Chapter 3
General Methods for the Extraction, Purification, and Measurement of Steroids by Chromatography and Mass Spectrometry

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3.1 Introduction

Steroids consist of an essentially lipophilic (or hydrophobic, non-polar) cyclopentanoperhydrophenanthrene nucleus modified on the periphery of the nucleus or on the side chain by the addition of hydrophilic (or lipophobic, polar) groups. Although steroids are widely distributed in nature and many thousands have been synthesised in the laboratories of pharmaceutical and chemical organisations, this chapter concentrates primarily on the methodology for the analysis of steroids of biological importance to human subjects and in particular on the methods for the analysis of the very low concentrations of steroids found in human biological tissues or formed during *in vitro* or *in vivo* studies. This does not, however, imply that the techniques discussed here may not find applicability in other areas of steroid analysis. This chapter neither discusses specifically the saturation analysis techniques including immunoassay-radioimmunoassay (RIA), enzymeimmunoassay (EIA), which are explained in Chapter 4, nor the analysis of cardenolides, sapogenins, alkaloids, brassinosteroids or ecdysteroids, which present their own analytical challenges but

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are of less interest in a clinical context. Further details on basic principles of mass spectrometry (MS) are discussed in Chapter 2.

During metabolism, steroids generally become more hydrophilic by reduction, further hydroxylation and esterification (conjugation) with glucuronic or sulphuric acid. Bile acids (containing a C24 carboxylic acid group) may be linked through a peptide bond to glycine or taurine. Despite the addition of these polar groups, the essential non-polarity (hydrophobicity) of the steroids means that they are all, to varying degrees, soluble in organic solvents and can thus be extracted from aqueous media by a solvent or solvent mixture of suitable polarity.

In vivo secreted steroid hormones are carried in the bloodstream bound to plasma proteins – specifically to binding globulins, which have high affinity but low capacity (e.g. cortisol binding globulin, sex hormone binding globulin, vitamin D binding globulin, etc.) or non-specifically to albumin, which has low affinity but high capacity. Some steroids (e.g. cholesterol) may be incorporated as an integral part of the structure of the plasma lipoproteins, and these lipoproteins may form part of the structure of cells and tissues from which steroids may need to be extracted.

Steroid hormones exert their *in vivo* effects by binding to specific receptor proteins, which although found in the cytoplasm of responsive cells, may be derived from nuclear receptors that bind avidly to the nuclear chromatin when the active steroid is bound but not when the active steroid is absent. It is well established that sex steroid hormones bind to nuclear receptors, which then act as transcription factors to control biochemical processes. Mounting evidence now indicates that steroids may also influence physiological events more rapidly via non-genomic mechanisms (e.g. Balthazart et al., 2006 and reviewed by Losel et al., 2003; Wehling and Losel, 2006). Direct and indirect action of steroids in mitochondria are new areas of research with relevance to oxidative phosphorylation (Gavrilova-Jordan and Price, 2007; Haider, 2007).

3.1.1 Analysis of Steroids

The presence in tissues and biological fluids of binding proteins and their interaction with the steroid of interest are important considerations when deciding how best to develop a method for the analysis of a particular steroid. The initial question to consider is whether it is possible to assay the steroid directly in the medium without prior treatment; and today, this would appear to be a major objective in the development of tests for routine use in clinical laboratories for diagnostic purposes since such procedures are simple and avoid the need for extraction. Immunoassay tests are often conducted with serum or plasma processed without any extraction or purification of the steroids. Tandem mass spectrometry looks attractive for the analysis of steroids, often with little pretreatment of the sample, but the cost of such apparatus at the present time and the expertise needed may mitigate against its routine use, although leasing arrangements and simplification of operation may

overcome these difficulties. However, when reaching a decision whether to use a direct assay, the specificity of the final quantitation and the requirements of the assay must be taken into consideration. A simple non-extraction assay may provide in many circumstances a semi-quantitative estimate of the concentration of the analyte, which may be of considerable value to clinical colleagues. Although this result may not be accurate, so long as it is precise and reproducible, it may still have value. There have been many reviews of methods of steroid analysis, too many to list here, but readers may find two (Grant and Beastall, 1983; Shackleton et al., 1990b) useful as an introduction to early literature in this area. There are a number of reviews of steroid extraction and separation published since 1999, which may be of use (Wolthers and Kraan, 1999; Shimada et al., 2001; Marwah et al., 2001; Nozaki, 2001; Volin, 2001; Appelblad and Irgum, 2002; Sjövall, 2004; Pujos et al., 2005; Setchell and Heubi, 2006); while some of these reviews restrict their coverage to specific steroids or groups of steroids, they are still useful as the methodology described is almost certainly applicable with modification to other steroids. The relevance of steroid hormone assay in the clinical laboratory has also been reviewed (Holst et al., 2004).

Any analytical procedure has three (or four) major steps:

1. Extraction of the analyte from the matrix.
2. Pre-purification.
3. Quantitation. In some cases, qualitative analysis is sufficient to meet the objectives of the analysis. The detection of synthetic anabolic steroid metabolites in urine of a sports person is thus often sufficient to define the abuse of anabolic steroids (Kicman and Gower, 2003). The list of prohibited steroids continues to grow (van Eenoo and Delbeke, 2006; Borges et al., 2007), and new 'designer' steroids are being found, creating new analytical challenges to doping control laboratories (Nielen et al. (2006)). This topic is discussed in greater detail in Chapter 9.
4. Quality assurance (QA) – both internal and, where possible, external–particularly in laboratories where large numbers of steroid assays are carried out. Excellent external quality assurance for steroid assays is provided to hospital clinical biochemistry laboratories in the UK, and this scheme is discussed in detail in Chapter 13 (see also discussion of a similar scheme for vitamins D metabolites in Chapter 11). It cannot be emphasised too often that good quality assurance is essential and that the maintenance of high standards of output from an analytical laboratory requires scrupulous attention to all quality control safeguards, which should include full information about the patient from whom the sample was taken so that unexpected or unusual results can be more easily detected (Jones and Honour, 2006).

Although blood, urine, and saliva have been the biological fluids most often examined for the presence of steroids, there is increasing interest in steroids in brain (so-called neurosteroids). In addition to the analysis of steroids in tissues and biological fluids, there is an interest in the presence of steroids in unexpected matrices, which are not specifically discussed here. These include

- Nutritional supplements (liquid and solid) that are widely used in sport but have given rise to positive tests in doping control (De Cock et al., 2001; Geyer et al., 2004; Parr et al., 2004; Tseng et al., 2005; Maughan, 2005; Martello et al., 2007; Parr et al., 2007). Methods for the measurement of anabolic steroids in the sports area are explained in more detail in Chapter 9.
- Human hair in which the concentrations of steroids along the hair fibres can be related to the time of exposure (Cirimile et al., 2000; Dumestre-Toulet et al., 2002; Wheeler, 2006; Gambelunghe et al., 2007; Rambaud et al., 2007).
- Water and sewage effluents for the presence of potential endocrine disruptors and as means of assessing contamination (e.g. Sumpter and Johnson, 2006; Szucs et al., 2006) or marine sediment analysis (Hajkova et al., 2007; Li et al., 2007).
- Animal tissues (e.g. Daeseleire et al., 1992) and feeds (e.g. Gonzalo-Lumbreras et al., 2007) for *inter alia* the presence of banned growth promoting steroids.

Clearly, the formulation of medications containing steroids is of considerable importance as is the need to demonstrate purity therein (Kotiyan and Vavia, 2000; Gorog, 2004, 2005), and this application of steroid analysis in the pharmaceutical area is discussed in Chapter 12. Noppe et al. (2008) have reviewed the measurement of steroid hormones in edible matrices.

All the analytical steps are interconnected, and it is obvious that the greater the specificity of the quantitation procedure, the less extraction or pre-purification necessary. High specificity quantitation procedures can thus be used with minimum pre-purification, and there are many examples of methods, which have been developed, that rely on this specificity. The specificity of liquid chromatography (LC) coupled to tandem mass spectrometry has allowed the assay of 12 steroid hormones in 200 μL of human serum, following a simple protein precipitation. On-line extraction/purification occurs prior to LC separation (Guo et al., 2006), and further specificity relies on multiple reaction monitoring (MRM). Such procedures may well provide valuable data, but it is very important to be fully aware of any short cuts, which may have been taken, and ensure that the method is only applied in situations for which it was developed. It is, however, not an uncommon practice that methods developed for one situation are applied uncritically to other situations in which they may not be valid.

3.1.2 Internal Standards

Purification prior to quantitation inevitably leads to loss of analyte, and the more extensive is the purification, the greater is the loss sustained. There must, therefore, be included in any quantitative method some means of assessing losses through the extraction and purification procedures. This is usually done by using an internal standard or radioactive (or stable isotope-labelled) recovery marker, which is added to the matrix at the start of the analysis. There are several requirements which must be considered in the selection of internal standards, which is of course constrained by the choice of the quantitation procedure to be used. The internal standard must,

after addition to the matrix, be distributed (e.g. bound to any protein, etc.) in the same way as the analyte. This is usually achieved by incubating for a period at 37°C, perhaps while gently shaking the fluid under analysis. A note of caution should be expressed here. Steroids are hydrophobic compounds and do not dissolve significantly in aqueous media and are thus added to the matrix dissolved in ethanol, methanol, or other polar solvent which is miscible with water. The volume of such a solvent should be as small as possible in comparison with the volume of fluid used for analysis to prevent denaturation of any protein. Sometimes, the steroid is added to a glass container, the solvent evaporated off and the fluid for analysis added. This practice is not to be recommended since steroids, when added in this way, can be adsorbed to the surface of the glass and may not subsequently be dissolved in the matrix, even when the glass has been inactivated by prior treatment with dimethyldichlorosilane. In addition, some steroids can be destroyed when evaporated to dryness on glass surfaces, particularly when the glass has previously been cleaned by treatment with chromic acid, a practice which used to be common but is now no longer so.

The internal standard chosen ideally must be indistinguishable from the steroid analyte during the process of extraction and purification but must be recognisable at the final quantitation stage. Internal standards can also fulfil a further function apart from their use as a means of assessing recovery. Steroids that are present in very low concentrations may become susceptible to irreversible loss by adsorption to glass surfaces, destruction by metal surfaces, and oxidation. The presence of a larger quantity of internal standard can prevent or minimise such losses. In such cases, the internal standard is also acting as a carrier. For gas chromatography–mass spectrometry (GC-MS) methods, the ideal internal standard is a deuterated or carbon-13 form of the analyte, and such standards should contain at least two but preferably three extra stable isotopes – this process leads to the so-called isotope dilution mass spectrometry (IDMS) procedure, and examples are given below. A number of publications describe methods for the synthesis of labelled steroids and bile acids with different isotopes (carbon and hydrogen) at positions around the steroids with up to eight hydrogens replaced with deuterium (Wudy, 1990; Zomer and Stavenuiter, 1990; Shoda et al., 1993; Furuta et al., 1999, 2003; Sulima et al., 2005; Kiuru and Wahala, 2006; Numazawa and Handa, 2006); however, addition of too many atoms of isotope can lead to chromatographic separation from the natural steroid. Insertion of deuterium into the steroid molecules is usually effected by acid-catalysed deuterium exchange and thus care must be taken during any extraction or pre-purification to avoid acid conditions, which can lead to loss of deuterium. Microwave-assisted synthesis of deuterium-labelled oestrogen fatty acid esters–a technique which may have other similar applications – has been reported by Kiuru and Wahala (2006). Isomers are also acceptable internal standards (e.g. 3β, 5α-tetrahydroaldosterone in the determination of 3α, 5β-tetrahydroaldosterone - Honour and Shackleton, 1977), but only if it is clear that the isomer is not found in the fluid under analysis. Chromatographic separation of analyte and internal standard may cause loss of the carrier effect, and the minimal separation of isotope-labelled standards has a positive advantage in this respect.

A chemical analogue of the analyte can also be used; for example, Δ^1-testosterone can be used in the measurement of testosterone by gas-liquid chromatography (GLC). Close chemical analogues used in this way must have physicochemical characteristics similar to the analyte, but must be distinguished from it before or during quantitation. Steroids labelled with deuterium are less readily available, but good sources to consider are Cambridge Isotopes (Cambridge Isotope Laboratories, 50 Frontage Road, Andover, MA 01810-5413), and recently the National Measurement Institute (Canberra City, ACT2601, Australia). Labelled free and conjugated metabolites are also available (Gartner et al., 2003; Gaertner et al., 2007). Other sources are given in a later section of this chapter (Section 3.9.3).

The use of tritium-labelled standards for use in steroid assays, not involving saturation analysis, has been described, and in such circumstances, a second internal standard has been necessary during the final separation and quantitation step. An example of such use of two internal standards is described (Seamark et al., 1980) in an early method for the measurement of vitamin D metabolites in human plasma by GC-MS. An alternative to actual addition of the standard to the fluid under analysis is to add the analyte to a second sample and process each side by side, the recovery being assessed by the difference between the two results. Such procedures are not recommended. As an added precaution, in some situations, two different internal standards can be used and the analyte quantified by relation to each separately. In such a case, the analyte concentration should be the same irrespective of which standard is used – where this is not so, it suggests that the standard that gives the lower result is incompletely resolved from a contaminating peak.

3.2 Extraction

Early methods of steroid analysis usually involved extraction as the first step and such extractions were normally carried out using solvents. The main drawback of liquid–liquid extraction is emulsion formation, though centrifugation will often overcome this situation but is time-consuming. Standing extraction tubes in dry ice to freeze the aqueous phase is a useful way of decanting an upper organic layer. While the modern trend has been to move away from such extractions because of the need to restrict use of toxic and/or flammable solvents and the problems of disposal, such extractions should not be completely discarded and there may well be situations where such procedures can still be useful. Indeed, a recent publication (Hill et al., 2007), describing the measurement of pregnanolones in third trimester pregnancy plasma, used ether for extraction, freezing the aqueous phase in solid CO_2/ethanol, and pouring off the ether layer. After evaporation to dryness, the residue was further purified by partitioning between *n*-pentane: 80% methanol in water (1:1) – (Ghulam et al. (1999) and Shu et al. (2003)) who describe similar extraction procedures in two assays for serum/plasma corticosterone. 1-Chlorobutane has been used for extraction of serum DHEA metabolites (Labrie et al., 2007). It is not the intention of this chapter to provide recipes for the extraction of specific steroids,

which can be found in the succeeding chapters in this book where more detailed reviews of the analysis of specific steroid groups are given, but merely to give an outline of the general principles of extraction and to draw attention to some of the problems which may be encountered during steroid analysis.

3.2.1 Solvent Extraction

When deciding upon the best solvent for such extractions, consideration must be given to the polarity of the steroid of interest and the interaction of the steroid to binding proteins. The extraction solvent must ideally do two things; it must totally disrupt the binding of the steroid to protein and must extract the steroid of interest quantitatively and leave behind in the aqueous medium other steroids and non-specific interfering substances. In practice, of course, this is never possible and by its very nature such solvent extraction will also extract a number of other steroids of similar polarity and thus similar structure to the analyte which may well interfere in the final quantitation. Depending upon the relative concentration of the steroid analyte in comparison to that of the potentially interfering steroids, it may be necessary to interpolate further purification steps prior to quantitation. For steroids which are incorporated into the lipoproteins, such as cholesterol and vitamin D, it may be necessary to add chemicals to disrupt the lipoprotein structure prior to extraction since without this disruption the recovery of steroid analyte may be very low (e.g. Axelson, 1985). Bile acids and their conjugates bind to protein and while it may seem sensible to extract these acids from acidic aqueous media, better recoveries are often obtained from alkaline media, perhaps because of the disruption of protein binding at pH11 and above. Solvent extraction of bile acids from tissues may be particularly difficult (reviewed by Street et al., 1983).

The more polar steroids such as the glucuronide and sulphate conjugates and sometimes even the polyhydroxylated C21 steroids are not always effectively extracted even by very polar solvents. A very early extraction procedure (Edwards et al., 1953) overcame this difficulty by the addition of ammonium sulphate at saturation concentrations to urine and acidification to pH2 with 10%(v/v) HCl prior to extraction with ether:isopropanol mixture (3:1, v/v). This procedure was quite effective, and majority of steroid glucuronides and sulphates were extracted to a large degree by this procedure. Although these steroid ester conjugates are most commonly found in urine, they are also present in plasma (especially, C19 glucuronides and sulphates and C21 sulphates) and may be extracted with varying efficiency into polar organic solvents. Addition of ammonium sulphate has also been efficacious in improving the extraction of vitamin D from plasma/serum (Hollis and Frank, 1985). Because there is a spread of polarity amongst the steroids, it is possible, by careful choice of solvents for extraction, to provide a considerable degree of selectivity for the steroid of interest, although such selectivity is seldom exclusive and there are many examples of the use of this type of selection. Figure 3.1 shows the range of solvent polarities in comparison to the polarity of various steroid groups.

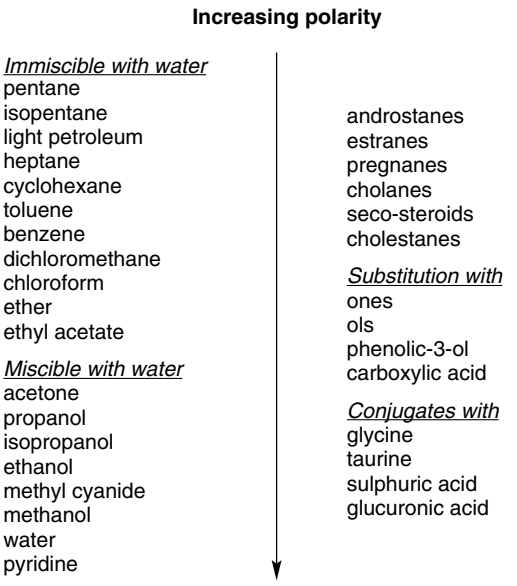

Fig. 3.1 Solvents available for steroid extraction from biological fluids.

When discussing the extraction of steroids, it must be remembered that many steroids often bind very tightly to glass and it is therefore advisable to silanise all glassware prior to use by treatment with dimethyldichlorosilane (1% v/v in toluene) or similar reagent washing afterwards with methanol. It is probably obvious, but still needs emphasising that solvent extraction generally precludes the use of plastic, silicone grease, etc. Considerable care must be taken to exclude all plastic since the occurrence of plasticisers (phthalates) in extracts may interfere in the final analysis. Steroids bound to glass are often difficult to dislodge and this may give rise to problems. It is often the practice, when assessing extractions or assay efficiency, to add radiolabelled or unlabelled steroid in solvent, evaporate the solvent to dryness, and add the medium of interest (plasma, urine, etc.), incubate and hope that the added steroid is distributed in the matrix in the same way as the endogenous analyte. In many cases, however, the steroid that has been added in this way is absorbed largely to the glass of the container and does not dissolve in the aqueous medium. Even subsequent solvent extraction may not totally dislodge the steroid which is bound to the glass and thus any corrections which are made to the final analytical result as a result of the recovery of added standard will give rise to a falsely high value. It is preferable if such procedures are used to ensure that the steroid binding is minimised by prior silanisation of all glassware and that a small amount of polar solvent (ethanol, methanol etc.), which is totally miscible with water is added, to dissolve the steroid prior to the addition of the aqueous medium or alternatively that the standard is added directly to the aqueous medium in a suitable solvent in sufficiently small volumes not to disrupt any binding or to denature any enzyme. Glassware may also present other hazards and it is advisable not to evaporate solvents and leave small quantities of steroids in the dry state for long periods. Some steroids (e.g. the secosteroids)

are particularly susceptible in the dry state to oxidation which can be substantially reduced by storage in suitable solvent.

