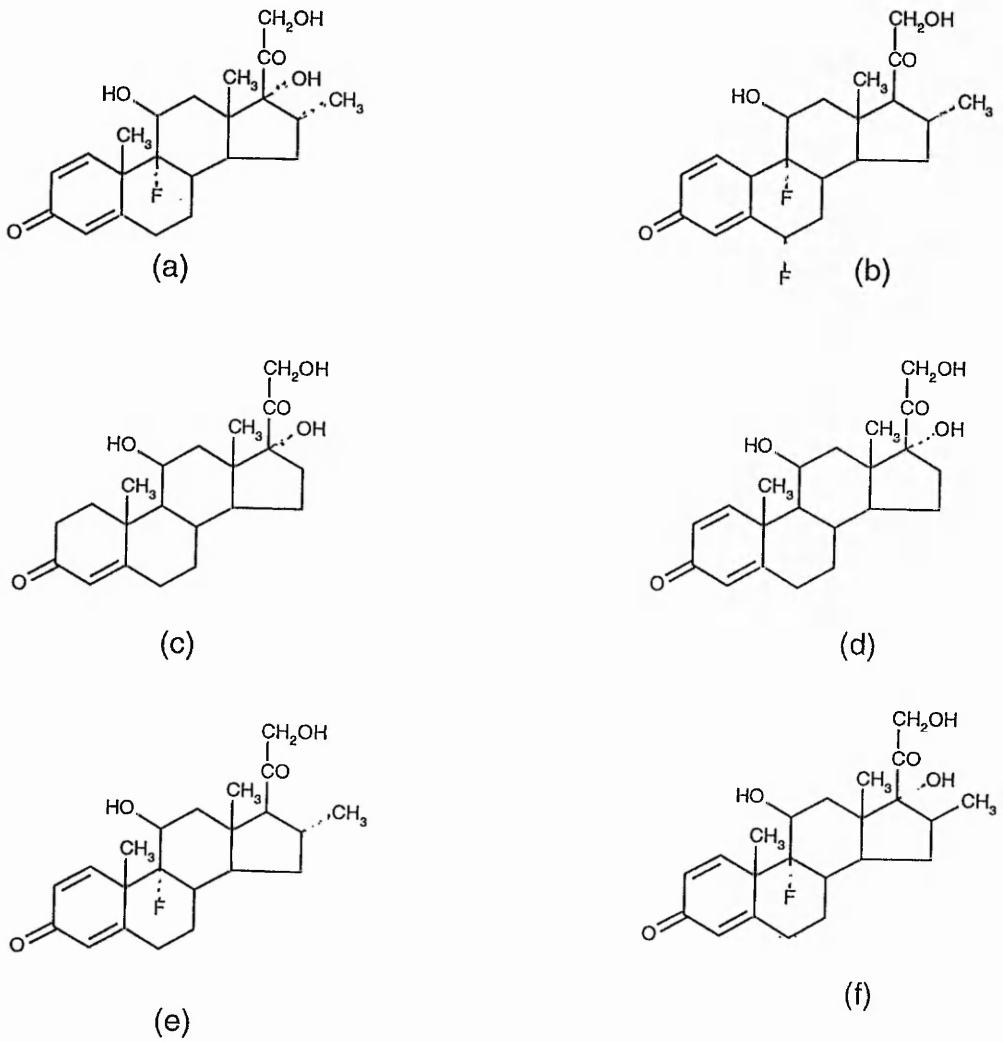

Figure 3.3. Corticosteroids investigated for IAC-HPLC-MS analysis: (a) dexamethasone, (b) flumethasone, (c) cortisol, (d) prednisolone, (e) deoxymethasone, (f) betamethasone.

support material. Conditions under which the Sepharose column could not function. A 5 cm IAC HEMA column was used to increase sample protein loading capacity and pro-long the lifetime of the HEMA supported antibodies. The column displaying no deterioration in performance after 200 injections over a three month period, a fact which reinforces the conclusion that the HEMA support was very robust.

The retention behavior of the anti-dexamethasone antibodies bound to the HEMA support was investigated for a range of related corticosteroids using UV detection. On loading the anti-dexamethasone antibody bound HEMA column with the full range of corticosteroids (Figure 3.3) in buffer, followed by elution with methanol/propionic acid, all the steroids investigated were found to be retained to some extent on the IAC column. The length of time taken to flush the column with loading buffer was varied to determine whether this affected the binding of the corticosteroids.

The results, given in Table 3.1, showed that washing the IAC column with buffer reduced significantly the amounts of some of the corticosteroids, notably cortisol and prednisolone, which were detected in the methanol/propionic acid eluting phase. A typical chromatogram for the elution of deoxymethasone after 7 mls of loading buffer was utilised to

Table 3.1 Specific and non-specific binding of selected corticosteroids on the anti-dexamethasone antibody/HEMA IAC column.

Corticosteroids	% recovery of corticosteroids on the IAC column following flushing with different volumes of washing buffer.			
	5 ml	7 ml	10 ml	15 ml
Dexamethasone	80	79	74	73
Cortisol	69	22	nd	nd
Prednisolone	65	24	nd	nd
Deoxymethasone	73	35	20	20
Betamethasone	71	37	22	21
Flumethasone	81	76	71	72

0.2 - 0.8 µg of each corticosteroid loaded onto IAC column,

nd = none detected

wash the HEMA IAC column is shown in Figure 3.4. Increased washing with the aqueous loading buffer further reduced the amounts of these two corticosteroids until, after 10 minutes washing of the column, no residual analyte was detected in the eluting band of the IAC column. This is shown graphically in Figure 3.5. for deoxymethasone, a corticosteroid which has weak interaction with the anti-dexamethasone antibodies bonded to the column. Under similar conditions the recoveries for deoxymethasone and betamethasone were 20-22 % and those for dexamethasone and flumethasone were >70 %.

Note that the recovery of dexamethasone through the IAC-HPLC system went from 74 % to 71 % over a period of three months, again demonstrating the robustness of the HEMA supported antibodies. This was determined by comparison of peak areas for dexamethasone standards analysed by the IAC-HPLC-UV system and a normal HPLC-UV system.

These data suggested that there was considerable non-specific binding on the IAC column, in addition to specific antibody-antigen binding, but these non-specific interactions between the column and the corticosteroids were sufficiently weak that adsorbed compounds could be removed quantitatively by washing with aqueous buffer.

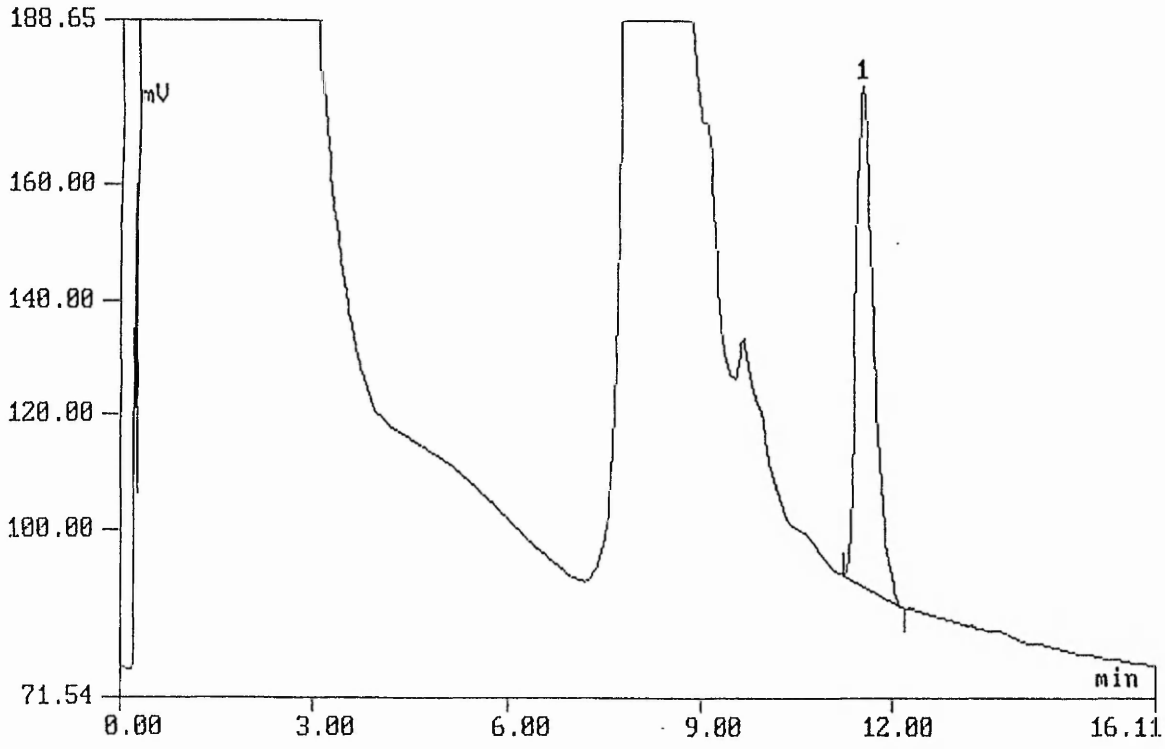

Figure 3.4. Typical chromatogram of dexamethasone after washing with 7 mls of loading buffer.

The contribution of the support material to the non-specific binding was investigated for the adsorption of the corticosteroids on HEMA using the same sample loading, washing and elution procedures as before. The system shown in Figure 3.1. was used with the anti-dexamethasone antibody/HEMA IAC column being replaced with a HEMA column blocked according to the manufacturer's instructions with 0.1 M Tris base (pH 9.0).

The results, given in Table 3.2, confirmed that non-specific binding by HEMA was occurring for all the corticosteroids, but that washing with 7 ml of loading buffer was sufficient to remove the support bound analyte. This is shown graphically in Figure 3.6 for deoxyamethasone, when the complete removal of the steroid from the HEMA column contrasts with the retention of analyte by the IAC column (Figure 3.5).

If the amount of each of the corticosteroids retained on the IAC column containing anti-dexamethasone antibodies, after washing with 10 mls or more of aqueous buffer, is assumed to be due to specific binding by the anti-dexamethasone antibodies and not due to non-specific adsorption, then cross reactivity for the corticosteroids may be determined. The calculated cross-reactivities, for the IAC column relative to dexamethasone, are similar to those determined by enzyme linked

Table 3.2. Non-specific binding of selected corticosteroids on HEMA support.

Corticosteroids	% recovery of corticosteroids on the HEMA column following flushing with different volumes of washing buffer			
	5 ml	7 ml	10 ml	15 ml
Dexamethasone	62	30	0	0
Cortisol	54	28	0	0
Prednisolone	69	36	0	0
Deoxymethasone	57	25	0	0
Betamethasone	64	56	0	0
Flumethasone	61	28	0	0

nd = none detected

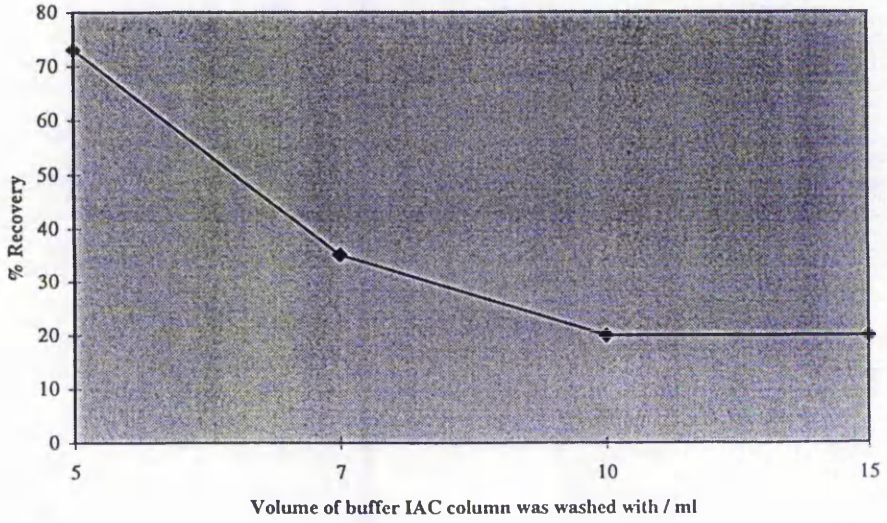

Figure 3.5. % recovery of deoxymethasone after different volumes of loading buffer was used to flush the IAC column.