Because of the occurrence of steroids of different types ranging from oestrogens through corticosteroids to bile acids and bile alcohols, there is no single procedure which efficiently and selectively removes all the steroids from the medium in which they are found. A single secreted steroid may be metabolised to innumerable metabolites of differing degrees of polarity which may then be conjugated with polar acids to assist in excretion. The reverse may happen, and steroid polarity may be decreased by the esterification of hydroxyl groups with fatty acids; for example, cholesterol esters are important constituents of the lipoproteins. Esters of oestrogens have also been described (Hochberg et al., 1991; Larner et al., 1992, 1993; Tikkanen et al., 2002), and the process of de-esterification, saponification, by incubation with alkali, usually KOH, is widely used as a first step in the extraction of a number of steroids from food and other material, although liquid chromatography–mass spectrometry (LC-MS) analysis of oestrogen esters (Miilunpohja et al., 2006) and cholesteryl esters (Liebisch et al., 2006) is now available without prior saponificiation. The wide variety of steroids and the metabolism, conjugation and/or esterification which they can undergo is not the only problem facing the analyst. Choice of the method must also take into account the medium selected for analysis, and, in this context, steroid binding and the problems this presents have already been mentioned. The physical characteristics of the matrix is clearly of importance and methods adopted for the analysis of granular or powdery food material will obviously not be appropriate for the analysis of a steroid in sunflower oil or metabolites of corticosteroids in human urine or plasma. Procedures for the extraction and measurement of cholesterol (Fenton, 1992), vitamin D (Rizzolo and Polesello, 1992; Jones and Makin, 2000) and anabolic steroids in meat (Marchand et al., 2000; Feduniuk et al., 2006; Xu et al., 2006; Nielen et al., 2007) and nutritional supplements (van Thuyne and Delbeke, 2004, 2005; Martello et al., 2007) have been reviewed.

Steroids share their essential non-polar nature with a large number of other lipids and methods, which effectively extract a wide range of steroids, and are likely also to extract a large amount of non-steroidal lipid material. Such lipid material must not be ignored since it can well interfere in subsequent separation or quantitation procedures. Lipid can be removed from the extracts by partitioning with organic solvents. This was an important step when isolating steroids from brain tissue (Ebner et al., 2006).

Steroids exist in hair in an intercellular space between the cuticle and cortical cells. Attention has been given to hair steroids as indicators of steroid abuse. The hair lipids include squalene, wax esters, triglycerides, free fatty acids, ceramides and cholesterol sulphate. Steroids can be extracted from hair using solvent mixtures such as chloroform with methanol (Choi and Chung, 1999). Analysis of steroids in hair as a means of detecting ingestion of anabolic steroids has been reviewed by Kintz (2004 – Kintz et al., 2006; Gambelunghe et al., 2007). Fatty acids can of course be easily removed by an alkaline wash but this may also remove acidic steroids such as oestrogens or bile acids. Saponification, apart from liberating steroids from their esters, also has the added advantage of hydrolysing many neutral lipids

which can then be removed by alkali or acid washes. It may not, however, be possible to remove potentially interfering lipid material by simple washings of solvent extracts and further separatory procedures, such as the use of magnesium oxide mini-columns as described by O'Shannessy and Renwick (1983), may be necessary. Despite these precautions, significant amounts of non-specific non-steroidal neutral lipid material may be present in supposedly clean steroid extracts and this may not always be appreciated since this interfering material usually does not have any recognisable characteristic which immediately betrays its presence. While in most instances, such material can be ignored, there are situations where it can assume importance, for example, in immunoassays where steroids extracted from biological matrices may not always behave in the same way as standard steroids used for the standard curve (e.g. Jawad et al., 1981). While many of these techniques are more than 20 years old, they should not be ignored and may still have applications to particular problems of today, especially when combined with more modern technology (e.g. Makin et al., 2002; Huang et al., 2007).

For the quantitative extraction of neutral unconjugated steroids from aqueous media such as urine, bile, plasma/serum, saliva, *in vitro* incubation media, etc., the use of an equal volume or excess of polar solvent such as ethanol, methanol, acetone or methyl cyanide is very effective. Tissue extraction is usually carried out either after or during homogenisation (e.g. Andersson and Sjövall, 1985). Such extraction systems can also be used to extract steroid conjugates with varying degrees of efficiency when used in combination with acid or alkaline pH and added salts. These solvents have the added advantage that they also disrupt steroid protein binding by denaturing the protein. In the case of cholesterol or vitamin D, this denaturation may have to be taken further by the addition of ammonium sulphate (Hollis and Frank, 1985) or pentylamine (Axelson, 1985) or other material to disrupt the lipoprotein. The protein can then be removed by centrifugation, leaving a 50:50 solvent:water mixture which can be further extracted by the addition of suitable solvent such as chloroform which is immiscible with water. This causes the formation of two layers, the bottom of which contains the unconjugated steroids which can be removed. Use of chloroform:methanol mixtures for such extractions produces exactly the same end result and such extractions have been widely used over the last 50 years (Bligh and Dyer, 1957). Many modifications of this procedure have been introduced which may well have advantages for particular purposes but do not greatly improve the general applicability of the original procedure for steroid extraction. Acetonitrile is widely used as an extraction solvent for vitamin D metabolites in a similar fashion and the aqueous extract after removal of the protein by centrifugation can be used for solid-phase purification using Sep-Pak silica cartridges (see Chapter 11). On-line deproteinisation during high-performance liquid chromatography (HPLC) can be achieved using a polymer-coated mixed functional silica column (Okumura et al., 1995).

Careful choice of solvents can provide a considerable degree of selectivity. It is clearly a relatively simple task to use solvent extraction to discriminate between non-polar neutral steroids and their conjugates with glucuronides and sulphates. It is not so simple to rely on solvent extraction to discriminate between steroids of similar polarity but it is possible to achieve quite simple separations by judicious choice of

solvent. There are numerous examples of such solvent selectivity in the literature, the use of isopentane to separate aetiocholanolone (96% extracted) and 11β-hydroxy-aetiocholanolone (2.5% extracted) (Few, 1968) and the separation of the oestrogens, oestradiol and oestrone (>95% extracted) from oestriol (4% extracted) using benzene:light petroleum (1:1, v/v)) (Brown, 1955). It might be argued that such examples of solvent selectivity, being 35–50 years old, are not appropriate for a book on modern methods of analysis but if such procedures, which are simple and quick, provide a solution to today's problems their age should not debar them from use.

There are other examples of such solvent selectivity. It has become fashionable in the age of the rapid and simple immunoassay method, which when applied directly to plasma without any tedious extraction or separation procedure, gives a very rapid result to rely too heavily on the advertising message on the side of the reagent pack without much critical appraisal. A good example of such reliance and the value of a simple extraction prior to assay is reported in the excellent paper by Wong et al. (1992) who carefully investigated the nature of the steroids in neonatal plasma that interfered in a "non-extraction" immunoassay for 17-hydroxyprogesterone, an important assay used in the early diagnosis of congenital adrenal hyperplasia (CAH), where a falsely high result could have severe consequences. The interference was demonstrated to be due to 5-en-3β-ol steroids conjugated with sulphuric acid, particularly 17-hydroxypregnenolone sulphate, which are formed in the fetal adrenal cortex (Shackleton, 1984) and are present in neonatal plasma. A simple extraction of the plasma with a relatively non-polar solvent, isopropanol:hexane (3:97,v/v), removed 17-hydroxyprogesterone leaving behind the interfering steroid sulphates. Two immunoassays for plasma 17-hydroxyprogesterone have been evaluated (Nahoul, 1994), comparing results with an in-house method which used ether extraction followed by column chromatography on LH-20 – both assays were found to give high values and it was concluded that neither was suitable for use with infants or women in the follicular phase of their menstrual cycle. Analysis of 17-hydroxyprogesterone by LC-MS/MS has a wider range for accuracy than RIA (Etter et al., 2006). Direct assays for testosterone are entirely inappropriate in the first 6 months of life (Fuqua et al., 1995). Urine free cortisol measurements with direct RIA measurements can have poor specificity and recovery (Gray et al., 2003; Horie et al., 2007). These examples illustrate very well the need to remember the value of a simple extraction or chromatographic step, which can provide a much needed increase in the selectivity of a method (Davison et al., 2005; Schirpenbach et al., 2006) and also demonstrate the need to remember that assays developed and validated using one medium (adult male plasma) may not be valid when applied to other media (neonatal or female plasma).

Urine is a complex medium which contains a wide variety of metabolic products of the secreted steroid hormones (androgens, oestrogens, corticosteroids, preganediols, cholesterol, etc.) which may be unconjugated or conjugated with glucuronic or sulphuric acids. A comprehensive analysis of urine presents, therefore, a considerable analytical challenge. While the analysis of urine for clinical diagnostic purposes is not as popular as it once was, the use of steroid profiles by capillary GLC can still provide very valuable information. Of interest also is the analysis of urine for the identification

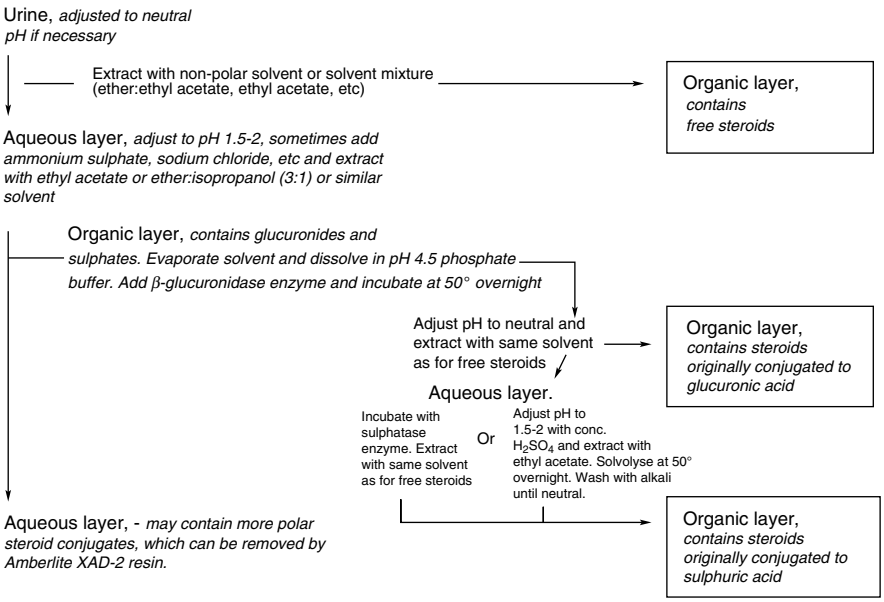

Fig. 3.2 Scheme for the extraction and separation of steroid groups from urine without chromatography.

of the metabolites of administered steroids, such as 4-hydroxyandrostenedione, an aromatase inhibitor, which may be of value in monitoring the treatment of oestrogen-dependent breast cancer (Foster et al., 1986; Poon et al., 1992). Figure 3.2 illustrates a non-chromatographic approach to the extraction of steroids from urine using solvent extraction and subsequent hydrolysis of the conjugates. A method of solvent extraction has been described for the extraction of testosterone and epitestosterone that uses an aqueous two-phase system composed of 1-butyl-3-methylimidazolium chloride and dipotassium hydrogen phosphate (He et al., 2005). In a similar manner, the efficiency of extraction of steroid sulphates using ion-pairing with a number of reagents has been evaluated (Cawley et al., 2005).

3.2.2 Solid-Phase Extraction

Although many of today's publications concerned with steroid analysis still usefully employ liquid-liquid extraction as an initial step in the purification of steroids of interest, it is in general true to say that over the last 20–25 years the most important step forward in steroid analysis has been the advent of solid-phase extraction (SPE), particularly the availability of microparticulate silica coated with octadecasilane packed into syringes or cartridges. The use of Sep-Pak C18, for the extraction of steroids was initially described by Shackleton and Whitney in 1980. A survey of methods

for steroid analysis published in 1993 indicated how popular such solid-phase procedures had become in that more than 70% of publications used such extractions. Although subsequent surveys have confirmed this trend, solvent extractions are still used today. Extraction of steroids from aqueous media has been carried out using neutral (Amberlite XAD-2 – Bradlow, 1968, 1977) and ion-exchange resins (DEAE-Sephadex – Derks and Drayer, 1978a), and such procedures are still used successfully even today, particularly for the extraction of polar steroids (e.g. Deboer et al., 1992; Poon et al., 1992); Dowex AG resins have been used in conjunction with adsorption cartridges for the purification of catechol and guaiacol oestrogens (Saegusa et al., 1993). An interesting but limited comparison of the uses of solid-phase extraction (SPE) and their value in contrast to solvent extraction in the pre-HPLC stage of the analysis of plasma cortisol and corticosterone has been published (Hariharan et al., 1992).

There are a wide variety of solid-phase materials available for use in the extraction of steroids and examination of the chapters in this book and the catalogues of suppliers of these materials will confirm this. The field is bedevilled by the use of trade names which sound nice but are not helpful when considering their method of action. Extrelut, Chem-Elut, VacElut, Bond-Elut, Tox Elut might appear to be different trade names for the same thing but they are not. SPE material appears to fall into two distinct groups. Firstly, those systems based on Keiselguhr (Celite – a diatomaceous earth) treated in various and sometimes unspecified ways (e.g. Chem-Elut, Analytchem & Extrelut, E.G. Merck) to inactivate the material and then sieved into different size ranges (e.g. Perona and Pavan, 1993; Saegusa et al., 1993; Suzuki et al., 1993). The material is then packed into syringes, cartridges, etc. of various sizes and shapes made from a variety of different plastics. The aqueous medium is poured onto the material, which takes up the water, and the steroids are then eluted with organic solvents. This process would appear to be a simple liquid–liquid partition chromatography process similar to the celite partition column chromatography of the past. Since these columns have a finite capacity to absorb water, it is possible inadvertently to overload and if aqueous material passes through the column, steroids of interest will also pass through still dissolved in the aqueous matrix. Tox Elut (Varian), a similar type of system but using a more granular material designed for the analysis of drugs of abuse has a dye incorporated into it, which indicates how far down the column the added aqueous medium has reached. Such systems are still occasionally used for preliminary purification before quantitation (e.g. Ibrahim et al., 2003; Fiet et al., 2004; Davison et al., 2005).

The second type of SPE material is based upon microparticulate silica either used directly or modified in an ever increasing variety of different ways. Some of the non-polar, polar and ion-exchange sorbents, which are in use today and can be obtained from a variety of sources (e.g. Biotage IST, Biotage GB Ltd., Duffryn Industrial Estate, Ystrad Mynach, Hengoed CF82 8RJ, UK and Analytchem International, Harbour City, CA, USA) are illustrated in Fig. 3.3, which is taken from the catalogue of Biotage IST, as an example of what is available. These sorbents can be packed into syringe-like reservoirs or pre-packed cartridges (e.g. Sep-Pak, Millipore-Waters) of different sizes, which can cope with differing loads. These SPE systems are based on a variety of absorption and partition. Most steroid

BIOTAGE RESIN AND SILICA BASED SORBENTS
Table adapted and updated from 2004 Catalogue of Sample Preparation Products and Services

Sorbent	Description	Structure	
Resin based sorbents: primary interactions are strongly NON-POLAR			
EVOLUTE ABN	Surface modified polystyrene divinylbenzene		
ENV+	Hydroxylated polystyrene-divinylbenzene		
101	Polystyrene-divinylbenzene		
Silica based non-polar sorbents: primary interactions are NON-POLAR			
Non-endcapped sorbents exert stronger polar and ionic secondary interactions than endcapped sorbents			
C18*	Octadecyl	-Si-$C_{18}H_{37}$	
MF C18	Octadecyl monofunctional	-Si-$C_{18}H_{37}$	
C8*	Octyl	-Si-C_8H_{17}	
C6	Hexyl	-Si-C_6H_{13}	
C4	Butyl	-Si-C_4H_9	
C2*	Ethyl	-Si-C_2H_5	
CH†	Cyclohexyl	-Si- 	
PH*	Phenyl	-Si- 	
CN*	Cyanopropyl	-Si-$(CH_2)_3CN$	
Silica based mixed-mode sorbents: primary interactions are NON-POLAR and IONIC			
HAX	C8 and strong anion exchange		
HCX	C8 and strong cation exchange		
HCX-3	C18 and strong cation exchange		
HCX-5	C4 and strong cation exchange		
HCX-Q	C8 and weak cation exchange		
Multimode	C18, strong anion and cation exchange		
Silica based ion exchange sorbents: primary interactions are IONIC			
Silica based anion exchange sorbents			
			Secondary interaction (aqueous matrix)
NH2	Aminopropyl	-Si-$(CH_2)_3NH_2$	Weak non-polar
PSA	Primary secondary amine	-Si-$(CH_2)_3NH(CH_2)_2NH_2$	Weak non-polar
SAX	Quaternary amine	-Si-$(CH_2)_3N^+(CH_3)_3$ Cl^-	Weak non-polar
PE-AX	Quaternary amine	-Si-$(CH_2)_3N^+(CH_3)_3$ OAc^-	Weak non-polar
Silica based cation exchange sorbents			
			Secondary interaction (aqueous matrix)
CBA	Propylcarboxylic acid	-Si-$(CH_2)_3COOH$	Weak non-polar
SCX	Benzenesulfonic acid	-Si-  -SO_3^- H^+	Medium non-polar
SCX-2 (PRS)	Propylsulfonic acid	-Si-$(CH_2)_3SO_3$-H^+	Weak non-polar
SCX-3	Ethylbenzene sulfonic acid	-Si-$(CH_2)_3$-  -SO_3^- H^+	Strong non-polar
Silica based polar sorbents: primary interactions are POLAR			
			Secondary interaction (non-aqueous matrix)
CN	Cyanopropyl	-Si-$(CH_2)_3CN$	
SI	Silica	-Si-OH	Weak ion exchange
DIOL	2,3-dihydroxypropoxypropyl	-Si-$(CH_2)_3$-OCH_2-CH(OH)-CH_2(OH)	
NH2	Aminopropyl	-Si-$(CH_2)_3NH_2$	Weak ion exchange
PSA	Primary secondary amine	-Si-$(CH_2)_3NH(CH_2)_2NH_2$	Weak ion exchange
FL	Florisil	$MgO•3.6\ SiO_2•0.1$ OH	
AL-A	Alumina – acidic		
AL-N	Alumina – neutral		
AL-B	Alumina – basic		

* Endcapped sorbent available † Cyclohexyl is not endcapped

Fig. 3.3 Biotage resin and silica based sorbents. Table adapted and updated from 2004 Catalogue of Sample Preparation Products and Services

extractions are carried out using reverse-phase methodology where the silica has been modified by linking the silanol groups to hydrocarbons of varying chain length, the most popular being octadecane forming octadecasilyl silica (ODS or C18) (Shackleton and Whitney, 1980), although other chain lengths have been used (e.g. C_2) for extraction of steroids, such as oestrone (e.g. Ciotti et al., 1989), from serum. Depending upon the treatment used to form the ODS material, a proportion of silanol groups on the silica may still be unchanged. Further treatment, with a silylating material to produce trimethylsilyl ethers (TMS) of these polar groups, known as 'end-capping' can be carried out. The presence of untreated silanol groups leads to a significant adsorption as well as a reverse-phase partition and deliberately produced non-fully end-capped material (Bond-Elut C18-OH) has been put to good use by combining the extraction and subsequent separation of vitamin D metabolites on the same cartridge by changing solvents, so-called 'phase-switching' (Hollis, 1986), and this procedure is now incorporated into a commercial method for 1α, 25-dihydroxyvitamin D assay.

Comment has been made before about the value of washing organic extracts with alkali and similar purification can be achieved with amino (NH_2) columns in series with C18 extraction columns (Schmidt et al., 1985). These silica-based sorbent columns too have finite capacity but are now available, as mentioned above, in different sizes or alternatively the packing material can be supplied as such and appropriate amounts made up in columns of suitable sizes for the particular application. When setting up a method using these SPE columns, attention must be paid to the material in which the silica is packed. Not all the plastics in which the C18 material was packed (including cartridge, syringe and frits, etc.) react to solvents in the same way and material may be eluted from the plastic which is innocuous when the eluent is used in one method but devastating in another. Small SPE columns can be inserted into HPLC systems (Lopez de Alda and Barcello, 2001; Barrett et al., 2005; Kataoka, et al., 2007). Miniaturised fibre-packed injection needles have recently been used as a novel extraction device prior to GLC, although it has not yet been applied to steroid analysis (Ogawa et al., 2007; Saito et al., 2007).

Before being applied to the column, any steroid–protein binding must be disrupted and in the case of the silica C18 extraction procedures this is usually today carried out by the use of acetonitrile or methanol which is added in equal amounts, vortex mixed and the protein plug removed by centrifugation. The acetonitrile:water mix is then applied to the cartridge and the steroids can be eluted with methanol, after washing with various concentrations of water in methanol to elute polar material. Sometimes, the protein plug is re-extracted and combined with the original extract applied to the Silica C18 SPE system. This procedure is very effective usually leading to near-quantitative recovery. For some steroids, such as vitamin D itself and cholesterol, more rigorous procedures may be required in order to disrupt protein binding (reviewed in Porteous et al., 1987). There are numerous papers, which can be referred to, that use these SPE methods for the extraction of steroids and all are used in essentially the same way. The use of BondElut C18 cartridges for the extraction of bile acids from serum required dilution of serum with 0.1 M NaOH or 0.5 M triethylamine sulphate and heating for 30 min at 64°C prior to application (Rodrigues and Setchell,

1996). It will be noted from Fig. 3.3 that ion-exchange SPE material is now available, and these are now being increasingly used for steroid extraction (e.g. Strahm et al., 2008). For the separation of free and conjugated steroids, mixed mode anion-exchange SPE cartridges were used (Ebner et al., 2006). Further applications of ion-exchange SPE are to be anticipated. Solid-phase extraction was also used to remove lipids from brain extracts (Liere et al., 2004; Ebner et al., 2006).

Reverse-phase extraction using C18 (ODS) coated silica is by far the most popular material for SPE, although subsequent separatory procedures using SIL (untreated silica) or –CN (cyano, particularly useful for separating steroids with oxo groups) have proved valuable. Indeed, Sep Pak C18 cartridges have been used successfully to extract bile acids from serum, urine, liver biopsy extracts, bile, gastric juice and faeces (Guldutuna et al., 1993). ODS-coated silica has also been used for steroid extraction prior to HPLC by the use of a guard column, packed with ODS-silica, in place of the injection loop. Diluted samples were injected into the column, washed and then eluted onto the analytical HPLC column with methanol (Wade and Haegele, 1991a).

The Waters company are producing a range of OASIS cartridges. There are five available Oasis® sorbent chemistries, which are designed to meet just about all sample preparation needs. They are all built upon unique water-wettable Oasis® HLB copolymer and provide exceptional results (AbuRuz et al., 2003). The sulphonic acid (MCX) and quaternary amine (MAX) derivatives of Oasis® HLB provide dual modes of retention enabling greater cleanup selectivity and sensitivity for both acidic and/or basic compounds – even if the sorbent in the wells runs dry. Oasis® WCX (weak cation exchanger) and WAX (weak anion exchanger) are also derivatives of Oasis® HLB. These sorbents are specifically designed to offer the same benefits and features as HLB with the ability to retain and release strong acids (e.g. sulphonates) and bases (e.g. quaternary amines). All of the five patented Oasis® chemistries are available in several device formats (e.g. cartridges, 96-well plate, and µElution plate) to fit specific needs. These cartridges were used more recently in the processing of steroids from prostate (Higashi et al., 2005c), brain extracts (Ebner et al., 2006), bovine milk (McDonald et al., 2007) and rat biofluids and foetal tissue (Samtani and Jusko, 2007).

Other solid-phase extraction procedures for steroid extraction from biological media have been described such as the use of graphitised carbon ("Carbopak", Supelco, Poole, Dorset, BH17 7NH) for the extraction of oestrogens and their conjugates from urine, serum and amniotic fluid, but can also be used for separation of steroids (illustrated in Fig. 3.4, Andreolini et al., 1987). Cyclodextrin, with which steroids form inclusion compounds, has also proved valuable as a means of extracting steroids prior to GC-MS (De Brabandere et al., 1993) or HPLC (Wade and Haegele, 1991b). Oestradiol-17β, oestriol and 17α-ethinyloestradiol have been analysed in environmental waters by LC with fluorescent detection following on-line solid-phase microextraction using a polymer monolith inside a polyether ether ketone (PEEK) tube (Wen et al., 2006).

Automated liquid sampling handling systems are now being used to improve analytical efficiency. SPE can be incorporated in to such procedures and has been

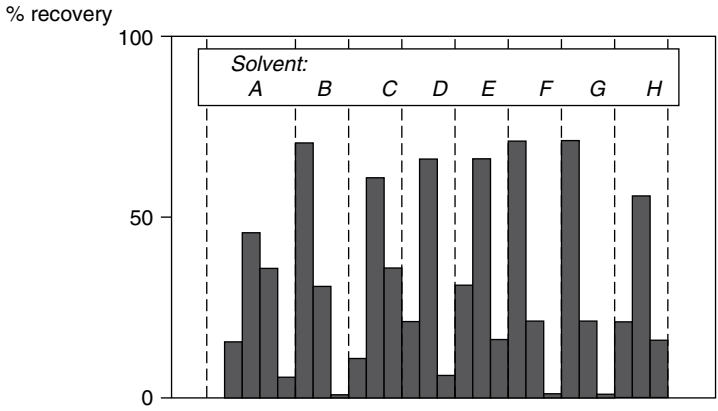

Fig. 3.4 Group separation of some steroids and their conjugates using graphitised carbon black cartridges (Carbopak, Supelco). These cartridges consist of a 6 cm x 1 cm i.d. cylindrical polypropylene tube which is one-sixth filled with 250 mg of carbon black with a particle size ranging between 20 and 125 μm. The absorbent bed is held in place by polyethylene frits. After removal of phospholipids by passing a methanol extract of serum or amniotic fluid through a C 18 Sep-Pak, the eluent in methanol:water (80:20) was percolated through the Carbopak cartridge and steroid groups were recovered by sequential elution with a variety of solvents figure illustrates the separation in this system of a number of different standard steroids identified as follows: solvent A, (methanol) elutes androsterone; solvent B, (100 mmol/1 of formic acid in methanol) elutes estradiol; solvent C, (chloroform: methanol, 60:40) elutes androsterone glucuronide; solvent D, (chloroform: methanol, 27:73, with 250 mmol/1 formic acid) elutes estradiol 3-glucuronide; solvent E, (chloroform: methanol, 60:40, containing 250 mmol/1 acid) elutes estradiol 17-glucuronide; solvent F, (chloroform: methanol, 10:90, containing 5 mmol/1 of tetramethylammonium hydroxide) elutes androsterone sulphate; solvent G, (chloroform: methanol, 80:20, containing 0.5 mmol/1 of tetramethylammonium hydroxide) elutes estradiol 3-sulphate; solvent H, (chloroform: methanol, 80:20, containing 5 mmol/1 methylammonium hydroxide) elutes estradiol 3-sulphate 17-glucuronide. (Reprinted with permission from Andreolini *et al*., 1987. Copyright 1987 American Chemical Society.)

tested for example in pharmaceutical applications (Tamvakopoulos et al., 2002) in urine steroid analysis (e.g. equilenin and progesterone – Rule and Henion, 1999; 6β-hydroxycortisol and cortisol – Barrett et al., 2005) and anabolic steroids – Haber et al., 2001) and for the measurement of low concentrations of oestrogens in natural and treated water, coupled to LC-MS (Rodriguez-Mozaz et al., 2004). In a similar fashion, solid-phase extraction can be incorporated into the LC system and thus are susceptible to automation. A recent example of this is the extraction of cortisol from human saliva using a Supel Q PLOT column for extraction in series with the analytical LC column (Kataoka et al., 2007).

A novel approach, coating absorptive material onto stir bars (Kawaguchi et al., 2006a), has been used for the extraction of estrone and oestradiol-17β (Stopforth et al., 2007a), testosterone and epitestosterone (Stopforth et al., 2007b) from human urine and oestradiol-17β from river water (Kawaguchi et al., 2004, 2006b) – the stir bars were removed and washed. The steroids were derivatised in the gas phase and then introduced into a GC-MS system by thermal desorption. Steroid conjugates, absorbed onto these bars could be treated in a similar way, de-conjugation taking place once the

bars had been removed. The use of these stir bars is not confined to GC-MS, and a similar procedure has been described for the extraction of urine spiked with oestriol, methyltestosterone and progesterone, and subsequent elution of the steroids for analysis by LC-MS (Huang and Yuan, 2007). A similar method using sol-gel coated polydimethylsiloxane/β-cyclodextrin stir bars has been described for extraction of oestradiol-17β from river water for subsequent LC-UV analysis (Hu et al., 2007).

3.2.3 Hydrolysis of Steroid Conjugates

Hydrolysis of steroid glucuronides is usually carried out using a β-glucuronidase preparation, although effective hydrolysis can also be achieved by oxidation with periodate that removes the glucuronide residue, leaving behind a mixture of free steroid and steroid formate. Hydrolysis of steroid sulphates is on the other hand usually carried out by acid solvolysis in ethyl acetate which is very effective for most steroids but does not always cleave the ester link of some bile acids. Parmentier and Eyssen (1977) described an alternative acid hydrolysis procedure using methanol:acetone (1:9,v/v) containing approx. 1% (v/v) HCl (18 h at 37°C) which they used to hydrolyse bile acid sulphates on C3, C7 and C12. A similar method was published in 1989 (Tang and Crone, 1989) which used anhydrous methanolic HCl for the hydrolysis of glucuronides, sulphates and phosphates. Enzyme hydrolysis of steroid sulphates can also be carried out using sulphatase preparations from a variety of sources (*Patella vulgata*, *Helix Pomatia* etc.) most of which also contain β-glucuronidase activity (Shibasaki et al., 2001), although such preparations do not always work efficiently (Epstein et al., 1983), and before use, they should be tested using a model steroid sulphate. Use of these enzyme preparations can also occasionally lead to further problems and it has been, for example, reported (Schmidt et al., 1985) that the recovery of DHEA from urine treated with *Helix pomatia* preparations drops dramatically as the concentration of enzyme increases. While enzyme hydrolyses such as those illustrated in Fig. 3.2 can be carried out without extraction, it has been noted by Schmidt et al. (1985), and many times in the past, that improved hydrolysis with less enzyme can be achieved after extraction, presumably because of the presence of interfering compounds in unextracted urine. In preparations containing both glucuronidase and sulphatase activity, sulphatase activity can be inhibited by incubating in pH 4.5 phosphate buffer. A study of the optimum conditions for steroid glucuronide hydrolysis was carried out by Ferchaud et al. (2000) who recommended the use of abalone entrails at pH 5.2 at 42°C. Hydrolysis of steroid conjugates, while rendering analysis of the steroid composition of these fractions simpler, does obscure information and it would clearly be better to analyse these conjugates without prior hydrolysis (e.g. Gaskell, 1990; Shackleton et al., 1990a). The advent of LC-MS has provided an ideal approach towards the analysis of intact glucuronides and sulphates, and this has been used for the analysis of intact urinary 17-oxosteroid sulphates and glucuronides using LC-MS (Jia et al., 2001) and serum androsterone glucuronide and androstenediol glucuronide using LC-MS/MS (Labrie et al., 2006). A more comprehensive approach

to this problem has been published by Antignac et al. (2005) who separated 14 steroid glucuronides and sulphates in a single run in their LC-MS-MS system. Figure 3.5 illustrates this separation. A similar extraction procedure using Bakerbond C18 cartridge followed by a quaternary ammonium cartridge (Bond Elut SAX) has been

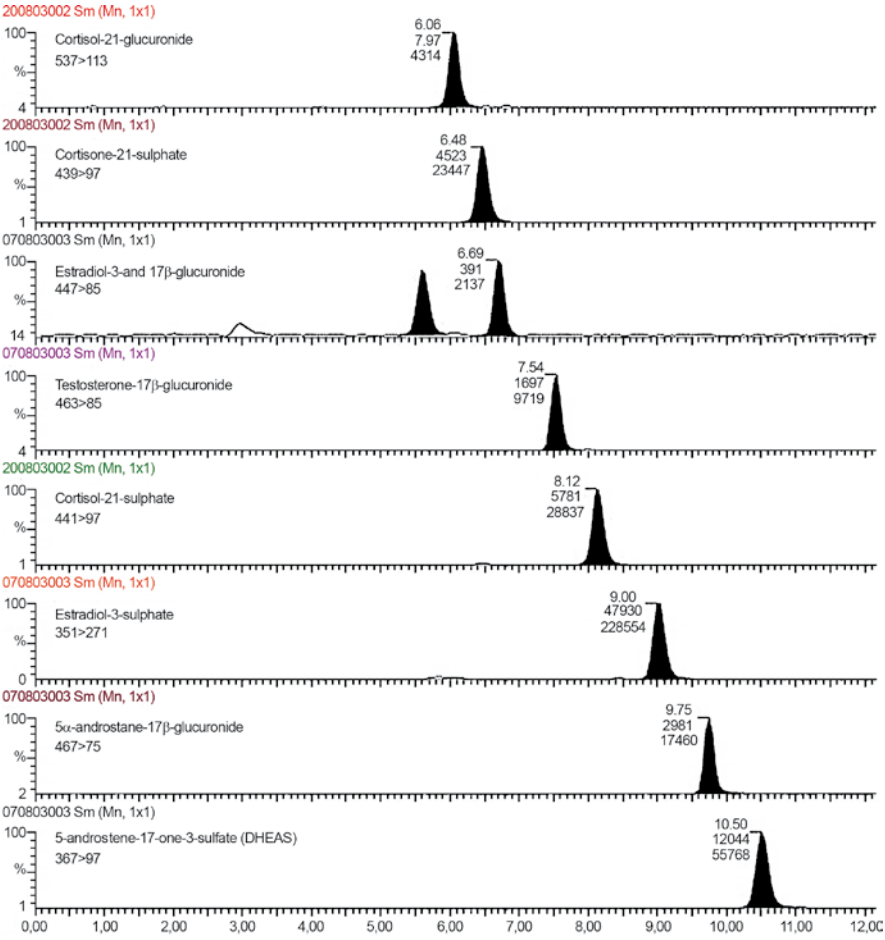

Fig. 3.5 Figure 3 from Antignac et al. (2005). Typical ion chromatograms obtained for a standard solution of steroid and corticosteroids phase II matabolites (10ng injected). Data were acquired using a Quattro LC® triple quadrupole analyzer (Micromass®, Manchester, UK) operating in electrospray ionization mode (ESI). Nitrogen was used as nebulization and desolvation gas, with flow rates of 90 and 600 L/h, respectively. Source and desolvation temperatures were 120 and 350 °C, respectively. The electric potential applied on the capillary was 3.5 kV, and the sampling cone voltage was optimized for each molecule. The LC–MS/MS experiments were performed using argon as the collision gas at a pressure of 4.0×10^{-14} mbar and a collision energy adapted for each compound. The ions selected in MSI and those monitored in MS2 after CID are shown in upper left of each panel. [Further data is given in Antignac et al. (2005) from which this Figure is taken with permission.]

used to extract 19-norandrosterone sulphate from human urine prior to LC-MS-MS (Strahm et al., 2007). Sodium cholate micelle capillary electrophoresis has been used to determine 16 oestrogens, DHEA and their glucuronide and sulphate conjugates in 100 μL of serum (Katayama et al., 2003).

Bile acids are excreted in more complex forms than most other steroids, being not only conjugated to glucuronic and sulphuric acids but also joined, via a peptide bond at C24 to either glycine or taurine. Hydrolysis of bile acid glycosides is influenced by the source of the enzyme, position of sugar moiety, enzyme activity and incubation conditions (Momose et al., 1997a), but is usually attempted using cholylglycine hydrolase (e.g. Paauw et al., 1996; Gatti et al., 1997). Other derivatives of bile acids have been described (Niwa et al., 1993). Hydrolysis to free acids therefore obscures even more information than normal. An increasing number of GC and LC-MS systems have been developed which allow bile acid conjugates to be analysed directly without hydrolysis (e.g. Street et al., 1986; Iida et al., 1992; Ikegawa et al., 1992; Yang et al., 1997; Tessier et al., 2003; Ando et al., 2006; Caron et al., 2006). In addition, a 2D HPTLC method with cyclodextrin/aqueous methanol solvent has been developed (Momose et al., 1998) as well as an ion-pair HPLC method using di-*n*-butylamine acetate as a mobile-phase additive (Sasaki et al., 2000). Analysis of bile acids is dealt with in greater detail in Chapter 10.

The common lack of reference metabolites in conjugated forms precludes validation of all urinary methods. A number of papers describe synthesis of conjugates using tissue and recombinant enzymes and chemical techniques for steroids (diMarco et al., 1998; Sasaki et al., Iida*[1] and Nambara, 2000; Kuuranne et al., 2002, 2003) and bile acids (Momose et al., 1997b; Gall et al., 1999; Lida et al., 2002; Kakiyama et al., 2005; Caron et al., 2006; Jantti et al., 2007).