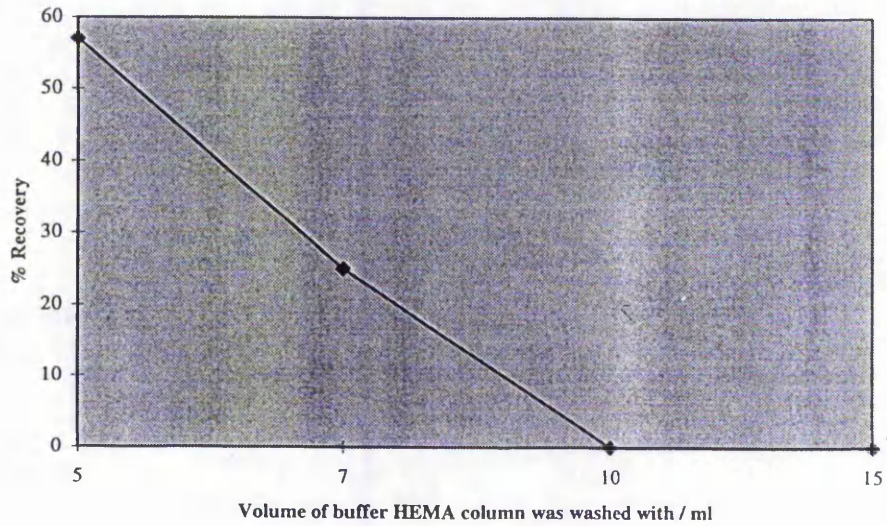

Figure 3.6. % recovery of deoxymethasone after different volumes of loading buffer was used to flush the HEMA column.

Table 3.3. Cross-reactivities for selected corticosteroids using IAC-HPLC.

Corticosteroid	Relative cross-reactivity / %	
	IAC-HPLC	ELISA
Dexamethasone	100	100
Flumethasone	96	96
Betamethasone	30	37
Deoxymethasone	27	21
Cortisol	0	1
Prednisolone	0	4

Antiserum batch: AD60

immunosorbent assay (ELISA) at the Horseracing Forensic Laboratories (Table 3.3.) [32]. The observed cross-reactivities suggested that the anti-dexamethasone IAC column would probably be suitable for the

quantitative determination of dexamethasone and flumethasone, and may also be useful to screen for deoxymethasone and betamethasone. There was no significant binding of cortisol and prednisolone. These two corticosteroids were therefore omitted from any future investigations.

3.3.2. IAC-HPLC-Particle Beam/Mass Spectrometry

The IAC-HPLC pre-treatment, using a switching loop to transfer the steroid containing fraction eluted from the IAC column to the HPLC column, was coupled *via* a particle beam interface to the quadrupole ion trap mass spectrometer (PB/QITMS).

The EI mass spectrum of dexamethasone obtained using the HPLC-PB interface (Figure 3.7.) produced a prominent fragment ion at m/z 312 which was selected for monitoring dexamethasone.

A problem with using the particle beam was that a high proportion of the analyte was lost during passage through the interface and this meant that the conditions for the particle beam analysis needed to be carefully tuned to achieve the levels of detection required for biological assay. To detect the corticosteroid dexamethasone by QITMS, a filtered noise field with a notch at 32.75-34.50 kHz was applied to the end caps to eject

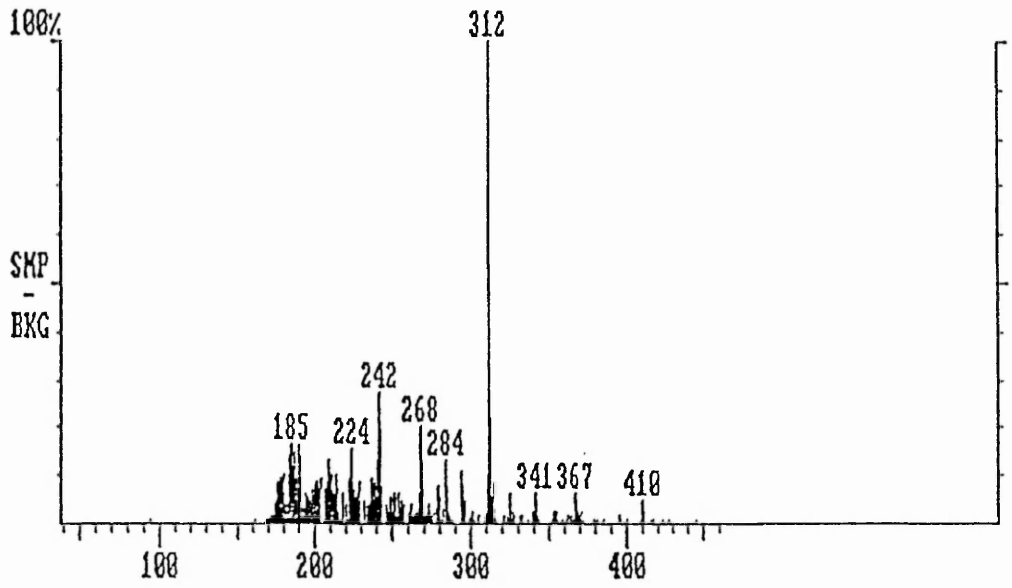

Figure 3.7. The EI mass spectrum of dexamethasone.

solvent and matrix ions from the ion trap during ionization. A long ionization time was used to allow a population of m/z 312 ions to accumulate in the trap. The ejection of solvent ions and the accumulation of a population of selected ions helped to increase the sensitivity of the detector.

Optimum transport of the corticosteroid through the PB interface was found to be achieved with a high percentage of methanol and the addition of a carrier, ammonium acetate, to the mobile phase. With a concentration of 0.2 M ammonium acetate buffer in the 80 % methanol/water mobile phase, 100 ng of dexamethasone could be detected by PB-QITMS with a >3:1 signal to noise ratio.

Dexamethasone standards spiked in equine urine in the range of 100-500 ng were analysed by IAC-HPLC-PB/QITMS. A linear response curve was obtained, with a correlation coefficient of 0.993. Selected ion monitoring (SIM) of the m/z 312 ion and the chromatographic retention time were used to confirm the presence of dexamethasone. The IAC-HPLC-PB/QITMS system had a limit of detection calculated at 3 $\mu g\ l^{-1}$ (signal : noise = 2:1) and sequential replicate injections of a spiked urine sample (10 $\mu g\ ml^{-1}$) showed a relative standard derivation of 7.4 %.

The relative standard derivation for a 10 μg l^{-1} sample injected over a period of seven days was 8.0 % (n=5). Figure 3.8. shows the chromatogram from a sample collected 1.5 hours after a single intra-muscular injection (20 μg kg^{-1}) of a Dextran preparation of dexamethasone to a horse. The concentration of dexamethasone in this sample was determined as 9 ng ml^{-1}.

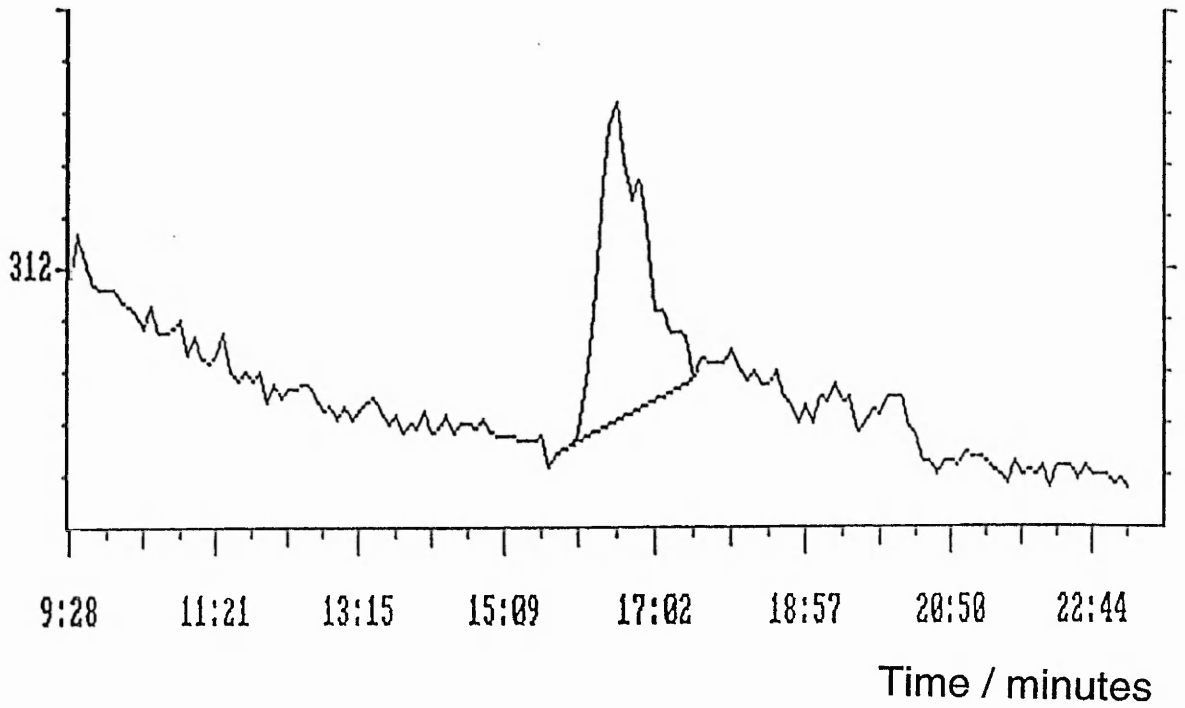

Figure 3.8. On-line IAC-HPLC-PB/QITMS determination of dexamethasone in a sample collected 1.5 hours after a single intra-muscular injection of a Dextran preparation.

Flumethasone gave a characteristic ion at m/z 350, as can be seen in Figure 3.9. and this ion was selected for monitoring the corticosteroids by IAC-HPLC-PB/QITMS so that their could be no confusion with dexamethasone. Flumethasone was determined using a notch set at 30.50 kHz - 28.50 kHz to trap the m/z 350 ions as the analyte eluted from the HPLC column. The instrumental conditions were otherwise the same as those for the dexamethasone determination. A series of flumethasone spiked standards were run in the range 100 - 500 ng, giving a linear response curve (correlation coefficient 0.989).

Selected ion monitoring (SIM) of the m/z 350 ion and the chromatographic retention time were used to confirm the presence of flumethasone. The IAC-HPLC-PB/QITMS system had a limit of detection calculated at 4 $\mu g\ ml^{-1}$ (signal : noise = 2:1). Replicate injections of a equine urine spiked sample (15 $\mu g\ l^{-1}$) showed a relative standard derivation of 6.9 %. This increased slightly to 7.1 % (n=5) when calculated for flumethasone samples injected over seven days. Figure 3.10. shows the chromatogram from a sample collected 1.85 hours after a single intra-muscular injection (4.8 $\mu g\ kg^{-1}$) of a Flucort preparation of flumethasone to a thoroughbred horse. The concentration of flumethasone in this sample was determined as 10 $\mu g\ l^{-1}$.

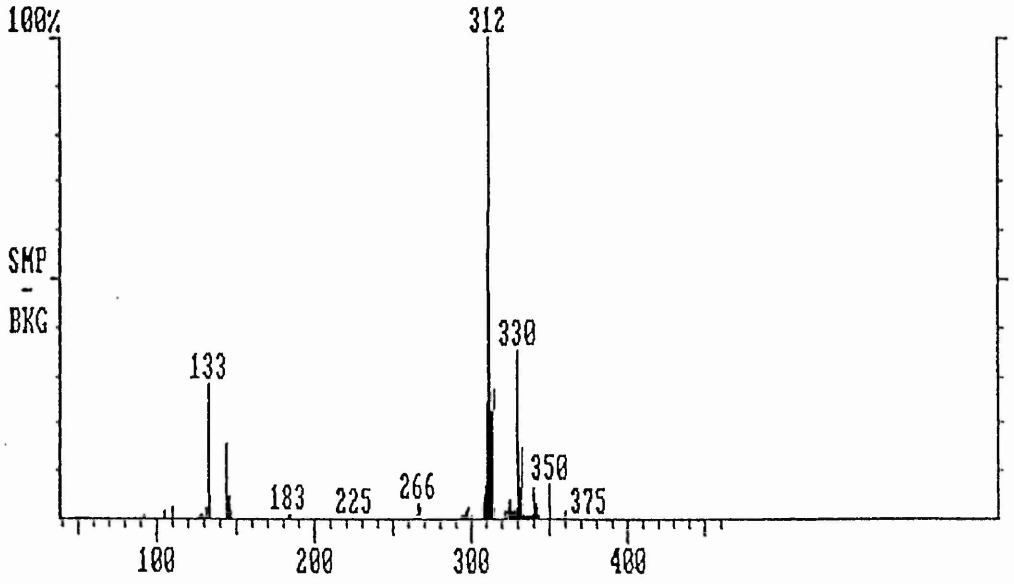

Figure 3.9. Mass spectrum of flumethasone analysed by PB/QITMS.

for an injection of a Flucort preparation (see experimental for chromatographic conditions).

Electron ionisation produced significant fragmentation of the corticosteroids under investigation and this, combined with the poor transmission of analyte through the particle beam interface, resulted in limits of detection of 3-4 $\mu g\ l^{-1}$ using selected ion monitoring, although full scan data required for confirmatory analysis may be acquired with little loss in sensitivity because of the ion storage capabilities of the ion trap. This compares with a detection limit of 0.1 $\mu g\ l^{-1}$ achieved in the work performed with IAC-HPLC-APCI-MS [Chapter 2]. The detection limits that would be required for analysis of corticosteroids at lower levels could be achieved by increased sample size, since the high selectivity of IAC allows large sample volumes to be handled without loss of analytical performance, as has been demonstrated by Stanley *et al* [19].

The on-line combination of IAC-HPLC-MS eliminated the need for the lengthy and complex extraction and derivatisation procedures which are necessary when analytes are determined using IAC followed by off-line derivatisation and GC-MS. The methodology is also simpler in terms of sample manipulation, with less manual intervention, and therefore there are fewer stages where analyte losses or contamination may occur.

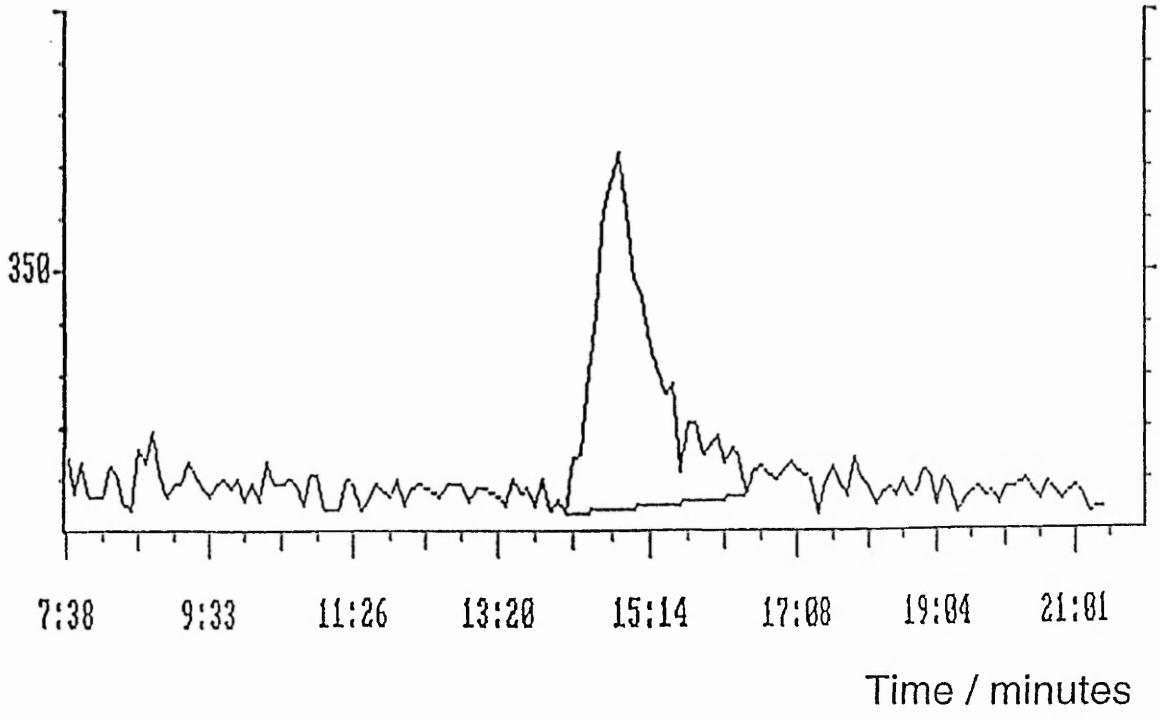

Figure 3.10. On-line IAC-HPLC-PB/QITMS determination of flumethasone in a sample collected 1.85 hours after a single intra muscular injection of a Flucort preparation.

The rigid HEMA polymer support allowed faster sample loading and elution of the IAC column, reducing analysis times compared to the soft gel supported IAC approach (Chapter 2). The packing of the HEMA stationary phase in conventional HPLC stainless steel columns allowed the use of IAC extraction with much more convenience, than IAC extractions performed with Pharmacia Sepharose columns.

3.4. CONCLUSION

On-line IAC-HPLC-PB/QITMS has been applied to the analysis of early post administration equine urine samples and without significant prior sample pre-treatment. HEMA (poly-hydroxyethyl methacrylate) was evaluated as a support material and proved to be more robust and convenient to use than the soft gel support, Sepharose. Automation of the method would be straightforward so giving a routine confirmatory analysis technique for flumethasone and dexamethasone in the equine.

Antibody cross reactivity and non-specific binding have been investigated for the HEMA anti-dexamethasone IAC column. The on-line IAC-HPLC-MS determination of dexamethasone and flumethasone in post administration urine samples showed high precision (6.9-7.4 %) with limits of detection in the range 3-4 $\mu g\ l^{-1}$.

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CHAPTER 4

THE DEVELOPMENT OF MULTIDIMENSIONAL IAC-HPLC AND IAC-HPLC-GC CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF TESTOSTERONE

4.1. INTRODUCTION

Different types of analysis have their own inherent problems. With the analysis of drug substances and their determination in biological matrices, the problems tend to be associated with the complexity of the matrix and the fact that the compounds may only be present at trace levels. The point that these compounds will frequently require derivatisation for GC analysis, or may be basic in nature and easily adsorbed onto active sites just adds to these. This is why multidimensional approaches, such as IAC-HPLC-GC [1-2], which can detect specific drugs at trace levels in complex matrices and reduce sample handling problems for the analyst, make sense.

4.1.1. Testosterone

Testosterone is a naturally occurring hormone in mammals and is the principal endogenous androgenic-anabolic steroid in humans and equines. An American doctor was first to observe that Soviet weightlifters were utilising testosterone as a performance enhancer [3], subjecting themselves to the rather painful side effect of requiring catheterisation [3], to provide a sample. The International Olympic Committee from then on

monitored the situation, finally banning testosterone use by sports people in 1983.

Anabolic steroids, such as testosterone, have various effects on the human/equine body with the results being generally more noticeable in the female, adolescent or castrated male, as the endogenous hormones are only naturally present in small amounts in these groups. The effects of such anabolic steroids have been summarised in the list below:

a) Increase red blood cell production,
b) Increase in appetite and body weight,
c) Stimulate growth of bones,
d) Stimulate growth of muscles,
e) Psychological effects.

The effects linked to performance enhancement has meant that, in human athletics, testosterone is the drug most commonly reported in steroid abuse, while in equine sports it's use is restricted.

4.1.2. Testosterone in the equine

Testosterone, in veterinary medicine, is used as an anabolic steroid for therapeutic purposes and as an androgen in the treatment of deficient libido in males and in suppression of oestrus in females. It is metabolised in the horse to testosterone sulphate and glucuronic acid and sulphate conjugates of the reduced metabolites [4].

There have been two preliminary approaches to the establishment of threshold values for monitoring abuse of testosterone in the equine. One approach is to determine the testosterone (T) to prasterone (P) ratio, T/P, for geldings and fillies [5]. The other is to determine the testosterone to epitestosterone (E) ratio [6]. Prasterone (also known as dehydroepiandrosterone) is produced by the adrenal gland and is a precursor rather than a metabolite of testosterone. It is therefore a suitable candidate as an endogenous reference steroid. *In vivo* experiments have not identified epitestosterone as a metabolite of testosterone and thus it too is a suitable reference steroid for the ratio approach.

The question of what detection approach should be adopted is still however a complicated one. The normal male horse produces large

amounts of testosterone, therefore the ratio approach is the only option. Castration removes the primary source of the steroid, but using immunoassay methods, low levels of testosterone have been reported in the gelding, probably generated by an adrenal source [7]. As there is no gonadal contribution to the circulating levels of testosterone in geldings though, quantification of endogenous levels of this steroid excreted in urine and establishment of an absolute concentration threshold, may present an alternative approach for the detection of testosterone abuse.

In the mare the situation is much more complex. Testosterone, epitestosterone and prasterone have all been detected at low levels. However, the concentrations of these compounds may be affected by physiological or pathological conditions (oestrous, pregnancy, ovarian/adrenal tumors). Therefore a ratio approach would seem sensible, since it may compensate for any fluctuation of testosterone concentrations due to physiological or pathological status.

Recent reports have demonstrated methods which may solve these difficult problems [8, 9] of determining the doping of testosterone in the equine, but there is still scope for trying new approaches and adding to them, approaches which have already been used for studying humans [10].

4.1.3. Approaches for detecting Testosterone doping in the equine

The presence of testosterone of exogenous origin in post-race urine samples constitutes a breach of the Rules of Horseracing. Current analytical determinations are dependent upon identifying the analyte and its metabolites in equine urine, supported by monitoring the testosterone to dehydroepiandrosterone ratio [5] (as stated above). This latter supportive data is necessary to distinguish between administered testosterone and the endogenous steroid [11].

The doped steroid however, may be determined by combustion isotope ratio mass spectrometry (C/IRMS). This technique involves the pre-spectrometric combustion of the carbon skeleton of the steroid to produce $^{12}CO_2$ and $^{13}CO_2$. The ratio of the carbon dioxide isotopes is then determined. If there is a measurable difference between doped (Synthetic) testosterone and endogenous testosterone, produced by the horse, then a positive result may be achieved. This is done by comparing the C/IRMS result for testosterone in the sample, with the C/IRMS result for a related compound (which will give a reference to what the $^{12}CO_2$ to $^{13}CO_2$ ratio should be for endogenous steroids), which is not a metabolite of the anabolic steroid.

C/IRMS studies for steroid measurements have been carried out on human urine samples [10, 12]. The reference compound used being epitestosterone. This method entailed lengthy pre-treatment of samples, with the steroids under investigation being derivatised for efficient gas chromatography, which effected the CO_2 isotope ratios determined. An improvement on this method, may be achieved by utilising a multi-dimensional chromatographic approach. This would reduce the pre-treatment time for samples and achieve the chromatographic resolution of the steroids required and eliminate the need for derivatisation.

There have been numerous publications describing the application of HPLC-GC for drug analysis [13-22] and some detailing the use of immunoaffinity chromatography (IAC)-HPLC coupled to GC [1, 2]. These have highlighted the potential of such a multidimensional approach for the confirmation of trace levels of compounds in complex biological matrices. Such an approach would be ideal for C/IRMS, however even the development of an IAC-HPLC enrichment method for samples before GC-C/IRMS would be a step forward. The investigation of multidimensional methods which could detect and confirm the presence of testosterone and resolve it from epitestosterone is also of interest in it's own right. Since such a technique could be used for detection and determination of testosterone in geldings. While separations of

underivatised anabolic steroids is also an area of great potential, with a number of methods described for determination of testosterone doping in the literature.

4.1.4. Contents of Chapter

The Chapter describes the initial development of an on-line IAC-HPLC clean-up for C/IRMS analysis. Also detailed is an automated on-line IAC-HPLC-APCI-MS technique developed and demonstrated for confirming the presence of testosterone. The IAC-HPLC-APCI-MS method utilising a PGC guard column as a novel IAC-HPLC interface. This work and the separations of anabolic steroids facilitated by porous graphitic carbon stationary phases are outlined. With the Chapter concluding with a description of work undertaken in developing an on-line IAC-HPLC-GC-QITMS method for testosterone.

4.2. EXPERIMENTAL

4.2.1 Materials

Testosterone, epitestosterone, nandrolone, epinandrolone, oestradiol, epioestradiol, dehydroepiandrosterone (DHA), sodium hydrogen orthophosphate, sodium dihydrogen orthophosphate, sodium azide and sodium acetate were purchased from the Aldrich Chemical Co. Ltd. (Dorset, UK). ^{14}C labeled testosterone was obtained from Amersham International (Amersham, Buckinghamshire, U.K.). Propionic acid, dichloromethane, ethyl acetate and methanol (Distol grade) were obtained from Fisons (Loughborough, UK). Water was obtained from a MilliQ system (Millipore, Bedford, MA, USA). All eluents were filtered through 0.45 μm filters (Millipore, Bedford, MA, USA). HEMA 1000VS-L was purchased from Presearch Limited (Herts, UK). Rabbit serum containing anti-testosterone antibody was provided by the Horseracing Forensic Laboratory (Newmarket, UK).

4.2.2 Standard and sample preparation

Stock solutions of the compounds analysed (e.g. testosterone) were prepared in methanol at a concentration of 1 mg ml^{-1}. Further dilutions

were prepared in methanol to give concentrations in the range of 0.2-500 ng ml^{-1} for standard analysis' and spiking experiments. Stripped urines (20 ml) [23] were spiked with drug and were then adjusted to pH 7.0 and centrifuged at 1500 g for ten minutes prior to analysis. The supernatant was removed and aliquots were introduced into the IAC multidimensional chromatographic systems.

4.2.3. Anti-testosterone IAC column

The crude rabbit serum samples were purified using a Protein G stationary phase (bed height 4 cm) packed in a C10 column (Pharmacia, Uppsala, Sweden). The appropriate fractions were collected and pooled. The antibodies were bound to HEMA and the HEMA was blocked using the method described by the manufacturer [24]. The HEMA was first swollen with water and then washed with 0.1 M NaOH/0.5 M NaCl, pH 8.3 coupling buffer. The anti-testosterone IgG solution (5 ml) was added to the HEMA (1 g) and coupling buffer (5 ml) and the resulting slurry was mixed overnight. The gel was washed and blocked with 0.1 M Tris base (pH 9.0). The HEMA anti-testosterone stationary phase was packed into a stainless steel HPLC column (50 mm x 4.6 mm). The IAC column was stored at 4°C in 30 mM phosphate buffer containing 0.5 M NaCl + 0.2 % sodium azide when not in use.