3.2.4 Immunoaffinity Extraction

In an ideal world, the initial extraction procedure would not be necessary as the quantitation method would be so specific that neither the biological matrix nor steroids with similar structures to that of the analyte would interfere. Unfortunately, although some direct immunoassays are described, there are very few of these analytical methods for steroids which cannot be improved by some form of extraction and/or pre-purification before quantitation, although there is a report of an immunoassay for 17-hydroxyprogesterone which was not improved by interpolation of a column chromatographic step (Lim et al., 1995). Extraction procedures, which removed only the steroid of interest from the matrix, would be of considerable

[1]Note that Lida, T and Iida, T appear to be the same person but are spelled differently in PubMed. In the text and reference list the spelling adopted by PubMed is used. Please check both spellings in the reference list.

value in some circumstances and one extraction method has shown considerable promise in this area. Use of columns packed with immobilised antibodies (immunoaffinity columns - IAC) for extraction can provide a means of selective extraction and purification (Glencross et al., 1981). The selectivity depends of course upon the specificity of the antibody for the analyte but has, for example, been successfully applied in the GC-MS analysis of oestradiol in human plasma (Gaskell and Brownsey, 1983), prior to immunoassay (Webb et al., 1985) or prior to HPLC by incorporating an antibody bound to Si60 in the injection loop of the LC system (Nilsson, 1983). In these methods, the antibody is chemically attached to a support (e.g. Sepharose or Sephacryl) and kits are available to carry out this procedure (e.g. Stanley et al., 1993). The immobilised antibody is then packed into a column and the matrix percolated through it. After washing, the analyte(s) can be released from the antibody and thus eluted from the column by altering the salt concentration. Highly specific antibodies obviously provide a selective extraction but there may be situations when a less selective, broad spectrum extraction is required. This can be achieved by using an antibody with broader specificity. Tsikas (2001) has reviewed the application of IAC prior to GC-MS and it has been used subsequently for clean up prior to HPLC-ToF (Time of flight) analysis of oestrogens in sewage-impacted urban estuary water (Reddy and Brownawell, 2005). The development of a multi-target IAC for oestrone, oestradiol and oestriol in urine prior to separation by micellar electrokinetic chromatography (MEKC) has also been described (Su et al., 2005). Testosterone in male urine has been successfully determined by partial filling micellar electrokinetic chromatography (PF-MEKC) after immunoaffinity SPE (Amundsen et al., 2007). Microemulsion EKC (MEEKC) has been used for the measurement of natural and synthetic oestrogens in pharmaceutical preparations (Tripodi et al., 2006) and scanning MEKC has been applied to the measurement of corticosterone and 17-hydroxycorticosterone in plasma and urine (Chen et al., 2004).

3.2.5 Extraction Using Molecularly Imprinted Polymers and Restricted Access Material

Since the first edition of this book, new forms of selective extraction have been introduced but not yet widely applied to steroid analysis. Firstly, molecularly imprinted polymers (MIPs Haginaka, 2001), which could be regarded as synthetic 'antibodies'. For example, oestradiol was extracted into an MIP packed into a microcolumn and subsequently eluted using microwave-assisted extraction (Bravo et al., 2005). Other examples again using oestradiol have been outlined (Dong et al., 2003; Szumski and Buszewski, 2004; Watabe et al., 2006). Specific extraction procedures for cholesterol, using molecularly imprinted cyclodextrin microspheres (Egawa et al., 2005) and sorbents (Pichon, 2007), have also been described. There is a comprehensive review of the literature (up to 2003) on MIP and its appli-

cations (Alexander et al., 2006). Use of MIP and RAM materials in SPE-LC-MS for monitoring oestrogen contamination of water has been reviewed by Rodriguez-Mozaz et al., (2007).

The second development uses the so-called restricted access material (RAM) coupled on-line to LC-MS (van der Hoeven et al., 1997; Petrovic and Barcelo, 2002; Petrovic et al., 2002; Christiaens et al., 2003, 2004; Souverain et al., 2004) or by the use of column switching – after absorption onto a C4-alkyl-diol silica RAM. The absorbed steroid was backflushed onto a conventional C18 column followed by ES-MS (Chang et al., 2003).

After extraction, it may still be necessary to carry out some form of purification before quantitation and this may be necessary for a number of reasons. Firstly, such purification removes potentially interfering compounds of similar structure which may on occasions be present in higher concentrations than the analyte itself. It may also be necessary to carry out a preliminary fractionation to separate steroids in particular group. There are a multiplicity of methods of purifying steroids after extraction from the biological matrix, all using some form of chromatography. Today, only two chromatographic techniques are however widely used, high-performance liquid chromatography (HPLC) and gas–liquid chromatography (GLC), both of which provide high resolution and thus considerable specificity which is further improved when these systems are coupled to a mass spectrometer (MS). Use of HPLC and the hyphenated techniques of HPLC-MS and GC-MS are discussed in separate sections below. Only a brief outline is given here of the other chromatographic techniques, which although less popular than they once were, may still have a role to play in certain situations and in countries where expensive HPLC and/or GLC apparatus is not available.

Steroid hormones circulate bound to albumin and/or specific binding globulin and it is accepted that the physiologically active steroid is the unbound portion which comprises around 10% or less of the total. Solvent extraction, with or without the use of protein denaturing agents and/or binding displacers disrupts the binding and thus extracts the total steroid, although there is one description of the use of 1 μL *n*-octane suspended from the tip of a microsyringe needle to extract free progesterone (Jeannot and Cantwell, 1997). In the clinical context, 'free' or unbound plasma/serum concentrations of steroids may be of value. Measurement of steroids in saliva (Simard, 2004), which is an ultrafiltrate may be a useful, albeit indirect, method of assessing free steroid in the circulation although a number of problems have been identified in salivary testosterone measurements (Granger et al., 2004). For direct separation of the free fraction, some sort of dialysis (e.g. Torma et al., 1995) or ultracentrifugation is still the method of choice today, usually combined with GC-MS as the concentrations involved are at least an order of magnitude lower than total concentrations (van Uytfanghe et al., 2005). A recent survey of androgen assays recommends the use of dialysis, ammonium sulphate precipitation or calculation (Vermeulen, 2005). A recent review on the measurement of androgens deals with the assessment of 'free' steroids as well as hair analysis (Wheeler, 2006).

3.3 Column Chromatography

This technique, as its name implies, relates to the separation of steroids on material packed into columns (usually glass, but can be inert plastic), which can be of any diameter and/or length. The basis of the separation can be:

- Partition chromatography, straight-phase (ordinary) or reverse-phase chromatography
- Absorption chromatography

In partition chromatography the steroid of interest is separated by its relative solubilities in mobile phase (usually, an organic solvent) and a stationary phase (usually, a hydrophilic water based solvent). The solvents can be reversed and the hydrophilic phase can be used as the mobile phase in which case the system becomes reversed-phase (RP) chromatography. In this technique, the stationary phase is mixed with an inert support, usually Celite (a diatomaceous earth) which has been washed and sieved, and the mixture is carefully packed into the column and the mobile phase allowed to pass through by gravity usually from a reservoir attached to the top of the column. Note that the method of packing such columns can exert considerable influence on the final resolving power (Edwards, 1969a). The mobile phase can remain the same throughout the separation (isocratic elution) or it can be varied (gradient elution). The variation of the solvent is achieved by pre-column mixing from two or more separate reservoirs and can be achieved in a number of different ways. These columns may have considerable resolving power which to some degree is determined by their diameter and length and as a rule of thumb the narrower the diameter and the longer the length, the greater the resolution. They do not however approach the resolution which is achieved using HPLC. They are, however, cheap, relatively simple to set up, and capable of dealing with large quantities of material. Many excellent reviews of the use of column chromatography have been published in the past (e.g. Neher, 1964), and interested readers can learn more about this technique from these reviews, which, even though written more than 40 years ago, are still valuable today, particularly as very little recent research has been carried out on this technique. Both straight-phase and reverse-phase partition columns are still in use today. In an investigation of C19 steroid glucuronides in premenopausal women with non-classical congenital adrenal hyperplasia, androsterone and dihydrotestosterone (DHT) were separated by reverse-phase chromatography on Celite columns eluting with increasing concentrations of ethyl acetate in isooctane using ethylene glycol as the stationary phase (Whorwood et al., 1992) or, in the separation of aldosterone prior to RIA, eluting with increasing concentrations of ethyl acetate in *n*-hexane using 30% formamide in water as the stationary phase (Schirpenbach et al., 2006). A very similar technique using ethylene glycol as the stationary phase (Fig. 3.6) was used for the purification of 5α-androstane-3,17-dione from peripheral plasma by Milewich et al., (1992). The same reverse-phase Celite chromatography, in mini-columns as illustrated in this figure, continues to be used (Bixo et al., 1997; Morineau et al., 1997; Silvestre et al., 1998; Ibrahim et al., 2003; Sieber-Ruckstuhl et al., 2006) – even one paper describing the separation of estradiol, testosterone and

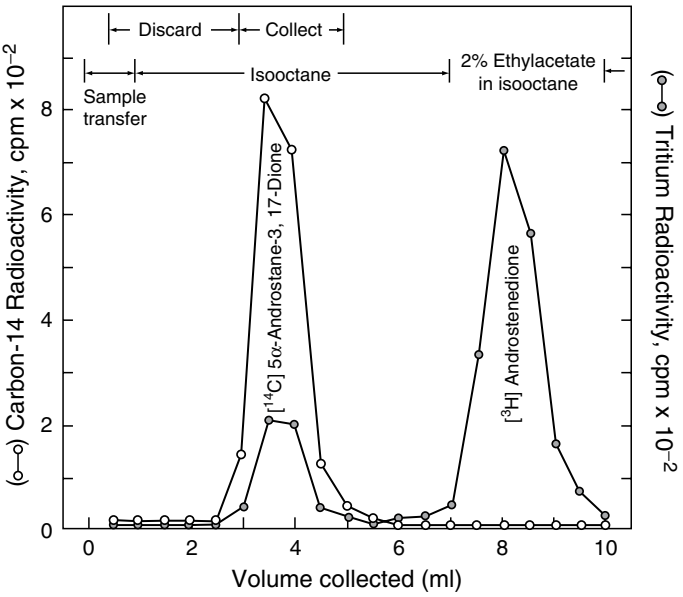

Fig. 3.6 [4-^{14}C]5α-Androstanedione (~2000c.p.m.) was separated from [1,2,6,7-^{3}H]androstenedione (~2000c.p.m.) on a Celite–ethylene glycol column by elution with iso–octane, A mixture of Celite (2.0g) and ethylene glycol was packed into 5ml disposable serological pipettes and conditioned with 5.5ml iso-octane. Samples were added to the column dissolved in iso-octane. After discarding the transfer volume (2 × 0.5ml) and the initial 1.5ml of iso-octane eluent, the next 2ml contained ~98% of [4-^{14}C]5α-androstanedione applied to the column. The bleeding of ^{14}C into the tritium channel was 30%. This chromatographic system was used to separate plasma 5α-androstanedione from more polar steroids. (Reprinted from Milewich *et al.,*1992, with kind permission from Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington OX5 IGB, UK)

androstenedione in the egg yolks from wild Louisiana alligators (Conley et al., 1997)! For quantitative analysis of allopregnanolone in serum or plasma by radioimmunoassay silica micro-column chromatography was necessary when the steroid levels were low (Bicikova et al., 1995). The method included permanganate oxidation of steroids to remove progesterone which was the main cross-reacting steroid. The separation and characterisation of pregnenolone-3-stearate in rat brain by HPLC required the ethyl acetate extracted steroids of brain to be purified further by silica gel chromatography (Shimada et al., 1997).

The column as an alternative can be packed with adsorbent material and steroids can be separated by selective adsorption to this material being eluted from the column by solvents of increasing polarity. Because these adsorptive materials contain varying degrees of water, the separatory process still involves partition to a small degree. Adsorbents which have been used are Florisil (magnesium silicate), aluminium oxide and silica. An example of the use of aluminium oxide columns is given in Fig. 3.7. Florisil has recently been used for purification of sterols in marine sediments after derivitisation prior to GC-MS (Li et al., 2007). The availability of microparticulate silica which can be pre-packed in cartridges or syringes has been

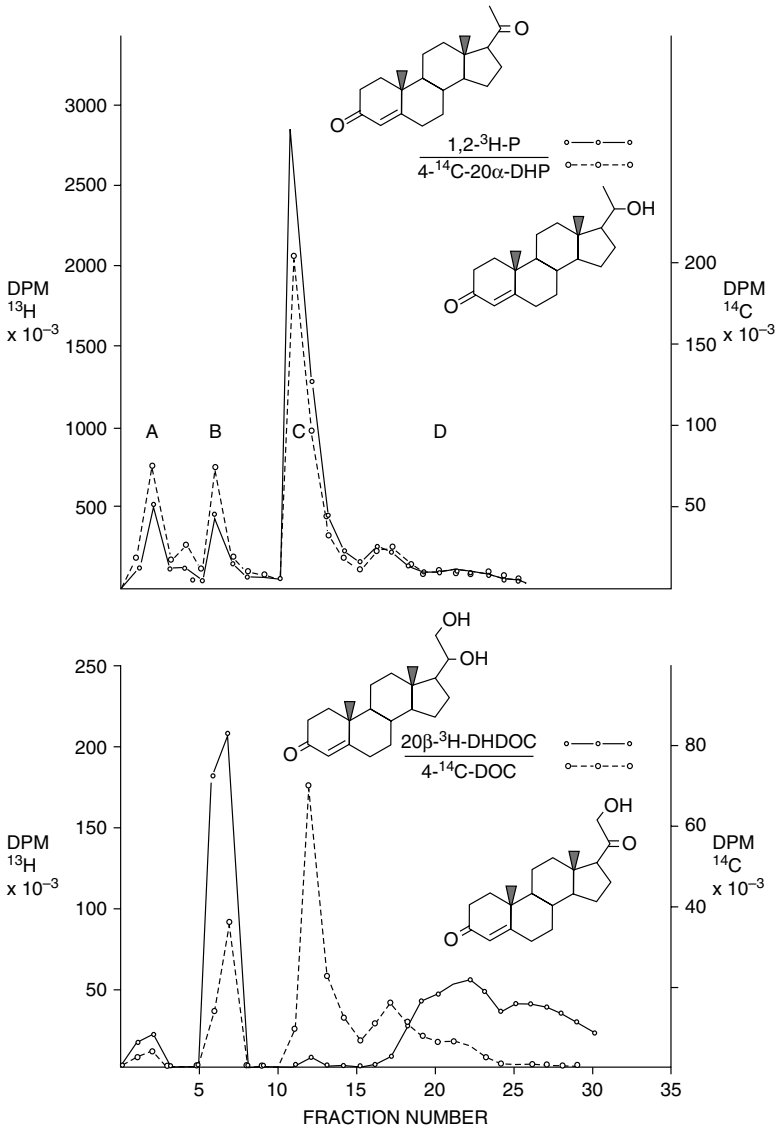

Fig. 3.7 Aluminium oxide separation of rabbit urinary metabolites of progesterone and 20α reduced progesterone (upper trace) and 11-deoxycorticosterone and 20β-reduced 11-deoxycorticosterone. Urine extracts were treated with a β-glucuronidase preparation in 0.2 M acetate buffer, pH 4.5, for 48 h. The aqueous solution was then adjusted to pH 2 with HCI and extracted with ether:ethanol (3:1). After careful drying, the extract was added to an alumina column: 20 g of alumina, deactivated by the addition of water (5%, v/w), added as a slurry in benzene to a glass column (2 cm i.d.). The column was then eluted with ether:ethanol (3:1), 50 ml, ethanol, 20 ml, 50% aqueous (v/v) ethanol, 20 ml, 0.1 M sodium acetate buffer, pH 5, 100ml, and 1 mM sodium acetate buffer, pH 5, 100ml. Eluate fractions (10 ml) were collected and the radioactive content was determined by liquid scintillation counting of a small aliquot of each fraction. Fractions A-D represent: A: 21-deoxysteroids; B: 21-hydroxysteroids; C and D: steroid C-21 acids. (Reprinted from Senciall *et al*., 1992, with kind permission from Elsevier Science Ltd, The Boulevard, Langford Lane, kidlington OX5 1GB, UK.)

discussed in the previous section where ODS-treated silica has been used for the solid-phase extraction of steroids from biological matrices. Microparticulate silica can give excellent separations and has replaced previous column separations using ordinary silica (e.g. use of silica-DIOL column giving comparable separation of cortisol and 11-deoxycortisol to that achieved in mini-celite columns – Morineau et al., 1997). Once again, details of separations of steroids on columns of silica, Florisil and aluminium oxide are given in Neher (1964). Use of microparticulate silica can provide rapid and simple purification and if cartridges are used they may, after washing, be re-used many times and can thus be very cheap. However efficient such cartridges or columns are for small numbers of samples, it must be remembered that where large numbers of samples have to be processed use of any kind of column chromatography can be cumbersome and time-consuming since columns are by their very nature sequential.

The use of SPE cartridges has become very popular for the preliminary fractionation of vitamin D metabolites, but one report (van Hoof et al., 1993) has attempted to resuscitate the use of paper chromatography, pointing out quite correctly that large number of samples can be processed in a single paper chromatographic run. This methodology was subsequently used prior to immunoassay of calcitriol (van Hoof et al., 1999). Similar considerations apply to the use of thin-layer chromatography (TLC) and both these techniques will be briefly discussed below. It is probably not correct to extrapolate from this simple demonstration of the use of paper chromatography for the separation of vitamin D metabolites to suggest that it has much of a role in modern steroid analyses. Separations of steroids using microparticulate silica can provide very satisfactory solutions to many of the problems facing the steroid analyst, and if batch separations are required TLC is most likely to be the preferable option to move to. Microparticulate silica cartridge separation of 11-deoxycortisol and cortisol after solvent extraction (see Fig. 3.8a), prior to immunoassay, has provided a useful method of measuring plasma cortisol levels in patients taking metyrapone (an 11-hydroxylase inhibitor), in whom the concentrations of 11-deoxycortisol rise and interact with the antiserum used in the cortisol immunoassay, achieving a comparable purification to that obtained using reverse-phase HPLC (Wiebe et al., 1991) and mini-celite and sil-DIOL colums (Morineau et al., 1997). Reverse-phase ODS silica has also been used for the separation of steroids and has been used to improve the specificity of an immunoassay for 3α-hydroxy-4-pregnen-20-one by separating the steroid using methanol:water (3:1) prior to immunoassay (Fig. 3.8b)). Microparticulate silica coated with ODS (e.g. Street et al., 1985; Payne et al., 1989) and attached to quarternary amine (Bond-Elut SAX, Scalia, 1990) have been used for the fractionation of steroids into conjugate groups prior to further analysis.

One form of column chromatography which is still widely used is ‘size exclusion’ or ‘gel filtration’ chromatography based on Sephadex, a cross-linked dextran, which has found considerable application (Murphy, 1971) in the separation of steroid groups, such as sulphates, glucuronides etc. Sephadex was designed for use with hydrophilic compounds in polar aqueous solvents but modified Sephadex (e.g. hydroxyalkoxy-Sephadex) lipophilic materials, such as LH-20 (a beaded, cross-linked dextran which has been polyhydroxypropylated) and Lipidex (hydroxyalkoxypropyl-Sephadex), are now available for gel filtration of hydrophobic compounds, such as steroids.