4.2.4. Instrumentation

4.2.4.1. HPLC

A six-port injection valve (7010 Rheodyne), fitted with a 20 µl or a 5 ml sample loop, was connected to a HPLC gradient system (Milton Roy) which delivered 50:50 DCM/methanol mobile phase to the porous graphitic carbon (PGC) (Hypercarb, Shandon Scientific) HPLC column (150 mm x 4.6 mm i.d. 7 µm particle size) at 1 ml min^{-1}. The flow from this column was monitored using UV detection (Perkin Elmer LC-85) at λ = 240 nm.

4.2.4.2. GC-MS

A Hewlett Packard 5971 GC-MSD was used to investigate the separation of the underivatised anabolic steroids. An on-column injection of the compounds was made onto a BP-17 (30 m x 0.25 mm, 0.25 µm film thickness) column. Programmed as follows, 80 °C (hold for 1 min) to 230 °C at 10 °C min^{-1} then to 260 °C (hold for 5 mins) Spectra were acquired over the range m/z 50 to m/z 350.

4.2.4.3. IAC-HPLC sample pre-treatment for GC-C/IRMS

The IAC-HPLC-UV instrumentation (Figure 4.1.) consisted of a Waters module 6000A HPLC pump (Bedford, MA, USA) (pump 1) which delivered mobile phase to the IAC column *via* a six-port injection valve (Rheodyne 7010) containing a 5 ml stainless steel injection loop. The mobile phase flowed from the IAC column to V1, a six port switching valve (Rheodyne 7010) containing a 5 ml stainless steel switching loop. Valve V1 was connected to two Waters 501 HPLC pumps (Bedford, MA, USA) (pumps 2 and 3). which delivered DCM/methanol mobile phase to the PGC column. The flow from this column was monitored using UV detection (Perkin Elmer LC-85) at λ = 240 nm. Appropriate fractions were collected, blown down under nitrogen and reconstituted in 150 µl of ethyl acetate.

C/IRMS analysis on these samples was carried out by Dr. Paul Mason in the Department of Geological Science at the Open University. Sample aliquots (50 µl) were injected using an Optic 2 injector which was connected to the GC (Varian). GC analysis was carried out on a DPX-5 column (25 m x 0.32 mm, film thickness 0.5 µm). Programmed as follows, 60 °C to 300 °C at 10 °C min^{-1}. The effluent was passed into a

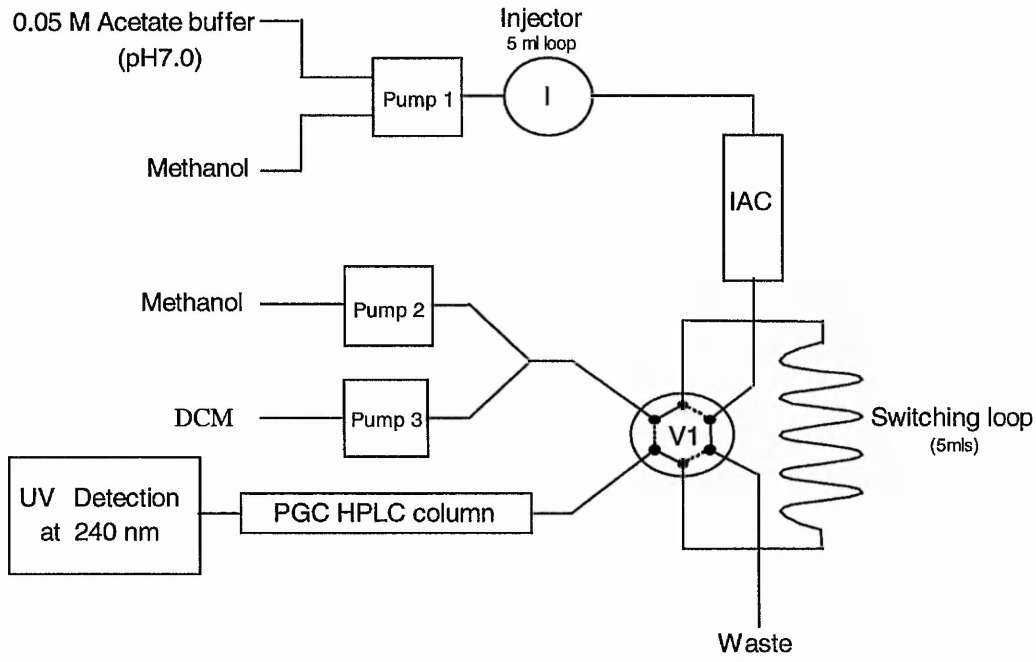


Figure 4.1. On-line IAC-HPLC configuration for C/IRMS sample pre-treatment.

Finnigan Mat Delta S combustion isotope ratio mass spectrometer with a source pressure of 1 x 10^{-5}.

Cryotrapping was performed using a 2 m length of 1/8 inch copper tubing connected at one end to a nitrogen cylinder, *via* a needle valve, and to a 'T'-piece (50 mm x 30 mm) located in the Varian GC oven at the other. Nitrogen gas flowed through the tubing to the 'T' piece through which a section of the GC column was threaded in the Varian GC oven. When Cryotrapping was to be performed, a coil of the copper tubing was immersed in a dewar of liquid nitrogen.

4.2.4.4. IAC-HPLC-UV

The IAC-HPLC-UV instrumentation (Figure 4.2) differed from the instrumentation described in section 4.2.4.3. in that the switching loop was replaced by a PGC guard column (10 x 4 mm) and an analytical PGC column was also connected to V1. The analytical separation was carried out on a 150 mm x 4.6 mm i.d. column packed with 7 μm PGC (Hypercarb, Shandon Scientific), and the effluent was transferred to a Waters Model 441 UV absorbance detector (λ = 240 nm).

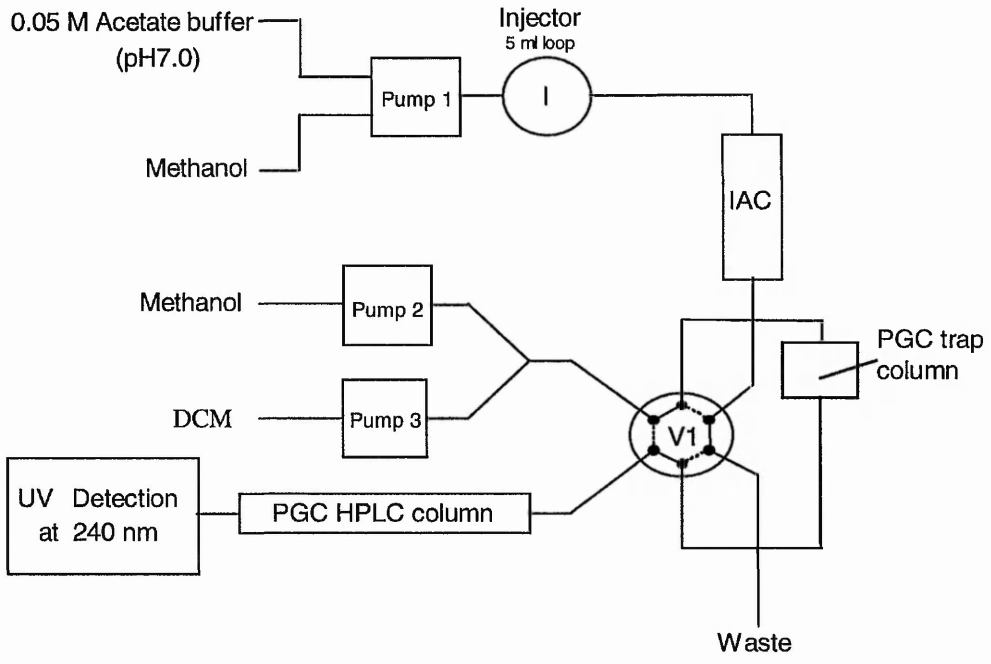


Figure 4.2. Schematic diagram of IAC-HPLC-UV system for testosterone determinations.

4.2.4.4.a. Chromatographic procedures IAC-HPLC-UV

The injected sample (5 ml) was delivered to the IAC column in acetate buffer (0.05M, pH 7.0) mobile phase at a flow rate of 1 ml min^{-1}. The IAC column was flushed with loading mobile phase, with the switching valve (V1) set to the load position, then the IAC column was eluted with methanol at 1 ml min^{-1}. At 15 minutes V1 was switched to the inject position and the recorder was started. The contents of the trapping column were then eluted and a methanol/DCM gradient, going form 0 % DCM to 100 % DCM over 10 minutes, which then pumped 100 % DCM for 7 mins, at 1 ml min^{-1}, with the suitable flowing on to the analytical PGC column for IAC-HPLC-UV analysis.

4.2.4.5. IAC-HPLC-MS

The IAC-HPLC-MS instrumentation (Figure 4.3.) consisted of a Waters module 6000A HPLC pump (Bedford, MA, USA) (pump 1) which delivered mobile phase to the IAC column *via* a six-port injection valve of a Gilson 231XL Sampling Injector (Anachem, Luton, Bedfordshire, U.K.). The injector of the Gilson instrument introduced the sample (5 ml) onto the IAC column, which was positioned across the injection valve.

The IAC column was then washed with 20 mls of loading mobile phase, using the Gilson, and the injection valve was then switch on-line with pump 1, which eluted the steroid fraction in methanol. The mobile phase transferred the eluted components from the IAC column to V1, a six port switching valve (Rheodyne 7010), fitted with a porous graphitic carbon (PGC) (Hypercarb, Shandon Scientific) HPLC column (10 mm x 4 mm i.d. 7 μm particle size) where the steroid was trapped. The valve was then switched to elute the fraction from the trapping column onto the (PGC) analytical column (150 mm x 4.6 mm i.d.) (Hypercarb, Shandon Scientific). A Hewlett Packard 1050 LC delivery system (pump 2), delivered a methanol/DCM gradient, going form 0 % DCM to 100 % DCM over 10 minutes, which then pumped 100 % DCM for 7 mins, to the column which was interfaced to a quadrupole mass spectrometer (VG Platform, Altringham, Cheshire) fitted with an atmospheric pressure chemical ionisation (APCI) interface. Testosterone was determined by selected ion monitoring of m/z 289 ion with a dwell time of 0.40 seconds.

4.2.4.6. IAC-HPLC-GC-MS

The IAC-HPLC-GC instrumentation (Figure 4.4) consisted of a Waters 6000A HPLC pump, which delivered both the loading (20 mM phosphate

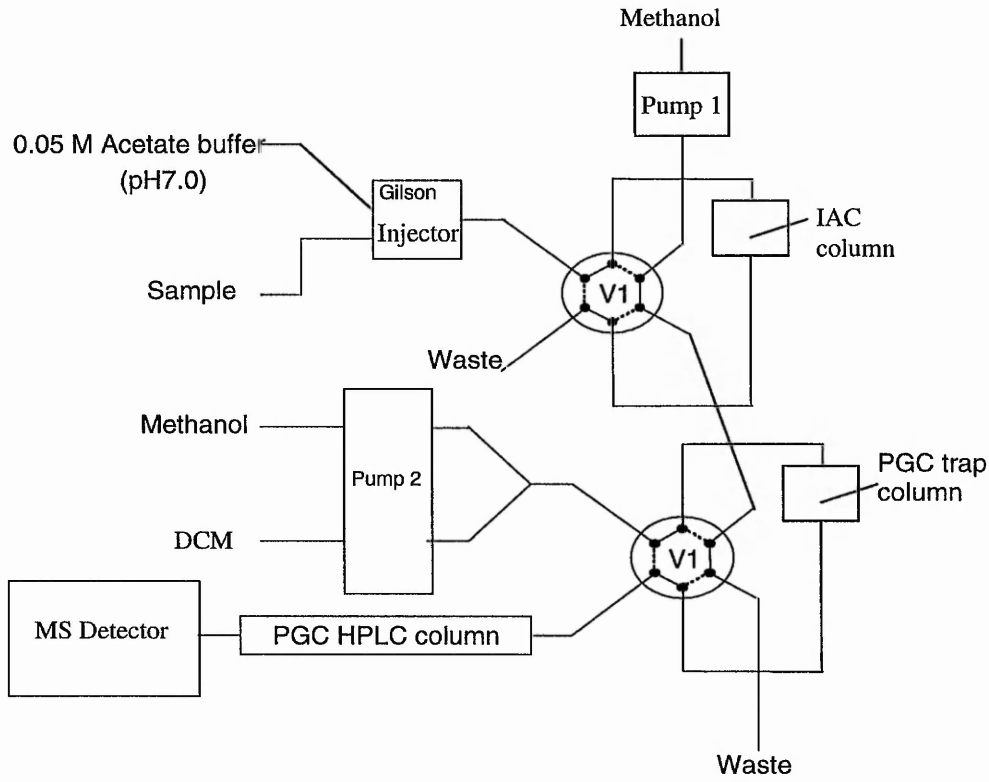


Figure 4.3. IAC-HPLC-MS instrumentation for testosterone determinations.

buffer + 0.5 M NaCl at pH 7.0) and elution (methanol) mobile phases to the IAC column at 1 ml min^{-1}. This pump was connected to the IAC column *via* a six port valve (7010 Rheodyne). A peristaltic pump (Watson-Marlow 501) was also coupled to a six port valve which pumped the sample onto the IAC column at 3 ml min^{-1}. Mobile phase flowed from the IAC column to a second six port valve (7010 Rheodyne), which enabled the band of interest to be transferred to the HPLC column. This valve was connected to a HPLC gradient pumping system (Milton Roy), which delivered DCM/methanol mobile phase to the porous graphitic carbon (PGC) HPLC pre-column (10 mm x 4 mm i.d. 7 mm particle size) at 300 µl min^{-1}. The flow from this column was monitored using UV detection (Perkin Elmer LC-85) at λ = 240 nm. Suitable fractions were transferred to the GC (Carlo Erba Fractovap 4160) by an on-column interface, incorporating a 10 m x 0.53 mm i.d. uncoated pre-column. The interface was constructed by adapting a commercially available GC-GC switching system (PCSS, SGE Ltd., UK). The pre-column was connected to a double 'T', which incorporated the early vapour exit and an additional carrier gas supply. Carrier gas to the pre-column was introduced *via* a mass flow valve down stream of a pressure regulator. A liquid CO_2-cooled 'T' piece, containing a short length of coated column (15 cm x 0.53 mm i.d. BP1 0.1 mm film thickness) was positioned at the head of the analytical column (30 m x 0.25 mm DB-17 0.25 mm film

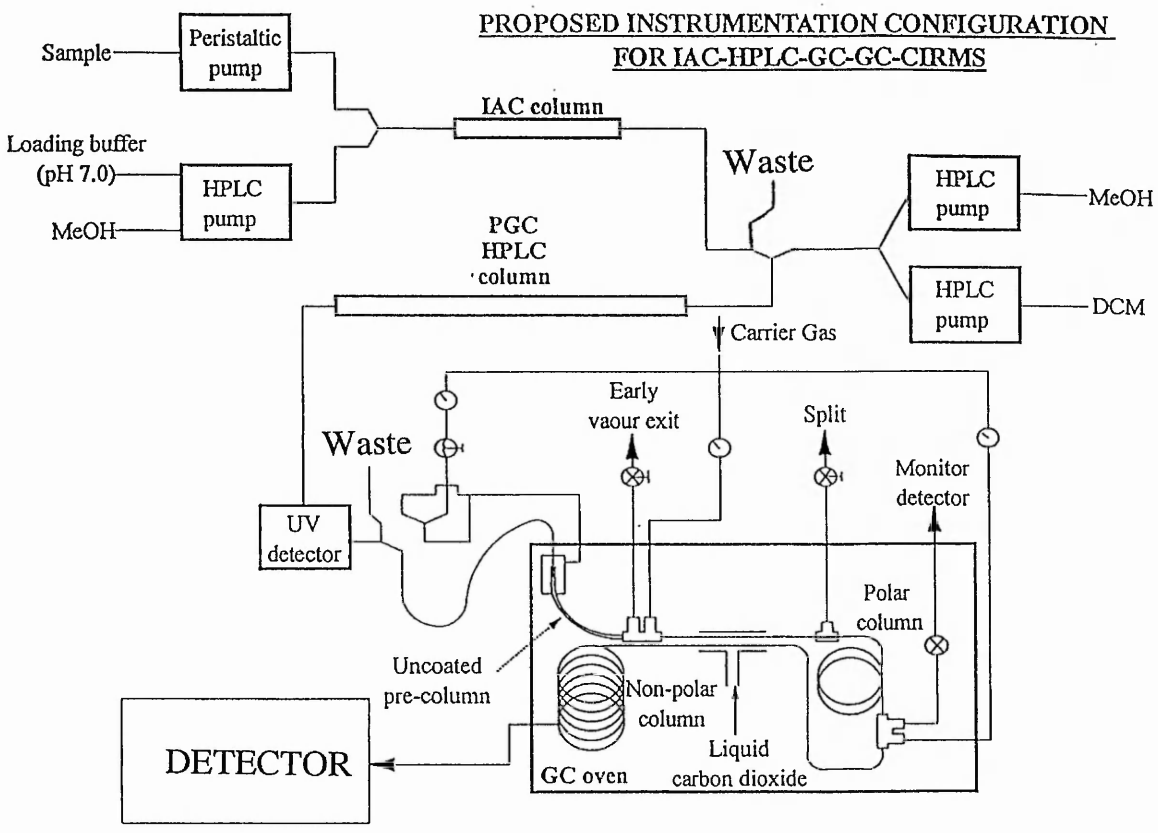


Figure 4.4. IAC-HPLC-GC instrumentation for testosterone determination.

thickness) for cryogenic focusing of the analyte(s) after elution from the pre-column. When transferring LC fractions to the LC-GC interface the GC oven temperature was 80 °C. On diversion the early vapour exit was open. 2 minutes after diversion the GC oven was ramped upto 230 °C at 10 °C min^{-1} and then up to 260 °C at 5 °C min^{-1}. The early vapour exit was closed and the cryotrapping begun. After 5 minutes the split was closed, the early vapour exit opened and the cold trap turned off. So allowing transfer of the testosterone to the GC analytical column. The GC effluent was monitored by FID detection, or was coupled to a quadrupole ion trap mass spectrometer (Finnigan MAT ITMS, San Jose, Ca). The QITMS was operated in electron ionization (EI) mode and a filtered noise field (Teledyne Scientific Instruments, Sunny Vale, CA) with a notch at 35.50-36.25 kHz was used to accumulate analyte ions mass selectively during the ionization period (50 ms).

4.3. RESULTS AND DISCUSSION

4.3.1. Evaluation of IAC column.

IAC sample clean-up for the endogenous steroids in equine urine samples was achieved by anti-testosterone antibodies bound to a HEMA support in a 4.6 mm i.d. HPLC column. The immunoaffinity chromatography

utilised the discriminating nature of the antibodies, which provided tremendous selectivity and allowed the antigen-conjugate that the antibodies have been raised against to be selectively enriched from the complex biological matrix of horse urine, so eliminating the need for further pre-treatment of the sample. The HEMA support proved to be a robust and easy to use support material for on-line IAC-HPLC, with the higher back pressures and flow rates possible through the IAC column in the multi-dimensional system allowing more rapid sample throughput. As with the corticosteroids described in the previous chapter, testosterone was eluted from the IAC column with 1 M propionic acid in methanol/water. The HEMA supported antibodies were again packed into a typical 4.6 mm diameter HPLC column which was convenient and easy to use. Experiments with ^{14}C labeled testosterone established that the analyte eluted from the IAC column in a 3 ml band.

Elution of testosterone from the IAC column with methanol was also evaluated, since this mobile phase would be more compatible with normal phase HPLC, which in turn is better suited than reverse phase HPLC for introduction into an LC-GC interface. An experiment with ^{14}C labeled testosterone establishing that the analyte eluted from the IAC column in a 4 ml band.

4.3.2. HPLC of Testosterone and Epitestosterone

The HPLC separation was the crucial intermediate step in the multi-dimensional chromatographic approach. It had to be compatible with the reverse phase IAC stage and with interfacing to GC. The steroid fraction eluted from the IAC column must be focused into a narrow band at the head of the HPLC column [25-29], preferably with no intermediate step or dilution. It also must be easy to couple with GC. Where the use of highly volatile solvents in the mobile phase, such as those used in normal phase chromatography would be preferred. A separation of epitestosterone and testosterone must also be achieved, since these two compounds are difficult to resolve by GC without prior derivatisation.

A mixture of testosterone and epitestosterone was used to investigate a number of HPLC columns. An initial separation on an ODS column was achieved quite simply with a methanol/water mobile phase, as shown in Figure 4.5.. The reverse phase mobile phase would however be unsuitable for an LC-GC procedure and therefore a number of normal phase separations were investigated. Silica and amino stationary phases were both unable to separate the steroid. Then a PGC stationary phase was tried.

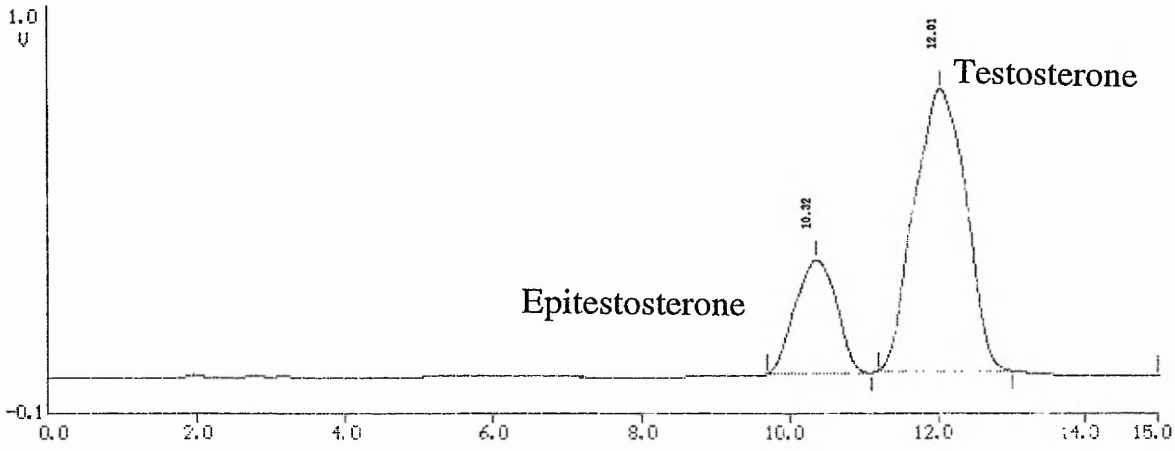


Figure 4.5. Reverse phase separation of testosterone from epitestosterone on an ODS column, eluting with 50 % methanol in water at 1ml/min, UV detection at 240 nm.

This was considered a good approach due, to the PGC stationary phase's ability to resolve diasterioisomers. The column also having the quality of being able to function in both reverse- and normal-phase modes of liquid chromatography. A 50 % methanol/dichloromethane mobile phase flowing at 1 ml min^{-1} resolved epitestosterone from testosterone the optimized separation being shown in Figure 4.6..

With the separation of the steroids achieved on a PGC column, attention was focused on the coupling of the IAC reverse phase step (eluting with methanol) with the normal phase PGC column. Experiments were carried out to determine how the steroid fraction eluted from the IAC column could be focused for the PGC separation.

Solutions of testosterone in methanol (5 mls) were injected on to the PGC column and eluted with methanol/DCM under the conditions described in Section 4.2.4.1.. The chromatogram pictured in Figure 4.7. was obtained. These experiments established that even when introduced in a large volume of methanol the steroids were focused at the head of the PGC column, and were eluted by the DCM/Methanol mobile phase without loss of chromatographic resolution. This meant that testosterone could be eluted from the IAC column in methanol and focused on the

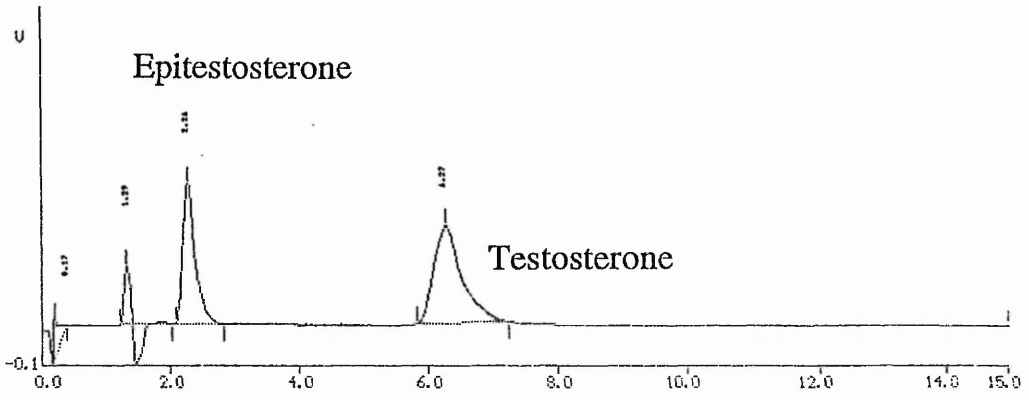


Figure 4.6. Normal phase separation of testosterone from epitestosterone on a PGC column.

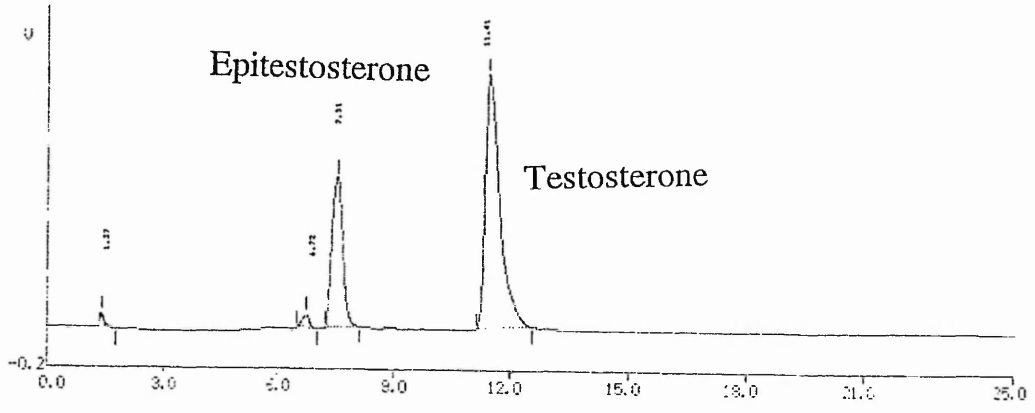


Figure 4.7. Normal phase separation of testosterone from epitestosterone on a PGC column after a 5 ml injection of the steroids in methanol.