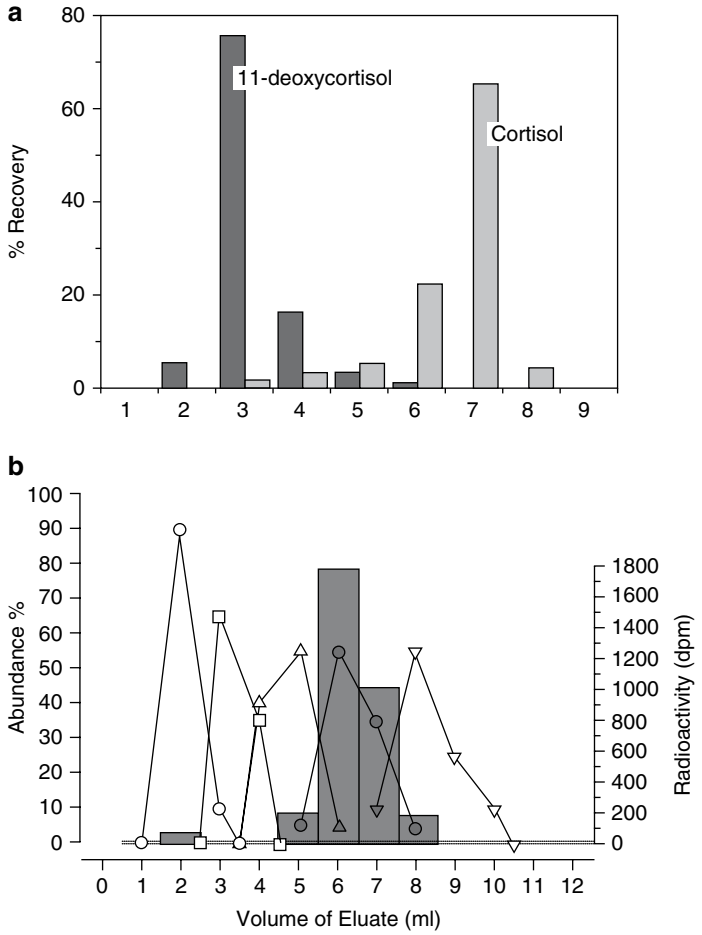

Fig. 3.8 Use of small SPE-C-18 columns or cartridges for the fractionation of steroids. (a) separation of cortisol and 11-deoxycortisol on Sep-Pak SIL cartridges. A mixture of radiolabelled steroids was applied to the cartridge, prepared as recommended by the manufacturers, and the column was sequentially eluted with 5 x 2 ml 1.5% methanol in chloroform (fractions 1-5) followed by 3 x 1 ml of methanol (fractions 6-8). Fractions were collected and the radioactivity was assessed by liquid scintillation counting (courtesy of W. H. Bradbury). (b) Separation of steroids by C-18 minicolumns (6 mm x 42 mm). [^{3}H]-3α-Hydroxy-4-pregnen-20-one (shaded bars), 4-pregnene-3, 11,20-trione (○), 3α-hydroxy-4-androsten-17-one (□), progesterone (Δ), 3α-hydroxy-4-pregnen-20-one (•), and 4-pregnene-3α,20α/β-diols (∇) were added to rat serum. After extracting with ether and washing with NaOH, the dried extract was taken up in 100 µl of methanol:water (3:1) and loaded on to the mini column. The columns were then eluted with methanol:water (3:1) at a flow rate of 0.3 ml/min, using a low vacuum. Fractions (each 1 ml) were collected and monitored by UV absorbance and scintillation counting for radioactivity. (Reprinted from Wiebe *et al.,* 1991, with kind permission from Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington OX5 1GB, UK.)

Sephadex LH20 columns for the fractionation of steroids was described by Wong et al. (1992) who extracted plasma with acetone:ethanol (1:1, v/v) and applied the dried extract, dissolved in methanol:chloroform (1:1, v/v) to an LH20 column, which was

then eluted with the same solvent, separating the steroids into three fractions; free steroids and steroid glucuronides, monosulphates and, after further elution with methanol, disulphates. Sephadex LH-20 has been used over many years for multi-component steroid analysis and has been incorporated into an automated pre-immunoassay chromatographic system for plasma pregnenolone and 17-hydroxypregnenolone (Riepe et al., 2001). A recent application was the separate measurements of 18-hydroxy desoxycorticosterone (DOC) and 18-hydroxy B from a single plasma extract (Fig. 3.9 – Riepe et al., 2003). Testosterone and many other steroid assays by RIA are notoriously unreliable in the newborn period without prior chromatography. Results for testosterone were up to 3.8 times higher with RIA alone compared with an analysis after Sephadex LH-20 eluted with methanol in benzene (Fuqua et al., 1995) although a safer solvent system would be recommended today. Lipidex is also widely used as a means of purifying steroid trimethylsilyl ethers formed by incubation with trimethylsilylimidazole (TSIM), the reagent being retained in the Lipidex, while the steroid derivative is eluted with hexane (Shackleton and Honour, 1976). Sephadex can also be modified to produce, for example, diethylaminoethyl(DEAE)-substituted Sephadex which acts as an ion-exchange column but still with size exclusion properties. A combina-

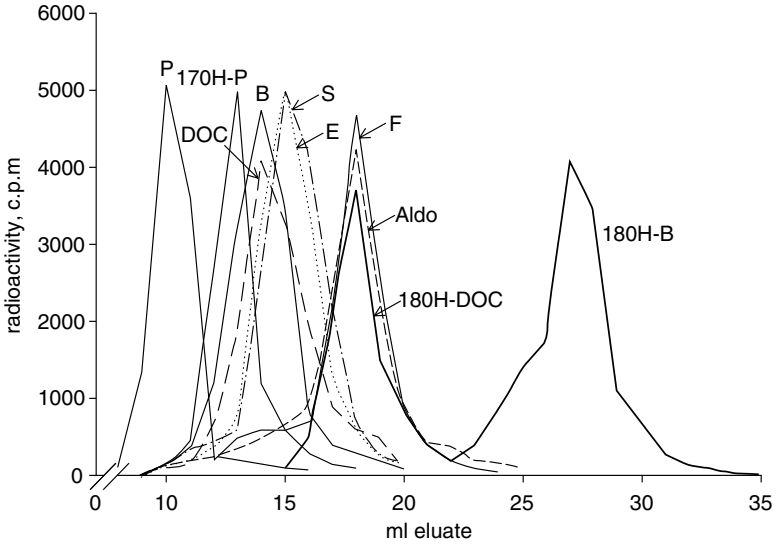

Fig. 3.9 Figure 1 from Riepe et al. (2003) [Chromatogram of tritiated steroids eluted from 45-cm Sephadex LH-20 columns using methylene chloride-acetone (1:2, v/v) as solvent. Peaks eluted: P, progesterone; 17-OH-P, 17-hydroxyprogesterone; B, corticosterone; DOC, 11-deoxycorticosterone; E, cortisone; S, 11-deoxycortisol; F, cortisol; Aldo, aldosterone; 18OH-DOC, 18OH-deoxycorticosterone; 18OH-B, 18OH-corticosterone. A Sephadex LH-20 chromatography system with 450 × 10 mm columns was used for steroid separation. Plasma extracts were redissolved in 1,000 µl of the solvent system and injected into Sephadex LH-20 packed chromatography columns. Elution was performed following gravity. From Riepe et al., 2003 with permission.]

tion of SPE, Lipidex 5000, Amberlyst A-26 and LH-20 chromatography has been used for the extraction of a variety of steroids from blood, liver and faeces prior to GC-MS after derivatisation (Al-Alousi and Anderson, 2002). QAE Sephadex and DEAE Sephadex have been used in a complex pre-GC-MS separation system for oestrogens in urine (Knust et al., 2007).

Urinary steroids can be fractionated into free glucuronide and sulphate fractions after extraction using Sep Pak C18 cartridges using columns of DEAE-Sephadex A-25 (see Fig. 3.10, Hämäläinen et al., 1991) as a preferable and more efficient alternative to the solvent separation procedures previously illustrated in Fig. 3.2. Adlercreutz et al. (2004) have developed a more complex separation system using a variety of ion-exchange resins in sequence to separate oestrogens and phytoestrogens in urine. The use of triethylaminohydroxypropyl (TEAP)-LH-20 as means of extensive and efficient fractionation of steroid groups prior to GC-MS has been described by Axelson and Sahlberg (1983), and this paper also references some of the early works in this field from Sjövall's laboratory. Bile acids can be separated into various conjugated fractions by the use of piperidinohydroxypropyl Sephadex LH20 (PHP-LH20) (e.g. Ikegawa et al., 1992) and the use of ion-exchange columns for steroid separation has been reviewed by Heikkinen et al.,(1983). Many other examples of the use of modified Sephadex for the separation of steroids have been

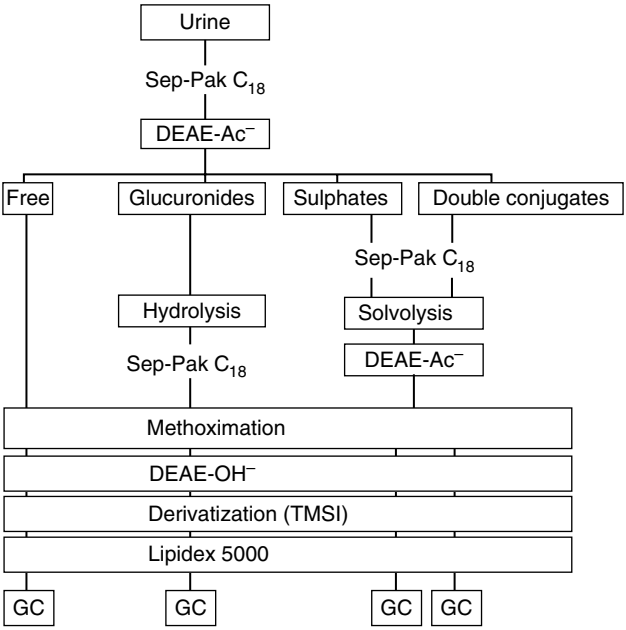

Fig. 3.10 An example of the use of modified Sephadex for the fractionation of urinary neutral steroids prior to GLC analysis (from Hamalainen *et al.*, 1991., with permission)

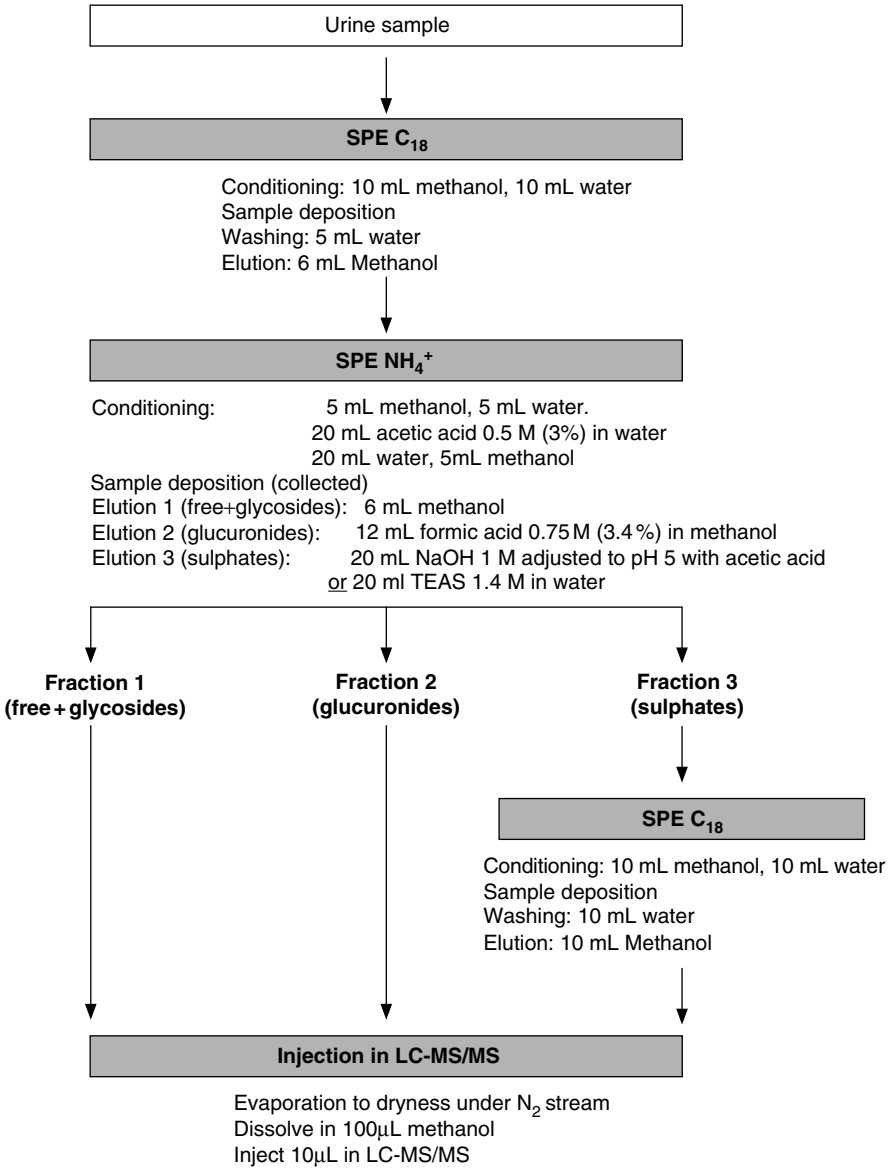

Fig. 3.11 Analytical protocol proposed for a complete separation of the free, glucuronide and sulphate steroids and corticosteroids. (from Antignac et al., 2005, with permission - Figure 4 [*Copyright 2005 Elsevier*])

described (e.g. Setchell and Shackleton, 1973; Archambault et al., 1984; Lisboa et al., 1991; Geisler et al., 2000; Wudy et al., 2002; Riepe et al., 2003; and see review by Sjövall, 2004). A similar separation to that illustrated in Fig. 3.10 can now be achieved using only C18 and NH_4^+ cartridges (see Fig. 3.11).

3.4 Thin-Layer Chromatography

The adsorptive material is coated onto a plate for thin-layer chromatography (TLC). Thin-layer plates can be either prepared in the laboratory or alternatively bought from a number of suppliers. While original TLC was carried out using glass plates, today the support is usually coated onto aluminium foil, which has the advantage that it is light, unaffected by solvents used for elution and areas of interest can be removed by cutting with scissors rather than the alternative procedure of scrapping off the adsorbent material. TLC of steroids is still widely carried out and the adsorptive materials used is usually silica gel although aluminium oxide has been used for the separation of C19 androgens. TLC has the advantage that a large number of samples can be processed in a single chromatographic run. The difficulty with TLC is the need to identify the areas on the plate which correspond to the steroid of interest. Steroids which are ultraviolet (UV) absorbing (e.g., delta-4-3-ones absorb at 240 nm) can be visualised with the use of UV light and adsorptive material is available which contains a fluorescent compound which enhances the UV absorbance of the steroid of interest. Steroids which do not absorb in the UV may have to be visualised by spraying part of the plate to identify the position of standards which have been run together with the samples of interest. There are a wide variety of methods of visualising steroids on TLC plates usually involving spraying and/or heating with a variety of reagents which may or may not be specific for particular types of steroids, and these have been admirably summarised by Edwards (1969b). If standards have not been run, a narrow side strip of the TLC plate can be removed and the steroids located. Fenske (2008) described a TLC method for plasma cortisol which involved dipping the plate into isonicotinic acid hydrazide and quantifying the cortisol by fluorimetry using a scanner.

A further advantage of TLC is that radioactive steroids can be identified by placing the plate in contact with x-ray film, producing an autoradiogram. Dalla Valle et al., 2004; Jang et al., 2007), although metabolites of [^{3}H]progesterone, for example, have been separated by TLC on silica gel, and located by cutting the plates into 1-cm bands and subsequent liquid scintillation counting (Carey et al., 1994). The availability of microparticulate silica has enabled the introduction of the so-called 'high-performance' TLC, which is claimed to have increased resolving power. Reverse-phase systems for TLC have been described but are no longer widely used. Although there are fewer examples of the use of TLC for steroid separation, it is still not uncommon (Fiorelli et al., 2002; Matsunaga et al., 2002, 2004; Robinzon et al., 2004; Waxman and Chang, 2006) The separation of hydroxylated metabolites of progesterone produced by the fungus *Aspergillus fumigatus* has been described (Smith et al., 1994) using Kieselgel 60 F254 (E.G. Merck) eluting with a solvent of ethyl acetate:petroleum (65:35 v/v) and catechol oestrogens, again on silica gel, eluting with benzene:heptane:ethyl acetate (5:2:3:, v/v) or chloroform:ethyl acetate (3:1, v/v) (Jellinck et al., 1991, 2006). The separation of five androstane isomers by TLC has been carefully

optimised for solvent system (Cimpoiu et al., 2006). Placental metabolites of synthetic corticosteroids were examined using TLC to address particularly the activity of HSD11B2 (Murphy et al., 2007). Jellinck et al. (2005) have separated 7α- and 7β-hydroxy DHEA by TLC on silica gel using xylene:ethyl actetate:chloroform (40:20:40) eluting solvent.

Vitamin D metabolites have also been separated by high-performance TLC (Thierry-Palmer and Gray, 1983) and the use of TLC in the separation of the vitamin D metabolites has been advocated as a useful method in countries where HPLC is not available (Justova and Starka, 1981).

Although TLC is still used for steroid separations, very little development has taken place in the last 30 years and it remains very much as it was in the 1980s. Some excellent reviews have been published but a useful introduction to methods available for a variety of steroids was given by Heftmann (1983) from which an introduction to the past literature can be obtained. TLC is still widely used to demonstrate purity of pharmaceuticals (Kotiyan and Vavia, 2000) and to demonstrate the reactivity of tissues and transfected genes (Lee et al.,1998; Godin et al., 1999; Gupta et al., 2001). Pharmaceutical impurities of less than 0.5% are possible without detection (false negative). Data from TLC experiments may thus need to be supplemented by more sensitive tests such as GC, GC-MS, HPLC, LC-MS (Engelbrecht and Swart, 2000; Gorog, 2004; Jellinck et al., 2005, 2006). Table 3.1 gives some examples of the use of TLC in the period 1995-2006, illustrating the continuing popularity of this simple and cheap technology and its wide application in the steroid field, from oestrogens to bile acids.