PGC column with no need for any intermediate dilution step, something which is commonly employed in other IAC-HPLC methods (see Chapter 2).

4.3.3. GC Separations of Steroids

Steroids are normally derivatised prior to GC analysis and in such cases testosterone is easily separated from it's epimer on a non-polar column. However this would complicate any on-line procedure and would make C/IRMS results difficult to interpret. Therefore direct analysis of the underivatised steroids was investigated on varying GC columns. A number of columns were investigated, HP1, CP sil 88, DB-5, DB-17, and all seemed to give similar resolution of the steroid mix.

Of the columns investigated the semi-polar (50 % methyl-, 50 % phenylsiloxane) DB-17 column gave the optimum resolution of the steroids, as shown in Figure 4.8. However, this column did not separate testosterone from epitestosterone. This meant that the prior HPLC separation of these compounds on a PGC column would be essential for a successful multidimensional procedure.

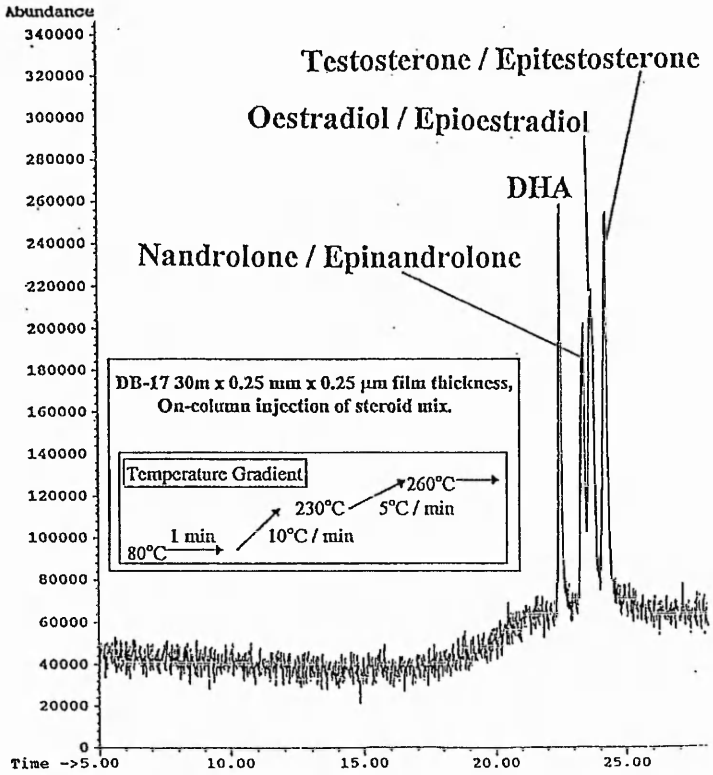
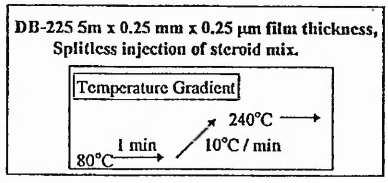


Figure 4.8. GC separation of steroid mix run on DB-17 column.



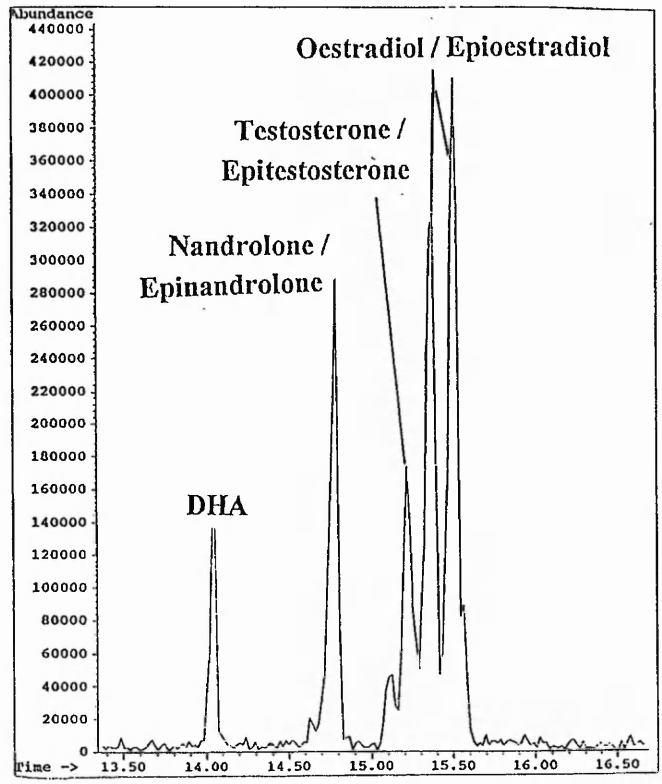


Figure 4.9. GC separation of steroid mix run on DB-225 column.

Towards the end of this study a brief investigation into the use of short GC columns was undertaken. With the aim of possibly developing a GC-GC separation. The column used for this was the polar DB-225 column. The use of short column GC for the analysis of underivatised steroids has been investigated [30]. However at the time it was felt that for C/IRMS analysis a low bleed column should be used. The separation achieved on the DB-225 column though was good (Figure 4.9.).

4.3.4 IAC-HPLC sample pre-treatment for GC-C/IRMS

In preliminary studies, IAC and HPLC were coupled together to provide off-line clean-up for GC-C/IRMS analysis. The conventional GC-C/IRMS system did not have the sufficient sensitivity for the determination of testosterone without prior concentration and gave poor peak shape of components after the sample passed through the combustion chamber. The objective of this preliminary work was therefore to determine whether IAC could provide the sample clean-up and analyte enrichment required to achieve meaningful results on the GC-C/IRMS system.

A cryotrapping system was employed to enhance the analyte response. This approach was unsuccessful at first, since trapping was attempted on

a section of the transfer line (uncoated column) connecting the GC column to the detector. However, when trapping was attempted on the end section of the analytical column a vast improvement in peak shape and sensitivity was observed (Figure 4.10).

A number of urine samples from a horse dosed with human chorionic gonadtrophin (HCG) (to increase natural production of testosterone and so raise levels of the steroid in the biological fluids of the animal) had been provided by the Horseracing Forensic Laboratory (HFL). The testosterone which was in it's conjugated form in the equine urine was converted to the free base at the HFL [23]. As well as these, some equine urine samples were spiked with synthetic testosterone at high levels (μg ml^{-1} levels). These were then subjected to on-line IAC-HPLC and the fractions collected were then analysed by off-line C/IRMS. The IAC-HPLC isolated samples gave some interesting results which are shown in Table 4.1.. δ values (the ratio of ^{13}C to ^{12}C) generated by the C/IRMS instrument seemed to show that engogenous equine testosterone (generated by dosing HCG) samples extracted by IAC-HPLC gave lower results than synthetic testosterone which was spiked in equine urine and then extracted. This suggests that the carbon isotope ratio make up of the synthetic testosterone and that of the testosterone generated by the equine were different, so boding well for future work. The actual figures

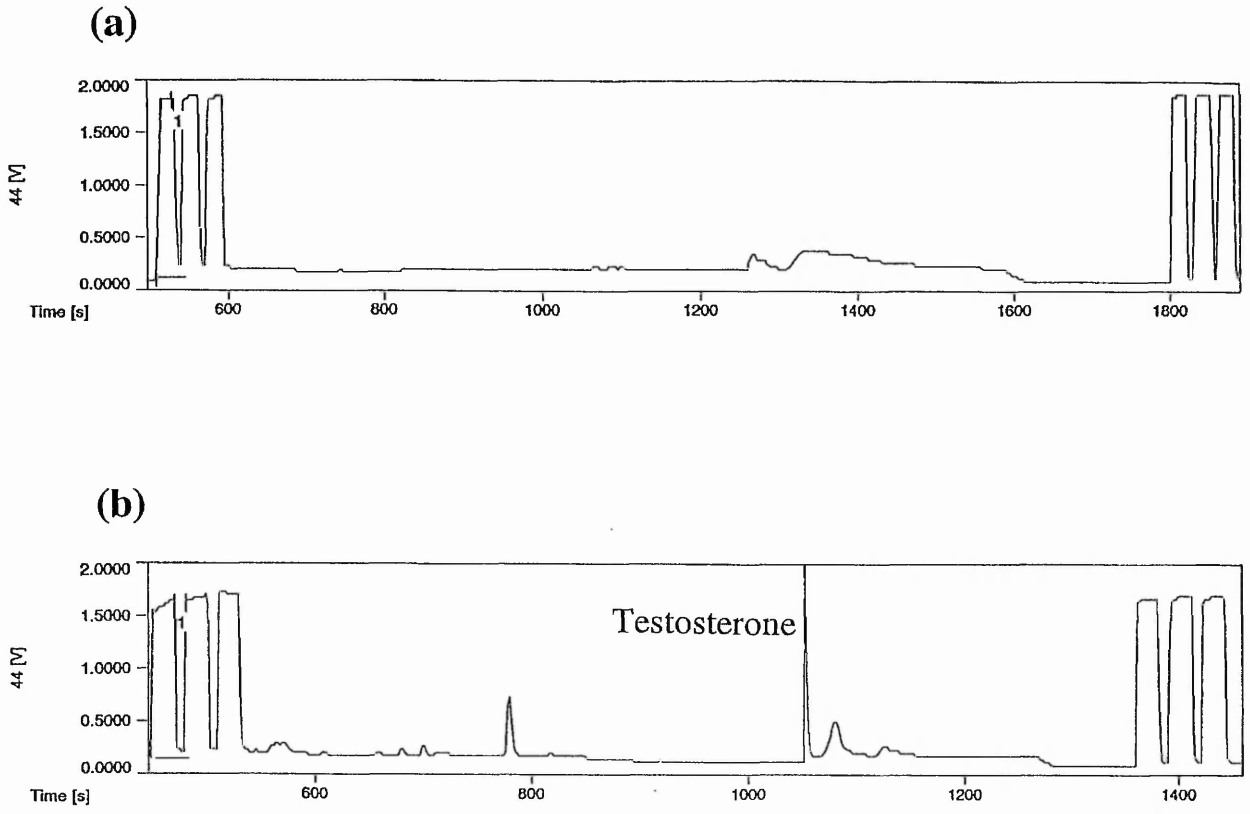


Figure 4.10. Chromatograms of testosterone standards analysed by GC-C/IRMS (a) without cryotrapping (b) with cryotrapping.

generated though had limited meaning as there were not enough data points and there were to many uncertainties in the process.

The main achievement of this work was the demonstration of the compatibility of the off-line clean-up with GC-C/IRMS. The IAC-PGC system helped to selectively enrich the sample so that data could be obtained on the GC-C/IRMS with cryotrapping. The work was inconclusive in determining if C/IRMS could be utilized as a technique for determining doping of testosterone, but a potential approach to future work had been highlighted.

Table 4.1. Results generated by GC-C/IRMS system.

Sample	**δ value determinations**
	1 **2**
Synthetic Testosterone spiked in equine urine	-29.43, -29.30
Endogenous Testosterone (induced by HGC)	-29.86, -29.74

4.3.5. IAC-HPLC-APCI-MS

The novel method of coupling IAC to a HPLC PGC column, facilitated by the PGC trapping column, with no requirement for a dilution stage, initiated investigations into the development of on-line IAC-HPLC-MS as an analytical method for detecting testosterone. The normal phase

separation would give more sensitivity when passing through an APCI interface and this method, if it proved effective for testosterone, could be adapted for other steroids.

Early studies of IAC sample clean-up using an anti-testosterone anti-body column, coupled directly to an analytical column *via* a PGC trapping column interface were carried out using UV detection. The testosterone was eluted from the IAC column in methanol and was focused on the trapping column. This was then switched on-line with the HPLC gradient system. The focused band of steroid being chromatographed with a methanol/DCM gradient, going form 0 % DCM to 100 % DCM over 10 minutes. This procedure allowed the detection of testosterone spiked in stripped urine at a level of 20 ng ml^{-1} as shown in Figure 4.11.. The IAC-HPLC-UV system gave a recovery of 94 % for testosterone. Replicate injections of a spiked sample urine (20 μg l^{-1}) showed a coefficient of variation of 2.2 %.