3.5 Paper Chromatography

Paper chromatographic systems, both straight- and reverse-phase Bush (1961), are seldom used today although careful scanning of the modern literature will still occasionally unearth the use of a paper chromatographic separation. Such separations have been used for the purification of radiolabelled steroids prior to use (Whorwood et al., 1992) and indeed may sometimes be used for the purification of a steroid prior to quantitation – in one such case, testosterone was purified by descending paper chromatography using a Bush A system (Bush, 1961) prior to immunoassay (Swinkels et al., 1992). It is interesting to note here that these authors are the same group that recommended the use of paper chromatography for the separation of vitamin D metabolites (van Hoof et al., 1993), although it is not clear precisely what advantage the use of paper chromatography has in this context, although these same authors have used it subsequently (Van Hoof et al., 1999). Androgens in seminal plasma were significantly lower by RIA after paper chromatography (Zalata et al., 1995). Measurement of cortisol in patients with chronic renal failure, who were given dexamethasone to suppress the cortisol

Table 3.1 Some examples of methods using of thin-layer chromatography for steroid separation since 1995

Application	TLC type	Eluting solvent	Ref
Testosterone metabolites	Silica gel (Whatman LK5DF)	$CHCl_3$:acetone(9:1) followed by CH_2Cl_2:EtAc: 95%EtOH (7:2:0.5)	Agrawal et al. (1995)
7-Hydroxylated metabolites of pregnenolone in mouse brain microsomes	Silica gel (Merck F254)	Ethyl acetate	Doostzadeh and Morfin (1997)
Steroidal alkaloid glycosides	Silica gel	$CHCl_3$:MeOH: ammonia soln (7:2.5:1)	Tanaka et al. (1997)
Analysis of androst-5-enediol in human prostate cancer cells	Not specified	Toluene:95% ethanol (9:1) x2	Miyamoto et al. (1998)
Bile acids and conjugates	? + Methyl-β-cyclo-dextrin	RP-HPTLC 2D	Momose et al. (1998)
C19 steroids formed in mammalian cells	Silica gel (Whatman) impregnated with $AgNO_3$	Toluene:acetone: $CHCl_3$ (8:2:5)	Godin et al. (1999)
Microsomal metabolites of DHA	Silica gel (F254)	2-D (1) Tol:MeOH (9:1) (2)$CHCl_3$:ether (1:1)	Schmidt et al. (2000)
Estradiol in pharmaceutical preparations	Silica gel 60F254	$CHCl_3$:acetone: IPA:gla. Acetic acid (9:1:0.4:0.1)	Kotiyan and Vavia (2000)
Isolation of aldosterone ester from heart	Florisil	Ethanol:$CHCl_3$ (2:98)	Gomez-Sanchez et al. (2001)
Estrogen metabolism in colorectal cancer cells	Silica gel Merck F254)	CH_2Cl_2:EtAc (4:1)	Fiorelli et al. (2002)
7-Hydroxylation of DHEA in Alzheimer's	Silica gel (F254)	$CHCl_3$:EtAc (4:1)	Weill-Engerer et al. (2003)
7-hydroxylation of DHEA in pig liver microsomes	Silica gel GF200 m	EtAc:Hx: gla Acetic acid (18:8:3)	Robinzon et al. (2004)

(continued)

Table 3.1 (continued)

Application	TLC type	Eluting solvent	Ref
DHEA metabolism in brain	Silica gel (F254)	Xylene:EtAc: $CHCl_3$ (40:15:45)	Jellinck et al. (2005, 2006)
HPTLC of androstenes	Silica gel (F254)	Bz:MeCN, Bz:EtAc Bz:dioxane	Perisic-Janjic et al. (2005)
Androgen metabolism in synovial cells	Silica gel (Merck F254)	Tol:MeOH (9:1) then at right angles $CHCl_3$:ether (1:1)	Schmidt et al. (2005)
Estradiol as aromatase inhibitor in breast cancer cells	Silica gel 60 F254	$CHCl_3$:EtAc (4:1) or CycloHx:EtAc (1:1)	Pasqualini and Chetrite (2006)
6β-Hydroxylation of testo. by CYP3A	Silica gel	CH_2Cl_2:acetone and $CHCl_3$:EtAc:EtOH	Waxman and Chang (2006)
Interconversion of 7α- and 7β-OH DHEA	Silica 60	$CHCl_3$:EtOH: H_2O (87:13:1)	Muller et al. (2006)
Optimisation of separation of 5 androgens	Silica 60	7 Different systems	Cimpoiu et al. (2006)
P450c17 assay in fish tissues	Silica Gel (Merck 5729)	CH_2Cl_2:EtAc:MeOH (85:15:3)	Zhou et al. (2005)
Purification of pyridine-carboxylate oxosteroid derivatives	Silica gel 60 F254 (Merck)	Not given	Yamashita et al. (2007a)
Metabolism of synthetic steroids by human placenta	Silica gel 60 F-254	$CHCl_3$:MeOH (95:5 or 97:3)	Murphy et al. (2007)
Microglia conversion of DHEA to androst-5-enediol	Silica gel	Xylene:EtAc:$CHCl_3$ (10:25:75)	Jellinck et al. (2007)
5α-Reductase activity	HPTLC Keiselgel 60 F254	$CHCl_3$:acetone (9:1) × 3 air-drying between each run	Bratoeff et al. (2007)
5α-Reductase activity	Silica gel	Toluene:acetone (8:2)	Jang et al. (2007)
Application	TLC type	Eluting solvent	Ref

(continued)

Table 3.1 (continued)

Application	TLC type	Eluting solvent	Ref
Cholesteryl ester hydroperoxide isomer analysis	Silica gel 60 F254	Hx:ether:acetic acid (70:30:1) blotting onto PVDF membrane prior to GC-EI(+)-MS	Minami et al. (2007) and Kawai et al. (2007)
DHEA conversion to 5-androstenediol	Silica gel	Xylene:EtAc:$CHCl_3$ (10:25:75)	Jellinck et al. (2007)
Steroid sulfatase activity - separation of E1 & E2	Silica gel G/UV-254 Alugram	13% EtOH in toluene	Stute et al. (2008)
DHEA metabolism in songbird brain	Silica gel	$CHCl_3$:EtAc (4:1)	Pradhan et al. (2008)
Estradiol and progesterone inhibit sulfatase activity	Silica gel 60 F254	$CHCl_3$:EtAc (4:1)	Chetrite et al. (2007), Pasqualini & Chetrite (2008)
Novel P450c17 in fish	Silica gel Merck 5729	CH_2Cl_2:EtAc:MeOH (85:15:3)	Zhou et al. (2007)
Brain microglia express steroid converting enzymes in mice	Silica gel aluminium sheets	$CHCl_3$:EtAc:xylene (62:21:17) and (68:23:9)	Gottfried-Blackmore et al. (2008)
5α-Reductase in normal and neoplastic prostate biopsies	Kieselgel 60 F254	EtAc:Bz (2:1)	Oliveira et al. (2008)
Separation of testosterone & esters	Variety of plates: silica, ODS and aluminium oxide	Variety of solvents in chamber for horizontal HPTLC at 20°C	Zarzycki and Zarzycka (2008) Zarzycki (2008)

production, were superior after paper chromatography than by direct RIA (Van Herle et al., 1998). It is our experience that paper chromatography using Bush systems is time-consuming and significant amounts of potentially interfering non-specific material is usually eluted from the paper together with the steroid of interest, giving rise to high blank values. While careful washing of the paper prior to chromatography can often reduce the blank values, this is not always the case and in our view there is very little to recommend the use of paper chromatographic systems for steroid separation today.

3.6 Gas–Liquid Chromatography

Gas–liquid chromatography (GLC or GC) is a partition system where the steroid solute is in the vapour phase. Because of the relatively high molecular weight of steroids and their derivatives, GLC has to be carried out at high temperatures, usually in excess of 200°C. The vaporised steroid, once introduced into the GLC column, is carried through the system by a gas, usually helium, because it is less dense than nitrogen, gives improved separation but is of course much more expensive to buy. Better separation can be achieved with hydrogen (Impens et al., 2001) but there are safety issues to be considered. For many years, the separation procedure with the greatest resolving power was gas chromatography (the early years of GLC were reviewed by Horning, 1968), originally carried out using packed columns but today capillary columns of glass or fused silica are more popular. The analysis of bile acids by GLC was reviewed by Batta and Salen in 1999, and since then they have extended the analysis of faeces to achieve a lipid profile (Batta et al., 2002). The measurement of glucocorticoids in biological fluids using, inter alia, GLC has been reviewed by Holder (2006).

3.6.1 Column Technology

Steroids of interest are separated by their relative solubility in the stationary phase which is a thermostable compound, liquid at the column temperature used, which is coated onto an inert support, usually Celite in packed columns or onto the walls of the tube in capillary columns. Wall-coated columns give better separation as would be expected from the increased number of theoretical plates produced by this methodology. There are a wide variety of stationary phases which have been used for the separation of steroids and the catalogue of any chromatography supplier will give lists of such material. Stationary phases are, however, usually substituted siloxane polymers which can be modified chemically. The commonest non-selective (i.e. separation is achieved purely by molecular weight which is related to vapour pressure) stationary phase was SE30 which has now been superseded by OV1 or OV101. Chemical substitution of other groups onto the siloxane polymer gives rise to further stationary phases which may have selective characteristics. For example, the use of cyano-substituted siloxane polymers is useful for the separation of ketones. It is necessary that these stationary phases be thermostable and that they are not eluted from the column. The elution of the stationary phase from the column, so-called column ‘bleed’, is less with capillary columns than packed columns and has been largely overcome by the use of chemically bonded stationary phases. There are a wide variety of stationary phases and pre-coated columns available and these are usually listed, together with their characteristics, in chromatography catalogues from a large number of commercial firms active in this area. Good examples of this are the catalogues

published by Chromatography Products and Supelco (Poole, Dorset, BH17 7NH) or Jones Chromatography (Hengoed, Glamorgan CF8 8AU). Complete resolution of all the steroids of interest may not be achieved using a single column, and it may be necessary to use a combination of columns. The separation of 6-hydroxy bile acids is an example, where a combination of CP-Sil-19 CB and CP-SIL-5 CB columns were needed (Batta et al., 1995).

Originally, the columns used for GLC were coiled glass columns of approximately 0.4 cm in diameter, 1–5 m in length, silanised by treatment with dimethyldichlorosilane, and packed with the stationary phase which was coated onto an 'inert' support which was usually acid-washed and sieved Celite. On many occasions, the 'inert' support was in fact not as inert as it was claimed to be and adsorption problems occurred leading to poor peak shape and sometimes loss of steroid. GC separation of bile acids has been reported using stainless steel capillary columns (Iida et al., 1995). The modern capillary columns, now usually wall-coated open tubular (WCOT) where the stationary phase is coated on the inside of fused silica columns of diameter of approximately 0.2 mm, although megabore columns (0.5–0.75 mm i.d.) with greater capacity, but reduced resolution, are also available. The thickness of the coating (from around 0.1 μm upwards) affects the capacity of the column, the greater the thickness, the greater the capacity. The effect of the thickness of phase coating are interdependent with those of changes in internal diameter and this relationship can be expressed as a 'phase ratio' (β), to which capacity has an inverse relationship. WCOT columns like their predecessors still suffer from the problem of column bleed at high temperatures and attempts have been made to overcome this problem by the use of chemically bonded stationary phase. This process can affect retention times of the steroids. Capillary columns can be of any length up to around 30 m, and at such lengths, they provide one of the most effective means of separating steroids with extremely high resolution. Short and micro bore columns are becoming fashionable for fast chromatography – a urine steroid profile using such columns can be obtained in less than 8 min with a 10-cm fine column compared with 40–60 min on a 30-m column. The GC needs an injector and flow control that withstands very high pressures when narrow columns are used. Short columns can enable faster separation but narrow bore columns can be easily overloaded with sample. Few publications have yet described the benefits for steroids (Rossi et al., 1994).

The increased resolution of capillary columns has reduced the need to use more than one stationary phase and for most applications a polar bonded-phase column is effective. When GC is used alone, polar columns are still necessary to fully separate all steroids but in GC-MS a single column, particularly when SIM is employed, is generally adequate in most situations. In our instruments, we use J & W (J & W Scientific, Folsom, CA 95630 - see www.chromtech.com) 15 m DB-1 polar bonded-phase columns of 25 mm i.d. and 25 μm film thickness, which are solvent rinseable. The upper temperature limit for sustained periods is 325°C, although some columns can be used to 400°C or more without significant column degradation. Some years ago, there was a need to separate methyl ether and trimethylsilyl ether derivatives of cortolic acids by gas chromatography without mass spec-

trometry. This was a case where only a polar column was effective in separating α-cortolic, α-cortolonic, β-cortolic and β-cortolonic acids. A Carbowax 20 M column (polyethylene glycol stationary phase) was used but like most polar columns, it had limited lifetime (Shackleton et al., 1980a). While Carbowax 20 M can still be purchased, superior performance is offered by J & W in the form of DB wax. These newer columns have bonded ethylene glycol stationary phase so are solvent rinseable, more robust and have less bleed than earlier columns.

3.6.2 Sample Injection

Apart from the qualities of the column itself, the length of time a column can be left untouched in an instrument is largely dependent on the injection system. In injection systems where only volatiles reach the capillary (solid injection, splitless injection with cold trapping), the first few centimetres of the column remain relatively clean for many injections over several weeks. In injection systems where solubilised material goes directly into the column (split injection, on-column injection), the first part of the column soon contaminates and must be cut off periodically. This results in a gradual shortening of column length which often does not affect resolution but may require data-system re-programming for automated instruments since the retention times of expected components change markedly.

The most generally applicable injection system for steroid derivatives is *splitless* injection with *cold-trapping*. This system allows a large injection volume (~2 mL) to be used. This vaporises in a removable glass insert in the heated injection port and the heavier (less volatile) components condense in the initial part of the column which is maintained at near ambient temperature (cold-trapping). During temperature programming, the solutes re-vaporise when they get to their volatilization temperature and are duly separated. This system keeps the column relatively clean since most of the involatile material is deposited in the injector glass insert. These inserts must be replaced every few days and for some separations silanisation of the insert is advisable. There are refinements to injection systems with pulsed and pressure features. Injection liners come in many shapes to affect maximum delivery of the sample depending on volatility, sample volume and sensitivity.

The *falling needle* solid injection system and its automated variant which employs a carousel containing a multitude of glass sample capillaries (Shackleton and Honour, 1976) are less commonly used. This system was favoured for many years before the introduction of the splitless system but it does have the disadvantage that in automated mode samples remain dry for many hours before analysis. Unstable derivatives are particularly vulnerable in this system. The solid injection systems have a great advantage in that the total amount of sample can be concentrated on the needle or in the injection vial. In syringe injection systems, particularly automated ones, the amount of sample that can be injected is usually only a small fraction of the solvent volume.

On-column injection from a syringe is particularly useful for minimizing gas chromatographic discrimination against high-mass components, but the heavy column contamination resulting mitigates against using the technique. There also seem to

be few reasons outside industrial analysis for using *split* injection since this both wastes much of the analyte solution and results in heavy column contamination.

3.6.3 Derivative Formation

GLC, as already mentioned, requires the steroid solute to be present in the vapour phase and capable of analysis without destruction at the high temperatures required for this. Androgens and oestrogens can be analysed by GLC without derivatisation since they are stable at the oven temperatures required but have long retention times and occasionally may be dehydrated. In addition, the presence of underivatised hydroxyl groups may impair the chromatographic resolution due to adsorption during chromatography. C21 steroids with the 17-hydroxycorticosteroid side chain undergo thermally-induced side-chain cleavage giving 17-oxo steroids and vitamin D metabolites undergo B-ring cyclisation, giving *pyro*- and *isopyro*-isomers (see Chapter 11 on vitamin D in this book). In order to improve chromatographic resolution and to prevent sidechain cleavage, chemical derivatisation is usually carried out prior to chromatography.

The majority of methods using GC (and GC-MS) for identification and measurements of steroids and metabolites with low detection limits require formation of derivatives prior to GC. A review of some useful derivatives, albeit in the context of GLC-mass spectrometry, is given by Brooks et al. (1983) and the place of GC-MS in the era of molecular biology is commented on by Wudy and Hartmann (2004). Halket has published a number of excellent reviews on this topic (Halket and Zaikin, 2003, 2004, 2005, 2006; Zaikin and Halket, 2003, 2004, 2005, 2006). Trimethylsilyl ether (TMS) and other alkylsilyl ethers (such as tertiary-butyldimethylsilyl ether) are suitable for hydroxyl groups and *O*-methyloxime (MO) for ketones. Enol-TMS ethers are formed on ketones with *N*-methyl-*N*-trimethylsilylfluoroacetamide-trimethylsilyliodosilane. These enol-TMS ethers are used in doping control for the detection of anabolic steroids (Saugy et al., 2000) and for corticosteroids (Choi et al., 2002). There have been some doubts recently about the reliability of this derivative procedure for anabolic steroids (Meunier-Solere et al., 2005) and oestrogens (Shareef et al. 2004, 2006). Pentafluorophenyldimethylsilyl-TMS ether (flophemesyl) derivatives give intense molecular ions in MS that are attractive for GC-MS analysis (Choi and Chung, 1999; Choi et al. 2001). Heptafluorobutyrates have been used for quantitative analysis of several steroids (Scherer et al., 1998; Wudy et al., 2000, 2001, 2002). Bismethylenedioxy-pentafluoropropionate derivatives have been used in the analysis of cortisol metabolites (Furuta et al., 2000a, b). The simultaneous analysis of fatty acids, sterols and bile acids as *n*-butyl ester TMS derivatives has been reported (Batta et al., 2002).

Hydroxyl groups can be derivatised in a number of ways but usually ethers are formed. Use of dimethyldichlorosilane, trimethylchlorosilane and bistrimethylsilylacetamide (BSA) or bistrimethyltrifluoroacetamide (BSTFA) will form trimethylsilyl derivatives on most of the steroid hydroxyls. Use of BSA or BSTFA alone will only form such derivatives on non-sterically hindered groups, whereas trimethylsilylimidazole (TSIM) will react with sterically hindered groups (such as the 25-hydroxyl in 25-hydroxyvita-

min D or the 17α-hydroxyl in the corticosteroids). Other silyl derivatives have also been used for the separation of bile acids (Iida et al., 1992, 2001; Batta et al., 1998). Other ester derivatives such as formates and acetates have also been used. The choice of derivative which is formed will depend upon the method of quantitation as well as the need for good chromatography. The use of tertiary butyldimethylsilyl ethers for quantitative GC-MS is very common since these derivatives give mass spectra without extensive fragmentation (Finlay and Gaskell, 1981; Masse and Wright, 1996). Vicinal hydroxyls can be derivatised to give cyclic alkyl boronate esters and the formation of these derivatives with unknown steroids gives an indication of the structure of the steroid (Brooks and Harvey, 1969). Oxo groups can be derivatised as oximes using methoxyamine hydrochloride in pyridine although enol-trimethylsilyl ethers can also be formed. The carboxyl group of steroid acids must also be derivatised prior to GLC and usually methyl esters are formed and there are simple methods available for this (e.g. Lillington et al., 1981). Mixed esters can also be used, such as the formation of cyclic boronate esters across vicinal hydroxyl groups and subsequent trimethylsilyl ether formation on the remaining hydroxyl groups, which has been used with vitamin D metabolites (Coldwell et al., 1984, 1990). The choice of derivatives for GLC must be a balance between the requirements of the chromatography and those of the detector.

3.6.4 GLC Detectors

Steroids separated by GLC are detected by one of the three main methods, the commonest of which is the flame ionisation detection (FID), which responds to all steroids with varying response factors. In order to use GC-FID for the quantitation of steroids, therefore, it is necessary to set up a standard curve or establish a response factor for the steroid of interest, assuming a straight-line response. Compounds containing nitrogen atoms can be detected with a nitrogen-phosphorus detector (NPD), but as most steroids commonly encountered do not contain nitrogen, using this detection system requires the formation of nitrogen-containing derivatives such as methyloximes (e.g. Vanluchene et al., 1990). This detection system can therefore be quite useful for the selective measurement and detection of steroids containing oxo groups. The third method of detection involves the use of electron capture, a potentially extremely sensitive detector, which requires electron-capturing moieties (usually, halogen atoms) in the analyte. With a few synthetic exceptions, steroids do not contain halogen atoms and thus derivatives (e.g. halogenated trimethylsilyl ethers, Pinnella et al., 2001) have to be made. A good example of the use of halogenated silyl ether derivatives is in a method for the measurement of DHA in plasma by GLC with electron capture detection after the formation of iodomethyldimethylsilyl ethers (Chabraoui et al., 1991). This report is also of interest in that it used reverse-phase Celite columns to purify the analyte after extraction with ether and aluminium oxide columns to purify the silyl ether derivative prior to GLC. Reports of methods using GLC with electron capture are becoming increasingly rare as these detection systems are difficult to use and are susceptible to detector contamination. GLC-EC, although extremely sensitive, is also very difficult to use quantitatively and although it was of

considerable value in early measurements of plasma steroid hormones, it has largely been replaced by interfacing the GLC with a mass spectrometer which when operated correctly can be as sensitive as electron capture and more selective. Pentafluorobenzyl and pentafluoropropionate derivatives have been used as electron capturing derivatives for oestrogens by GC-NI-CI-MS (Brandon et al., 1999; Kim et al., 2000; Evans et al., 2005). Other derivatives have been reviewed by Shimada et al., (2001).

GLC can be linked to mass spectrometers and the availability of simple bench-top spectrometers has reduced the cost of GC-MS. The application of MS to the analysis of steroids is discussed below. The use of capillary gas chromatography for the analysis of urinary steroid profiles is a very good example of the valuable use of GLC-FID using trimethylsilyl ether-*O*-methyloxime derivatives (Shackleton et al., 1980b - Figure 3.12) and when such systems are also linked to an MS, allowing identification of unusual peaks, they can be of immense value (e.g. Palermo et al., 1996; Shackleton, 2008). An example of such a profile for a normal man is given in Fig. 3.12). This is a typical example of such a profile, which provides useful and rapid information in

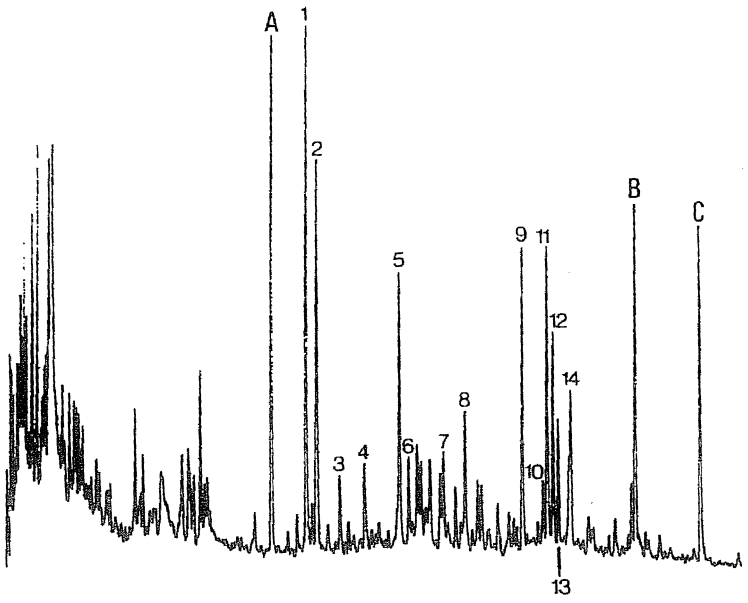

Fig. 3.12 Urinary steroid profile from a normal man 28 years of age. Urine samples were treated with β–glucuronidase and sulphatase enzymes and steroids extracted with Amberlite XAD-2 and Sephadex LH-20. Steroids were then derivatised forming pertrimethylsilyl ethers on all hydroxyl groups and *O*-methyl oximes on oxo groups. GLC was carried out using a 15–25m WCOT column with OV1 or OV101 and using a temperature programme from 160°C to 260°C at approximately 2.5°C/min. Three internal standards were used for the GLC: A, 5α-androstane-3α, 17α-diol; B, stigmasterol; and C, cholesteryl butyrate. Steroids (underivatised) are identified as follows: 1, androsterone; 2, aetiocholanolone; 3, DNA; 4, 11-oxo-aetiocholanolone+11-oxo-androsterone; 5, 11β-hydroxyandrosterone+17α-hydroxypregnanolone; 6, 11β-hydroxyaetiocholanlone; 7, pregnanetriol; 8, androstenetriol; 9, tetrahydrocortisone; 10, allotetrahydrocorticosterone; 11, tetrahydrocortisol; 12, allotetrahydrocortisol; 13, α-cortolone; 14, β-cortolone+β-cortol (from Shackleton et al., 1980b, with permission)

the clinical setting. A recent modification (Pujos et al., 2004) of this technique for urinary steroids involved solid-phase extraction and separation of the extracted steroids using a second SP cartridge into three fractions containing DHEA sulphate, corticosteroids and androgens. Each fraction was analysed separately, the corticosteroids being oxidised to androgens before analysis. It is not clear what advantage is conferred by this somewhat cumbersome methodology.

Some methods not requiring hydrolysis of steroid conjugates have been described. DHEA-S decomposes with loss of sulphuric acid at the elevated temperatures of the GC injector to give reproducibly one of the three isomers with double bonds in the A and B rings. Methyl ester formation before TMS ether will protect glucuronides for GC (Zemaitis and Kroboth, 1998). A method for the separation of the 3-glucoside and 3-glucuronide conjugates of bile acids was described (Iida et al., 1995). Applications of GLC systems, not using mass spectrometry, for the separation of steroids are decreasing, with the exception of the use of urinary steroid profiling, which is still a useful clinical tool, even when GC-MS is not used (Taylor, 2006). There are some useful reviews (Wolthers and Kraan, 1999; Shimada et al., 2001; Volin, 2001; Appelblad and Irgum, 2002).

3.7 High-Performance Liquid Chromatography

HPLC has become an increasingly important chromatographic technique for steroids because:

(i) High temperatures are not required.
(ii) Choice of stationary and mobile phases for optimal separation.
(iii) Material can be recovered from the column eluates for further analytical procedures.
(iv) The resolution achieved by HPLC is superior to TLC and paper chromatography,
(v) HPLC offers the potential and versatility for separation of intact conjugates.
(vi) Although most steroid metabolites are virtually without ultraviolet absorption, which is the most useful of current detectors, some further metabolites of steroids can be detected with a refractive index or an electrochemical detector or by the use of pre- or post-column reaction with compounds which cause enhanced UV absorbance, fluorescence etc.
(vii) Methods of linking HPLC to mass spectrometers have greatly improved, allowing the routine use of LC-MS and LC-MS-MS (see below).

Methods for HPLC separation of steroids up to 1987 have been reviewed by Makin and Heftmann (1988). Reviews by many authors (Volin, 1995, 2001; Marwah et al., 2001; Shimada et al., 2001; Gorog, 2004) consider a number of HPLC separations of steroids in relation to clinical applications and sensitivity, respectively. Diode array detection (DAD) improves the quality of UV data, but is less sensitive.

There have been a few papers since 1995 with new applications of HPLC (e.g. Hu et al., 2005) and Table 3.2 is a selection of LC methods in the period 1990-2008 but does not include LC-MS or LC-MS-MS methods, which are discussed later (see also review by Honour, 2006). LC-MS-MS (i.e. tandem mass spectrometry) seeks to use the flexibility of the detector to obviate the need for extensive clean up of samples. In essence, a short HPLC column is little more than an injection system and "dilute and shoot" analysis is expected. There are a number of dangers here that will be addressed elsewhere in this chapter.

3.7.1 Columns

The separation of steroids with HPLC can be effected by absorption, partition, ion-exchange, reversed-phase (RP) and reversed-phase ion-pair chromatography. High-performance silica and alumina columns give excellent separation of steroids. RP columns eluted with polar binary solvent mixtures, usually methanol or acetonitrile with water, are now used widely. RP columns using microparticulate silica coated with C18, C8, C2 and phenyl materials (listed in Fig. 3.3) have been used. The chromatography depends largely on partition so that selectivity will vary to some extent with the carbon chain length and the nature of the mobile phase. A risk with RP packings is that very non-polar material will accumulate on the columns and decrease separation. This can be prevented to some extent by the use of a guard column (30–70 mm in length) containing the pellicular equivalent of the analytical column. Guard columns are cheap and can be dry-packed. The first few millimetres of packing from the analytical column can also be replaced at intervals.

RP columns with 60,000–80,000 theoretical plates per metre are common. These offer excellent resolution and sharp peaks permit detection by UV absorption of around 1 ng of steroid injected onto the column. Typically, columns are 100–300 mm in length and around 4–5 mm internal diameter. Cyano and amino phases have been used to effect the separation of corticosteroids (Ando et al., 1986). HPLC of polar oestrogens has been achieved on ion-exchange columns (Musey et al., 1978). Micro-bore columns (<2 mm i.d.) may permit increased sensitivity by narrowing the elution peak, but depending on the volume of sample and the total mass of material in the extract, there may be a loss of peak shape and resolution.

The complete separation of naturally occurring mixtures of steroid hormones poses problems due to the wide range of polarities and the tendency for steroids of similar polarity derived from different metabolic pathways to elute in clusters. Careful selection of the stationary phase from the range of commercially available products can enable a system to be devised with high selectivity (O'Hare et al., 1976; Schoneshofer and Dulce, 1979). Silica packings to which are bonded octadecyl or diol groups are most popular for general use. Supports differ in particle size, porosity and levels of residual accessible silanol groups. Synthetic polymers may be more inert than silica. The physical characteristics of many packings have been studied with various solvent gradients. There seems to be no

easy means to identify the most suitable packing for a particular separation. Selective differences cannot be firmly attributed to alkyl chain length or to shape of the packing. Immobilised cyclodextrins, macrocyclic polymers of glucose, which have been used for the extraction of steroids because of their ability to form inclusion complexes (vide supra), have also been introduced as stationary phases (cyclobond) for steroid chromatography (Agnus et al., 1994; Zarzycki et al., 2006; Clifton et al., 2007 and see Table 3.2) and may have advantages to offer but that may depend on carbon load (Zarzycki et al., 2002). Carbon-coated zirconia was compared with porous graphitic carbon stationary phase for separation of equine conjugated estrogens giving separations superior to C18 and alkyl-bonded silica phases (Reepmeyer et al., 2005). In a similar fashion, graphitised carbon, which has been used for crude steroid fractionation (Andreolini et al., 1987, see Fig. 3.4), may also have use as a stationary phase for steroid HPLC since it is micro-crystalline and contains no unreacted silanol groups such as those on silica-based materials and thus may be considered to be a suitable inert material for RP chromatography.

Supports have variable and often incomplete coverage of residual silanol groups ('uncapped') which affects separation, peak shape and recovery. Some packings with about 5% of uncapped silanol groups are chemically reactive with steroids due to intramolecular hydrogen bonding. This leaves the phase acidic and may explain the instability of certain steroids in such systems. Aldosterone and 18-hydroxylated steroids are susceptible to a number of reactions on certain columns which can influence the quality of the HPLC result. Acid, such as may be found on uncapped HPLC supports, can lead to ring closure of such steroids with a bridge of C-18 to C-20 or C-21. In the presence of methanol, this may lead to the formation of methyl ethyl ketals. Other products, dimers and isomers are possible leading to the production of a number of peaks in the HPLC analysis of a single compound. These products can have retention times spread throughout a solvent gradient elution of a RP column. This may be disastrous in the interpretation of a metabolic study unless products are characterised by other means. Some supports are not recommended for aldosterone and related steroids, e.g. 18-hydroxycorticosterone (O'Hare et al., 1980). The extent to which a packing is not covered (end-capped) can be determined by a methyl red absorption test (O'Hare and Nice, 1981). Glycine-conjugated bile acids can be separated by using RP coated silica columns (Nambara and Goto, 1988) and Fig. 3.13 illustrates an example of such a separation carried out on a reverse-phase ODS silica column, using gradient elution with mixtures of acetonitrile:methanol:water using fluorescence detection of the 4-bromoethyl-7-methoxycoumarin derivatives formed pre-column (Guldutuna et al., 1993).

3.7.2 Mobile Phases

Chromatographic systems suitable for HPLC of steroids are based upon or can be tested with TLC (Hara, 1977; Cimpoiu et al., 2006). Useful separations of steroids can be achieved using isocratic chromatography on silica gel with binary solvents (Hara et al., 1978; Capp and Simonian, 1985). The separation of a range of steroids is best achieved with

Table 3.2 Some selected references (1990–2008) for HPLC methods of steroid analysis not involving mass spectrometry

Steroid Analyte(s)	Column	Solvent	Special conditions	Detection	Reference
Digoxin and metabolities (standards)	Spherisorb ODS II	Acetonitrile:water (20:7)		Electrochemical detection of 3,5-dinitrobenzoyl derivatives	Embree and McErlane (1990)
15 Bile acids and pregnanediol, pregnenediols	Finepak SIL C18	50 nM imidazole buffer, pH 6.0:THF (approx. 1:1) or :acetonitrile (25:75)	40°C	3a-OHSDH to give 3-oxoste-roids, forms dansylhydra-zones. Post-column detection by peroxy-oxalate chemiluminescence	Higashidate *et al.* (1990)
Some anabolic steroids (standards)	Bondex C18	Methanol:water (7:3)		UV at 254 and 280 nm	Noggle *et al.* (1990)
Studies on aromisation of 19-oxygenated 16-OH-androgens	Radial Pak C18	0.5% Phosphate buffer, pH 3.0:methanol (1:1)		Electrochemical	Numazawa *et al.* (1990)
Corticosterone in rat urine	Capcell PAK CN	MeCN:water (24.5:75.5)	40°C	Post-column mixing with 65% sulphuric acid at 65°C. Measures fluorescence (exc. 460 nm, em. 510 nm)	Sudo (1990)
Bile acids and conjugates	Develosil ODS-5 YMC-GEL C_8 mBondapak C_{18}	MeCN–0.5%$(NH_4)_2SO_4$ (or KH_2PO_4, pH4)±b-cyclodextrin in various mixtures (i.e. 5:18, etc.)		Pre-column formation of 3-(1-anthroyl) derivatives. Measures fluorescence (exc. 370 nm, em. 470 nm)	Shimada *et al.* (1990)
Urinary corticosteroids	Zorbax ODS	Methanol:water:0.01 M Na acetate (70:30:5)		Mixed post-column with lucigenin/ Triton X-100/0.28 MKOH and induced chemiluminescence monitored	Takeda *et al.* (1990)
Plasma cortisol and 11-deoxycortisol	Supelcosil-LC-18-DB	Gradient of increasing water in acetonitrile		UV at 242 nm	Underwood *et al.* (1990)
Serum bile acids	Reversed-phase	Gradient elution MeCN–MeOH–water mixtures		Pre-column derivatisation with 4-bromomethyl-7-methoxycou-marin and fluorescence detection (exc. 320 and em. 385 nm)	Wang *et al.* (1990)

(continued)

Table 3.2 (continued)

Steroid Analyte(s)	Column	Solvent	Special Conditions	Detection	Reference
Corticosteroids in serum	Shimpack CLC-ODS	MeOH:THF:water (26:18:56)	48°C	UV at 254 nm in series with fluorescence detector for E1, E2, E3 (exc. 285 and em. 310 nm)	Wei *et al.* (1990)
Prednisone, prednisolone and 20-reduced metabolities in perfusion media	2 Brownlee Spheri-5 RP18 columns in series	MeOH:water (57.5:42:5)	40°C	UV at 242 nm	Cannell *et al.* (1991)
Bile acids in human bile	Resin-based reverse-phase PLRP-S	Gradient of MeCN in NaOH	35°C	Pulsed amperometry	Dekker *et al.* (1991)
Free bile acids	Nova-Pak C18	2-propanol:MeCN:water (65:10:25)		Pre-column formation of pentachlorophenyl esters—UV at 230 nm	Ferreira and Elliott (1991)
Prednisone, prednisolone and major urinary metabolities	Zorbaz SIL	Methylene chloride:glacial acetic acid:MeOH (91.3:7.5:1.2)		UV at 254 nm	Garg and Jusko (1991)
3-Oxo bile acids in serum	Nova-Pak Phenyl	3% MeOH in 0.3% phosphate (pH 7.0) buffer:MeCN (8:5)		Pre-column formation of 2-anthroylmethyl oximes. Fluorescence (exc. 260 and em. 405 nm)	Goto *et al.* (1991)
Cortisol and cortisone in saliva	Keystone C8	MeCN:MeOH:water (10:40:50)	50°C	UV at 240 nm	Wade and Haegele (1991b)
	Keystone Hypersil	33% EtAc in hexane, 0.5 satd with water		Pre-column formation of 2-anthroyl esters. Fluorescence (exc. 305–395, em. 430–470 nm)	Haegele and Wade (1991)
Cortoic and cortolonic acids	Pecosphere CR-C18	MeCN:MeOH:water (34:34:32)		1-Pyrenylmethyl esters UV with photodiode detector	Iohan and Vincze (1991)

Anabolic steroids in tissues	Supelco C18	MeCN:0.01 M KH_2PO_4 (pH 3) (48:52 and 46:54). Gradient of MeCN in water. KH_2PO_4 (pH3):MeCN:MeOH: THF (60:21:7:12)		Electrochemical, UV and fluorescence depending upon steroid	Lagana and Marino (1991)
Prednisone, prednisolone and cortisol in plasma	Axxiom ODS	THF:water (25:75)		UV at 240 nm	McBride *et al.* (1991)
Bile acids and conjugates	YMC-GEL C8	MeCN:water (3:1)±5 mM methyl-b-cyclodextrin		Pre-column formation of 1-bromopyrenacyl esters. Measures fluorescence (exe. 370 nm, em. 440 nm)	Shimada *et al.* (1991)
Estetrol, estriol, cortisol and cortisone in amniotic fluid	Medipola-ODS	Phosphate buffer, pH 3.1:MeCN:MeOH (20:2:7)	40°C	UV at 245 nm for E and F, electro-chemical detection for estrogens	Noma *et al.* (1991)
Serum cortisol	TSK	MeCN:THF:19mMK biphthalate (pH 1.85)		Pre-column formation of sulphuric acid fluorophores (exc. 365 nm and em. 520 nm)—*cf.* Nozaki *et al.* (1992)	Nozaki *e t al.* (1991)
Serum steroid profile in Cushing's	NS-Gel C18	Gradient of MeOH in water	50°C	RIA	Ueshiba *et al.* (1991)
Steroids in human ovarian follicular fluid	ChromSpher	Gradient of MeCN–water–methanol		UV detection at 242 nm or 206 nm (estrogens)	Vanluchene *et al.* (1991)
Plasma cortisol and cortisone	Shandon Hypersil-C18	Water:THF:MeCN (80:10:8)+ 5 ml triethylamine/1 and citric acid to adjust pH to 6.5		UV detection at 242 nm	Hariharan *et al.* (1992)
Conjugated bile acids in urine	Bile Pak II	Gradient elution with MeCN–MeOH–10 mM phosphate buffer, pH 7.3		Post-column immobilised 3a-OHSDH/NAD. NADH released by reaction with bile acid measured by chemiluminescence using isoluminol microperoxidase and 1-methoxy-5-methyl-phenazinium methyl sulphate	Ikegawa *et al.* (1992)