The IAC-HPLC-UV system established the potential of the coupling of the anti-testosterone IAC column with the HPLC column *via* a PGC trapping interface. The combination of IAC-HPLC with mass spectrometry was then investigated using an atmospheric pressure

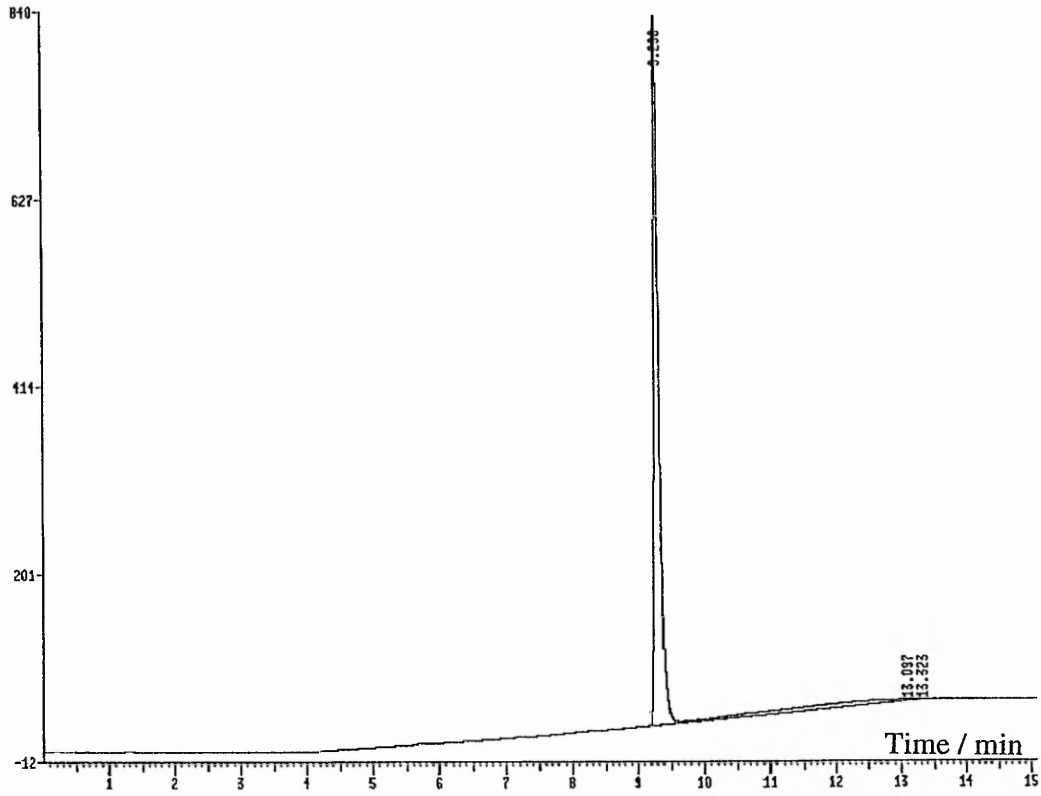


Figure 4.11. IAC-HPLC-UV chromatogram of testosterone spiked in equine urine at 20 ng ml^{-1}. Testosterone eluting at 9.29 mins.

chemical ionization (APCI) interface. The equipment used for this work although similar in function to that employed for the IAC-HPLC-UV system was altered so that the process could be conveniently automated and run overnight.

The spectrum of testosterone generated by APCI-MS is shown in Figure 4.12.. Under APCI conditions testosterone gave a protonated molecular ion, $[M + H]^+$ at m/z 289, which was selected for single ion monitoring.

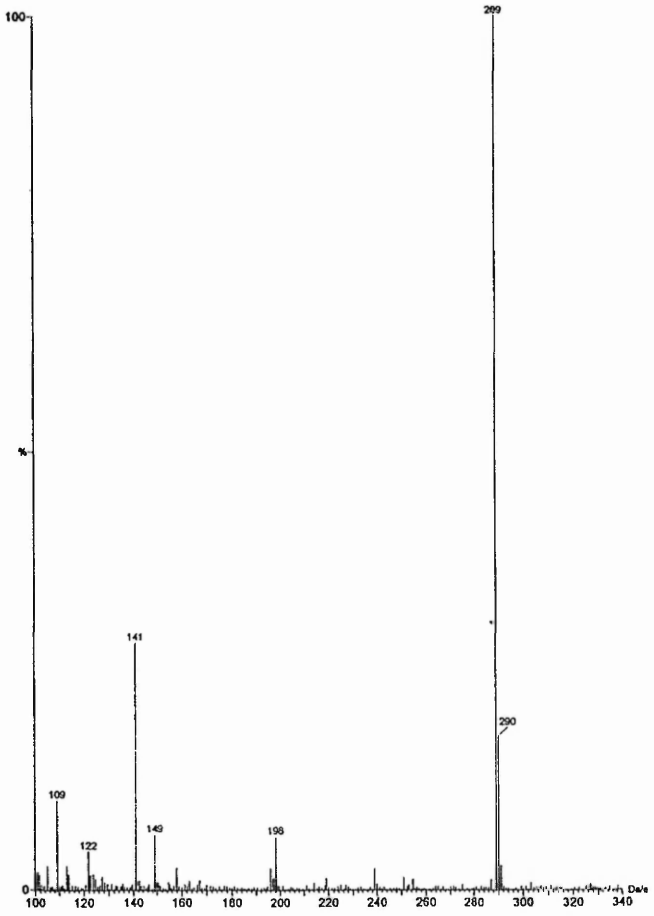


Figure 4.12. Spectrum of testosterone generated under APCI conditions.

Single ion monitoring (SIM) of the m/z 289 ion and the chromatographic retention time were used to confirm the presence of testosterone following IAC-HPLC clean-up. The IAC-HPLC-MS system gave a recovery of 92 % for testosterone and had a limit of detection calculated at 0.5 $\mu g\ l^{-1}$ (signal : noise = 3:1).

A good linear response, with a correlation coefficient of R = 0.996 was obtained for spiked urine samples in the range of 1-25 $\mu g\ l^{-1}$. Replicate injections of a spiked urine (5 $\mu g\ l^{-1}$) showed a coefficient of variation of 3.3 % for testosterone in stripped urine. Figure 4.13. displays the selected ion chromatogram (m/z 289) of a 6 $\mu g\ l^{-1}$ testosterone spiked in stripped equine urine.

The on-line combination of IAC-HPLC-MS eliminates the need for extraction and derivatisation procedures which are employed when using IAC followed by off-line GC-MS. This methodology is also simpler in comparison with other IAC-HPLC techniques in that there is no requirement for a dilution step during the transfer between IAC and HPLC.

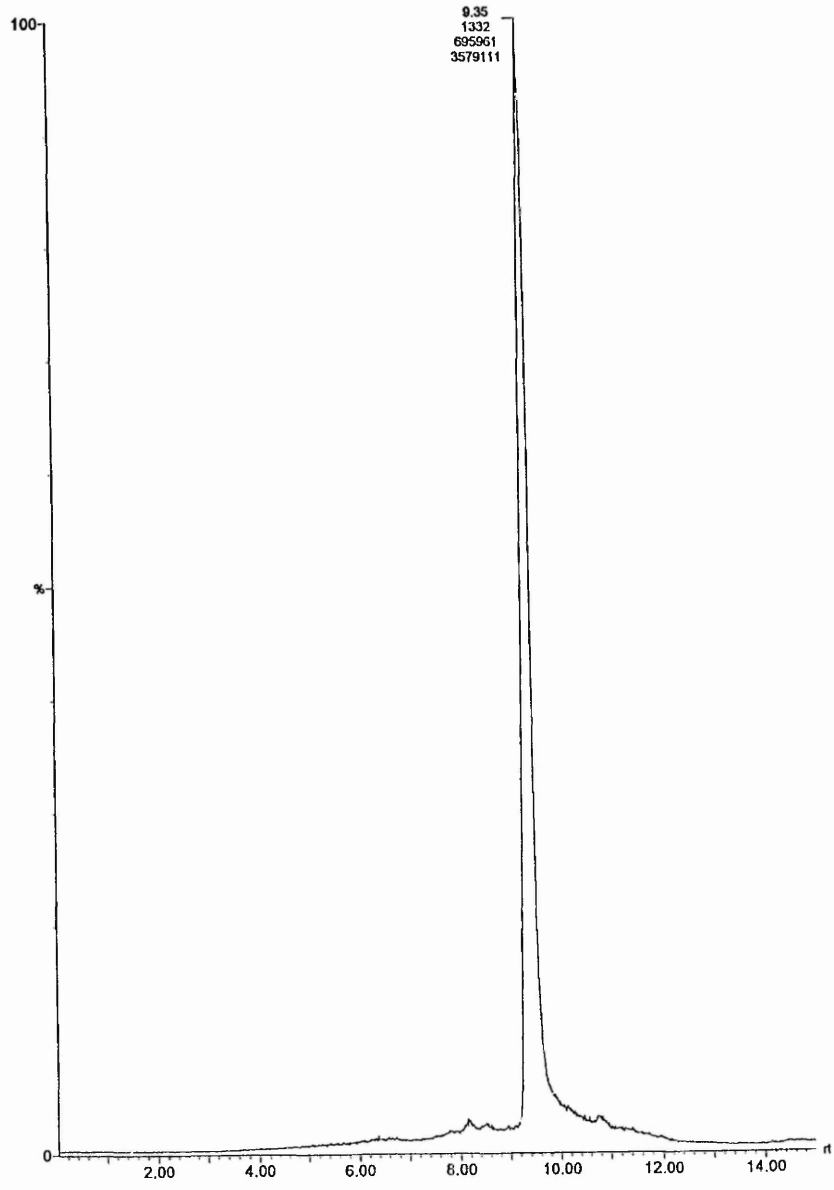


Figure 4.13. IAC-HPLC-APCI-MS Selected ion chromatogram (m/z 289) of a stripped urine sample spiked with 6 ng ml^{-1} of testosterone.

4.3.6. IAC-LC-GC-FID

The interface between the HPLC, PGC column and the GC was of the on-column type. Partially concurrent evaporation of the mobile phase being used to eliminate solvent and allow transfer of the analytes.

The early vapour exit (EVE) was opened during sample introduction from HPLC to GC, so permitting the venting of solvent vapour away from the analytical column, allowing the analyte to be condensed on the pre-column. Closure of the EVE after most of the solvent had evaporated, allowed the steroids remaining behind on the pre-column to be transferred to, and focused on, a the 15 cm length of coated column held within the cryogenically cooled 'T' piece cold trap. This concentrated analytes in a tight band at the head of the analytical GC column.

The splitter between the cryogenic cold trap and the head of the analytical column, enabled the flow rate through the pre-column (not compatible with the analytical column) to be maintained whilst the early vapour exit was closed. The analytes being retained on the cold trap even with the split open. Closure of the liquid CO_2 supply to the cold trap and the splitter, allowed the analysis to proceed on the analytical column.

The pressure at the mid-point double 'T' was set at 97 kPa, a pressure slightly exceeding the equilibrium pressure at the outlet end of the pre-column when the inlet pressure at the GC injector port was set at 106 kPa, with the split open. This helped to excluded the HPLC mobile phase from the analytical column. After transfer of analyte from the pre-column to the cryogenic cold trap, the early vapour exit was re-opened and the transfer line forward flushed with fresh HPLC mobile phase, whilst analysis proceeded on the GC column.

The supply of carrier gas to the injector and hence the uncoated pre-column was *via* a pressure regulator positioned upstream of a mass flow regulator. This set-up ensured that a positive flow of carrier gas was maintained through the pre-column during sample introduction despite the backpressure exerted by the evaporating mobile phase. In normal operation, when no HPLC fraction was being diverted to the GC, approximately 20 ml min^{-1} carrier gas was used to backflush the transfer line, whilst a flow of 8 ml min^{-1} was maintained through the pre-column. On transfer of the HPLC fraction to the GC, the entire flow from the mass flow valve passed through the pre-column to assist solvent evaporation.

The chromatograms obtained for 24 μg l^{-1} standard injections of testosterone made to the HPLC-GC system with cryogenic focusing, and

using FID detection, are shown in Figure 4.14. In this case 750 µl volumes of HPLC effluent (between 21-23.5 minutes after injection) were diverted to the GC.

Cryogenic focusing provided a convenient method to obtain a good chromatographic peak shape for testosterone, and such an approach, in general, ensures that GC resolution is not sacrificed by the process of transferring the steroids to the analytical column. Since most of the solvent is diverted away from the analytical column, cryogenic focusing of any solvent along with the analytes is a minimal problem.

The IAC-HPLC-GC-FID system gave a recovery of 90 % for testosterone and had a limit of detection calculated at 2.5 µg l^{-1} (signal : noise = 3:1). A good linear response, with a correlation coefficient of R = 0.991 was obtained for spiked urine samples in the range of 4-50 µg l^{-1}. Replicate injections of a spiked sample urine (10 µg l^{-1}) showed a coefficient of variation of 5.6 %.

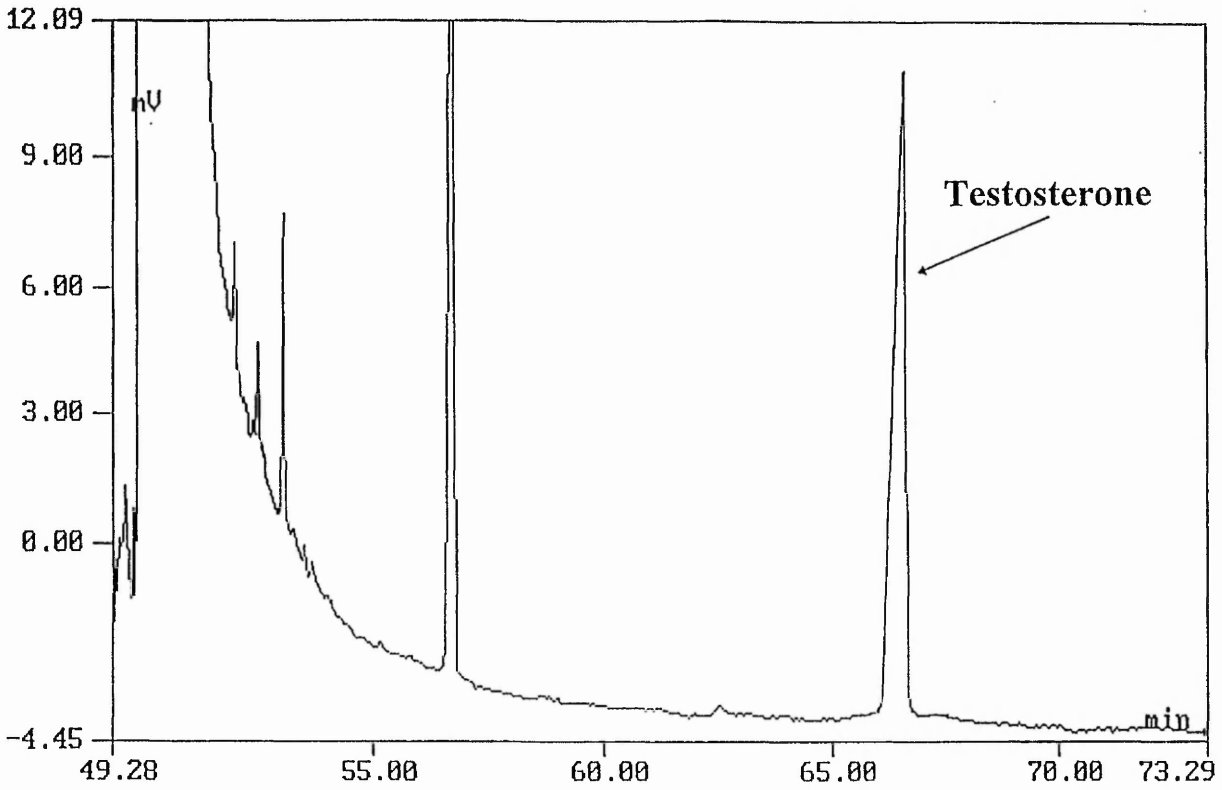


Figure 4.14. IAC-HPLC-GC-FID chromatogram of testosterone spiked at 24 μg l^{-1} in stripped equine urine.

4.3.7. IAC-HPLC-GC-QITMS

In drug analysis, as with other areas of trace analysis, it is common to use mass spectrometry in a confirmatory role as it was in Chapters 2 and 3. This allows the presence of a drug to be established unambiguously, both qualitatively and quantitatively, following a preliminary identification made using a cheaper, less specific procedure.

EI is widely used in MS confirmatory studies since the resulting mass spectrum serves as a structural fingerprint for a given compound due to the extensive fragmentation observed. This ionisation technique is highly compatible with GC sample introduction and the development of IAC-HPLC-GC-MS is therefore of interest.

The IAC-HPLC pre-treatment, using a PGC trapping column to transfer the testosterone containing fraction eluted from the IAC column to the GC interface, was coupled to the quadrupole ion trap mass spectrometer (QITMS). The EI mass spectrum of testosterone from a HPLC-GC interface, shown in Figure 4.15, produced a prominent molecular ion at m/z 288. To improve detection of testosterone by QITMS, a filtered noise field with a notch at 35.50-36.25 kHz was applied to the end caps to eject matrix ions from the ion trap during ionization. A long ionization time

was used to allow a population of m/z 288 ions to accumulate in the trap. The ejection of other ions and the accumulation of a population of selected ions helped to increase the sensitivity of the detector.

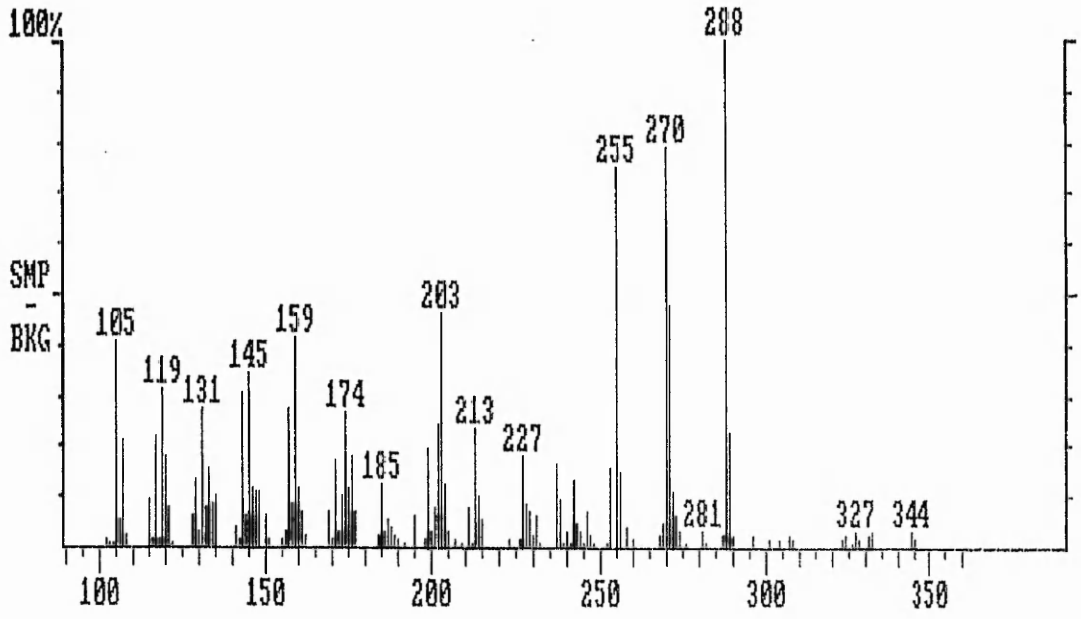


Figure 4.15. The EI mass spectrum of testosterone.

Testosterone standards spiked in stripped equine urine in the range of 10-100 ng were analysed by IAC-HPLC-GC-QITMS. A linear response curve was obtained, with a correlation coefficient of 0.986.

Selected ion monitoring (SIM) of the m/z 288 ion and the chromatographic retention time were used to confirm the presence of testosterone. The IAC-HPLC-GC-QITMS system had a limit of detection

calculated at 1 μg l^{-1} (signal : noise = 2:1) and sequential replicate injections of a spiked urine sample (10 μg l^{-1}) showed a relative standard derivation of 8.4 %, a typical chromatogram of one such injection is shown in Figure 4.16.

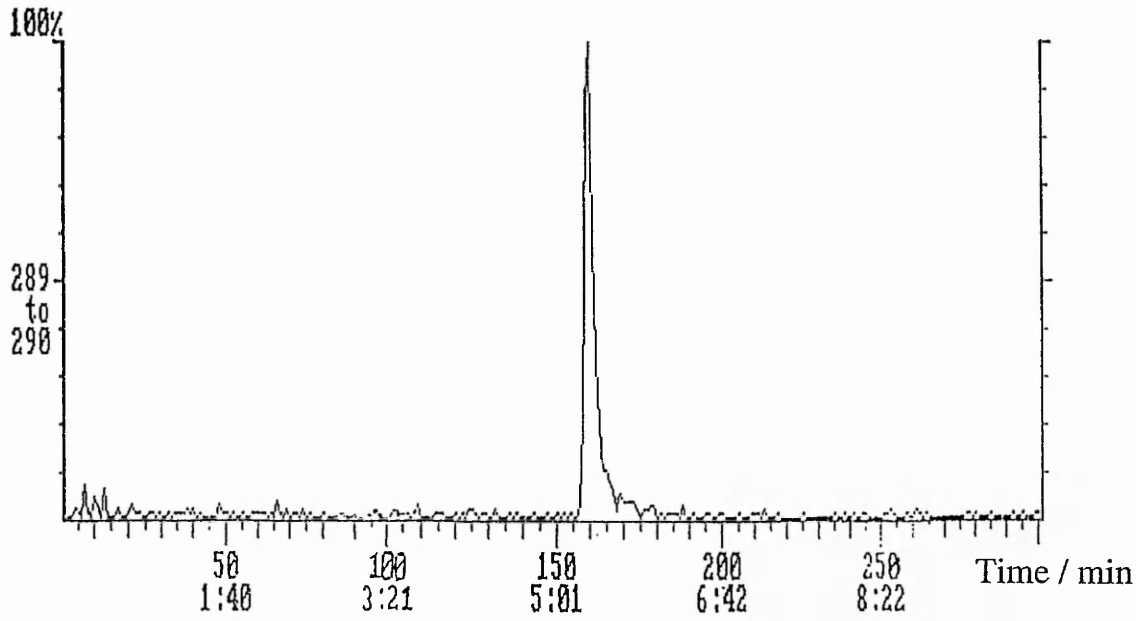


Figure 4.16. Selected ion chromatogram (m/z 288) of 10 μg l^{-1} of testosterone spiked in stripped urine analysed by IAC-HPLC-GC-ITMS.

4.4. CONCLUSION

An effective and novel IAC-HPLC clean-up method has been developed using a PGC trapping column. The PGC stationary phase has been shown to be an effective stationary phase for the separation of testosterone and epitestosterone. Unlike other IAC multidimensional approaches the system does not require a dilution step in transfer of fractions from the IAC column to the next stage, a tremendous advantage.

The on-line coupling of IAC-HPLC with both LC-MS, and GC-MS has been demonstrated. These techniques proving effective for steroid determination. The IAC-HPLC clean-up was also demonstrated as an off-line pre-treatment for GC-C/IRMS for testosterone in equine urine. This provided a good starting point for the development of a method to confirm the presence of testosterone doping in racehorses using combustion isotope ratio mass spectrometric measurements.

4.5. REFERENCES

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PUBLICATIONS

1. On-line immunoaffinity chromatography-high-performance liquid chromatography-mass spectrometry for the determination of dexamethasone. Colin S. Creaser, Stephen J. Feely, Edward Houghton, Mark Seymour and Philip Teale, Analytical Communications, 1996, **33**, 5-8.
2. Immunoaffinity as a sample clean-up step for detecting drugs at low levels in equine body fluids. M. A. Seymour, J. Byard, S. J. Feely, H. Marsh, K. Reeve, P. Teale, C. S. Creaser and E. Houghton, Methodological Surveys in bioanalysis of drugs. editor E. Reid, RSC Publications, 1996, 215-218.
3. Determination of Corticosteroids by Immunoaffinity Chromatography Combined On-line with HPLC-MS. Colin S Creaser, Stephen J Feely Edward Houghton, Mark Seymour and Phillip Teale, J. Chromatogr., (in press).

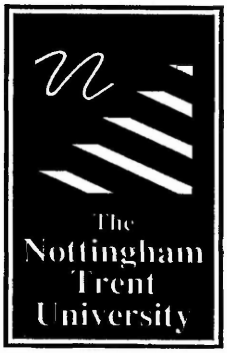
CONFERENCE PRESENTATIONS

1. On-line immunoaffinity chromatography-HPLC for the trace determination of dexamethasone, Colin S. Creaser and Stephen J. Feely, presented at The Royal Society of Chemistry Analytical

Division Research & Development topics Meeting, University of Hertfordshire, July 1994. (Poster)

2. Combined immunoaffinity chromatography-high performance liquid chromatography-mass spectrometry for the detection of dexamethasone in post administration equine urine samples, Colin S. Creaser, Stephen J Feely, Edward Houghton, Mark Seymour and Philip Teale, presented at the International Symposium of Analytical Chemistry, University of Hull, July 1995. (Poster)
3. Developments in the determination of corticosteroids using combined immunoaffinity chromatography-high performance liquid chromatography-mass spectrometry, Colin S. Creaser, Stephen J Feely, James Stygall, Edward Houghton, Mark Seymour and Philip Teale, presented at the 21st annual meeting of the British Mass Spectrometry Society, (UMIST) Manchester, September 1995. (Poster)
4. Advances in multi-dimensional IAC-HPLC for the analysis of corticosteroids, Stephen J. Feely, C. S. Creaser, E. Houghton and M. Seymour presented at The Royal Society of Chemistry Analytical Division Research & Developments topics Meeting, Nottingham Trent University, July 1995. (Oral)
5. Multi-dimensional chromatography combined with mass spectrometry for the determination of testosterone, Colin S. Creaser, Stephen J Feely, Edward Houghton and Mark Seymour, presented at the 22nd

annual meeting of the British Mass Spectrometry Society, University of Swansea, September 1995. (Poster)



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