(continued)

Table 3.2 (continued)

Steroid Analyte(s)	Column	Solvent	Special Conditions	Detection	Reference
Urinary free cortisol	TSK ODS-80	MeCN: water or phosphate buffer (pH 1.85) mixture		Pre-column formation of ethanol-H_2SO_4 fluorophorescence. Fluorescence (exc. 365 nm and em. 520 nm)-*cf.* Nozaki *et al.* (1991)	Nozaki *et al.* (1992)
Free and sulphated estrogens	Beckman ODS	2% Tetrabutyl-ammonium hydroxide (pH 3): 33% MeCN in water (6.5:93.5)		UV at 210 nm or fluorescence (exc. 280 nm and em. 312 nm)	Su *et al.* (1992)
3α, 5β-Tetrahydroaldo-sterone and cortisol in human urine	L-column ODS	MeOH: MeCN: 0.5 mM ammonium acetate (50:10:40)		Pre-column formation of quinoxalines by reacting with 1,2-diamino-4,5-methylenedioxy-benzene. Fluorescence detection (exc. 350 and em. 390 nm)	Yoshitake *et al.* (1992)
Serum cortisol and cortisone	Ultrasphere ODS	MeOH:THF: water (25.5:9.0:65.5) or MeOH:MeCN: 58 mM NaH_2PO_4 containing 6 mM heptanesulphonic acid		Photodiode array detection—UV absorption	Volin (1992)
Steroids with delta-4-3 oxo group	RP C18	MeCN:water (1:1); 0.01 M Tb nitrate, 0.1 M SDS in 20% MeCN		Post-column mixing with terbium nitrate in 0.1 M SDS or micellar LC. Detection by sensitised terbium fluorescence	Amin *et al.* (1993)
Ethinyl estradiol in rabbit plasma	Novapack C18	50 mM phosphate buffer, pH 3.6–MeCN–MeOH (10:7:3)		Electrochemical detection	Fernandez *et al.* (1993)
Free and conjugated bile acids in a variety of tissues	Ultrasphere ODS	Gradient of MeCN–MeOH–water		Pre-column derivatisation with 4-bromomethyl-7-methoxycou-marin.Fluorescence detection	Guldutuna *et al.* (1993)
Plasma cortisone and corticosterone	Shandon ODS-Hypersil	Water:THF:MeCN (80:10:8)+ 5 ml/l of triethylamine and citric acid, pH 6.5		UV at 242 nm	Hariharan *et al.* (1993)

Plasma corticosteroids	Zorbax ODS	Water:MeOH (25:75) containing 5 mM tetramethyl-ammonium hydrogen sulphate		Pre-column derivatisation with 2-(4-carboxyphenyl)-5,6-dimethyl-benzimidazole. Fluorescence (exc. 334 nm and em. 418 nm)	Katayama *et al.* (1993)
Plasma estrogens	Wakosil-5 C18	Water:methanol (1:9)		Same as above. Fluorescence (exc. 336 nm and em. 440)	Katayama and Taniguchi (1993)
Urinary free cortisol	RP 18	MeOH:water:n-propanol containing 20mM SDS (pH6) (18:80:2) and (38:60:2)		UV at 240 nm	Li *et al.* (1993)
Bile acids and glycine conjugates in human bile	YMC Gel C8	MeCN:MeOH: water (6:7:8)±2.5 mM y-cyclodextrin		Pre-column formation of 7-methoxy-1,4-benzoxazin-2-one-3-methyl ester. Fluorescence detection (exc. 280 and em. 320 nm)	Shimada *et al.* (1993)
Catechol and guaiacol estrogens	Inertsil-ODS-2	0.5% phosphate buffer, pH 3.0: MeCN (59:41)		Electrochemical detection	Suzuki *et al.* (1993)
Pregnanolone (standard)	Cyclobond I (b-cyclodextrin bonded phase)	MeOH:water (65:35)		UV at 241 nm—indirect absorption using testosterone as a probe	Agnus *et al.* (1994)
Hydroxylated metabolites of equilin in rat bile	Cosmosil 5C18-AR	0.5% acetate buffer, pH 5-MeOH-MeCN (60:9:30)		Electrochemical	Ikegawa *et al.* (1994)
Estrogens in human urine	Beckman ODS	MeCN:water (25:75) containing 14 mM cyclodextrin	40°C	UV at 280 nm	Lamparczyk *et al.* (1994)

(continued)

Table 3.2 (continued)

Steroid Analyte(s)	Column	Solvent	Special Conditions	Detection	Reference
Salivary cortisol	CN- or ODS		Post-column mixing with H_2SO_4	Laser-induced fluorescence	Okumura et al. (1995)
Serum cholesterol, cholestanol	-	-	1-Eicosanol as IS post-column Pt-catalysed reduction on-line	Pre-column derivatisation with 2-[2-(iso-cyanate) ethyl] -3-methyl-1,4-naphthoquinone. electrochemical detection	Nakajima et al. (1995)
Bile alcohols	NovaPak Phenyl and TSK-GEL ODS	MeOH- H_2O (83-85%)	2,4-Dinitro-phenyl hydrazones-formed after 3α-OHSDH-ase	UV at 364 nm	Une et al. (1996)
Serum conjugated bile acids	ODS	MeCN: MeOH (60:40) and H_2O Gradient	hydolysis with cholylglycine hydrolase	Fluorescent derivative with 2-bromoacetyl-6-methoxy-naphthalene	Gatti et al. (1997)
Free Glucucorticoids in plasma/urine	Nucleosil 120-C18	H_2O: MeCN (76:24)		UV at 254 nm	Hay and Mormede (1997)
Oestriol-3- and 16-glu. in pregnancy urine	YMC-Pak C4 (A) and Ph(B)	MeOH: 0.5% Tri Ethylamine at 40°C	Column switch A to B	Derivatisation with 6,7-dimethoxy-1-methyl-2(1H)-quinoxaline-3-propionylcarboxylic acid hydrazine. Fluorescence	Iwata et al., (1997)

In vitro hydroxy testosterones	Supelcosil LC-18	MeOH:H_2O Gradient	Cortexolone As IS	UV at 254 nm	Purdon and Lehman-McKeeman (1997)
Prenenolone-3-stearate in brain	-	-	Derivatisation with dansyl hydrazine or 4-(N,N-dimethyl aminosulfonyl)-7-hydrazino-2,1,3-benzoxa-diazole	Fluorescence	Shimada et al. (1997)
Urine free Cortisol	LiChrospher 100 C-18	MeOH: MeCN:H_2O (43:3:54)	Meprednisolone as IS	UV	Turpeinen et al. (1997)
21-oxo-steroids	RP		Pre-column formation of dansyl derivative	Post-column 1,1'-oxalyl-diimidazole-peroxyoxalate induced chemi luminescence	Appelblad et al. (1998)
Progesterone, 17-OH-Prog + 4 x 3-oxo-steroids	Wakosil 5C4	MeCN:H_2O (7:3)	Pre-column derivatisation with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propiono-hydrazide (BODIPY FL-hydrazide)	Fluorescence-chemiluminescence	Katayama et al. (1998)

(continued)

Table 3.2 (continued)

Steroid Analyte(s)	Column	Solvent	Special Conditions	Detection	Reference
Urinary cortico steroids	SIL	Propan-2-ol: Hx; (2:98), then (7:93)		Pre-column formation of 9-anthroyl Cyanide Fluorescent cpd	Neufeld et al. (1998)
Glucocorticoids in plasma/urine	Cosmosil 5SL (x2)	Diethylene dioxide: EtAc:CHCl3 Hx:pyridine (500:100: 100:1400:21)	9-Anthroyl nitrile + base catalyst to form 21-fluorescent esters	Fluorescence	Shibata et al. (1998)
Oestrogen in breast tumour tissue by RIA	Hypersil-5μ- ODS	MeCN -phosphate Buffer, pH 3.5		UV at 280 nm	Geisler (2000)
Urine 6β-OH-cortisol	Novapak C18	CH3COOH-MeCN-KH2PO4 50 mM at	25°C and 45°C	UV at 245 nm	Homma et al. (2000)
Progesterone and neuroactive metabolites in serum	Novapak SIL	EtOH in CH_2Cl_2		Fraction collection for RIA	Murphy and Allison (2000)
16a-Hydroxy-DHA and metabolites	5 m C2/C18 Pharmacia	H_2O: MeOH: MeCN	30°C	UV at 238 nm and 210 nm and radio activity	Schmidt et al. (2000)
Urine 2-OH-oestradiol-17-S in Pregnancy	Mightysil RP-18GP	5 g/L $NH_4H_2PO_4$ pH3 - 40oC		Electrochemical detection	Takanashi et al. (2000)

Alphaxalone and pregnanolone in rat plasma	Microsphere C18	25 nM acetate (pH3.9):MeCN (ca 40:60)		Pre-column formation of dansyl derives. Fluorescence detection.	Visser et al. (2000)
Oestradiol in plasma	CapcelPak ODS	MeCN:H_2O (85:15)	Pre-column formation of dansyl derivatives	Post-column chemiluminescence with H_2O_2 and bis(2,4-dinitro-phenyloxalate.	Yamada et al. (2000)
17-Ketosteroid sulphates and glucuronides	Luna C18	0.1 M NH_4Ac: MeCN (35:25)		Sonic spray ionisation Hitachi M8000 ion trap	Jia et al. (2001)
Steroids	CN column	MeOH in ethoxy nonafluoro butane			Kagan (2001)
Serum urso-deoxycholic acid	LiChrospher 100 C18	MeCN:H_2O (6:4)		Derivatisation with 2-bromo-2'-acetonaphthone UV at 245 nm	Nobilis et al. (2001)
Hydroxy testosterones	TSK-gel ODS- 80Ts	MeOH-THF		UV at 254 nm	Tachibana and Tanaka (2001)
Biliary bile acids	Luna C18	MeOH: MeCN: H_2O (53:23:24) +30 mM NH_4Ac pH5.6		Evaporative light scattering detection (ELSD)	Torchia et al. (2001)

(continued)

Table 3.2 (continued)

Steroid Analyte(s)	Column	Solvent	Special Conditions	Detection	Reference
Hydroxy testosterones	Hypersil-BDS C18	THF:MeCN- H_2O		UV	Whalley et al. (2001)
Hepatic estradiol metabolites	Hypersil ODS-5μm	CH_3COOH buffer pH4.5 -MeCN		Electrochemical detection	Cheng et al. (2001)
Microsomal oestradiol-3- and 17-glucuronides	Alltima-phenyl	MeCN: 50 mM NH4PO4 (pH 3) (35:65)		Fluorescence detection	Alkharfy and Frye (2002)
Plasma Oestradiol	CalcellPAK C8 UG-120	0. 0.1%TFA: MeCN (1:1) And 20 mM PO4 buffer(pH9): MeCN (1:1)		Electrochemical detection	Yamada et al. (2002)
Adrenal steroids in CAH	BDS-Hypersil	H_2O-MeOH		Fractions for RIA	Fernandes et al. (2003)
Testo and Epi-T	Hypersil C18	H_2O-MeCN		UV at 245 nm	Ganzalo-Lumbreras et al. (2003)
Cortisol and 6β-OH-cortisol in urine	Symmetry sheild RP8	NH_4 acetate (pH 4)-MeCN		Diode array at 244 nm	Rouits et al. (2003)
Plasma testosterone	C18	KH_2PO_4(pH 4)-MeCN-MeOH		UV at 240 nm	Ng and Yuen (2003)
Corticosteroids anabolics	Hypersil-ODS	Binary solvent Mixtures with SDS - micellar LC	Diode array 190-360 nm		Ganzalo-Limbreras and Izquierdo-Hornillos (2003)

Prednisolone and cortisol in plasma and urine	Hypersil SIL	CH_2Cl_2-H_2O-MeOH-THF-Gla CH_3COOH	UV at 240 nm	AbuRuz et al. (2003)
Androst-5,16-dienes and androst-4,16-dienes	Luna 5 µm, C18	H_2O-MeOH	UV at 210-215 nm	Decreau et al. (2003)
DHA and 7-OH-metabolites in brain	C18 ET250/4 Nucleosil 100-5 MeOH	MeCN-H_2O-NH_4HCO_3-	UV and radio activity	Kazihnitkova et al. (2004)
6β-OH-F and F in urine	Synergi 4 m Polar-RP 80A	Phosphate-acetic acid and Phosphate-acetic acid-MeCN	photodiode array	Furuta et al. (2004)
25- and 24,25-Hydroxy-vitamin D3 in serum	Ultrabase C18	MeOH:water linear gradients from (90:10) to (50:50)	Photodiode array detection	Quesada et al. (2004)
Synthetic corticosteroids	Synergi Max-RP	MeCN:H_2O (35:65)	Post-column chemiluminescence Using luminal + hexacyanoferrate III As catalyst	Vasquez et al. (2005)
16-Androstenes in boar testis	Phenomenex 5 mm C!8	MeCN-H_2O (85:15)	Radioactivity and UV at 200 nm	Sinclair et al. (2005)
Testosterone and epi-T from urine	µBondapak C18	MeCN-MeOH TRIS-HCl(pH6.9)	UV at 245 nm	He et al. (2005)

(continued)

Table 3.2 (continued)

Steroid Analyte(s)	Column	Solvent	Special Conditions	Detection	Reference
Cortisol and cortisone	Hypersil C18	Micellar medium with SDS		UV at 254 nm	Izquierdo-Hornillos et al. (2005)
Cortisol and cortisone in urine	Supelco DSC 18	MeOH: H_2O (63:37) at 30°C		UV at 254 nm	Gatti et al. (2005)
6β-OH- and cortisol Urine and plasma	Inertsil-PH-3	MeCN: H_2O gradient		UV at 245 nm	Hu et al. (2005)
DHEA and DHEA-S A-dione and Testo	C8-SIL	Ion-pairing		Circular diochroism at 295 nm	Gergely et al. (2006)
Oestradiol and Catechol oestrogens in Catfish	Luna C18	MeCN-$NH_4H_2PO_4$ (pH 3)		Electrochemical	Mishra and Joy (2006)
Reviewed in Honour (2006)					
Oestrogen catabolism	LiChroCART 250-4 RP18	MeOH:H_2O (8:2 to 9:1) Gradient		Derivatised with 2-(4-carboxyl-phenyl) 5,6-dimethyl-benzimidazole. Fluorescence detection Ex:336 nm, Emm: 440 nm	Delvoux et al. (2007)
Human fetal cord blood steroids	Supelcosil LC-18	30% MeCN in H_2O + 12 nM β-cyclodextrin at 29°C	Photodiode Array		Clifton et al. (2007)
Metabolomics		35% MeCN			Zarzycki et al. (2006)

Butane acid-DHEA-diester in rat plasma	Diamonsil C18	MeCN:H_2O - gradient from 75-98% MeCN and back to 75%	Pre-column dansyl derivatives - laser-induced fluorescence detection (Ex350 nm, Emm520 nm)	Peng et al. (2007)
Plasma cortisol in children	No details of HPLC assay are provided - merely that the samples were analysed by 'a central laboratory (Quest Diagnostics) using HPLC'			Chrousos et al. (2007)
Free cortisol/ cortisone ratio in human Urine	Varian Pursuit C18 coupled to Waters Atlantis C18	MeCN:H_2O 3:7 at 28°C	UV detection at 240 nm	Al Sharef et al. (2007)
Serum Cholesterol	Develosil C30-UG-3	MeCN:2-propanol (9:1) with 50 mM $LiClO_4$ at 20°C	LC-4C electro-chemical detector	Hojo et al. (2007)
Cholesterol in biological samples	Novopak C18	MeCN:2-propanol (9:1)	Jones oxidation and UV detection at 240 or 250 nm	Dong et al. (2007)
Steroids in animal feeding water	Several RP columns	MeCN:water (35:65)	UV-diode array detection (190-360 nm)	Muniz-Valencia et al. (2008a) see (2008b) in animal feed
Estrone in environmental and drinking water	RP C18 column	MeOH:water (55:45)	UV detection	Wang et al. (2008)
25-Hydroxy- and 24,25-di-hydroxy-vitamin D3 in serum	Using same automated method as previously described (Quesada et al., (2004))			Mata-Granados et al. (2008)

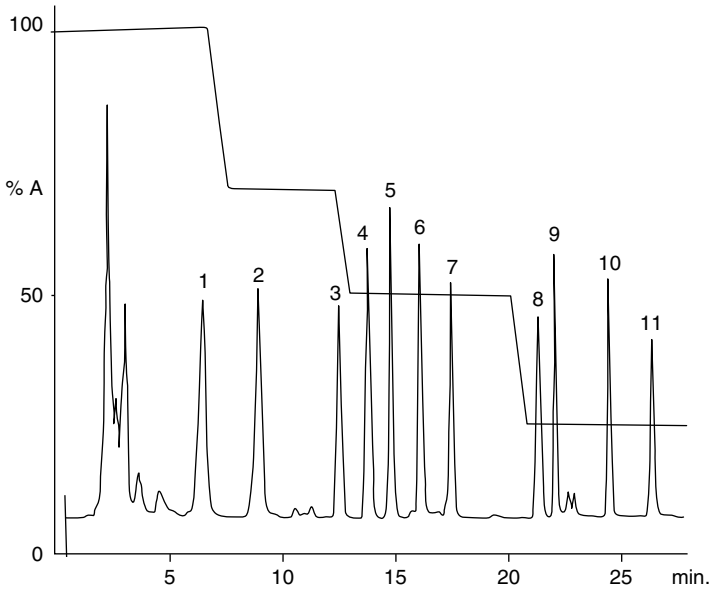

Fig. 3.13 HPLC of standard free and glycine conjugated bile acids: 1, glycoursodeoxycholic acid; 2, glycocholic acid; 3 glycochenodeoxycholic acid; 4, glycodeoxycholic acid; 5, ursodeoxycholic acid; 6, cholic acid; 7, glycolithocholic acid; 8, chenodeoxycholic acid; 9, deoxycholic acid, 10, lauric acid; 1l, lithocholic acid. HPLC was carried out on an Ultrasphere IPC18 column (250 mm x 4.6 mm) at 35°C using a solvent gradient from (A) acetonitrile:methanol:water (100:50:75) to (B) acetonitrile:methanol (100:50). The elution gradient from mobile phase A (100%) to B (drawn line) is marked in the figure. Pre-column derivatisation of bile acids to their 4-bromomethyl-7-methoxy-coumarin derivatives were detected by fluorescence (from Guldutuna *et al.*, 1993, with permission).

gradient elution and there exists methodology to assist optimisation (Nikitas and Pappa-Louisi, 2005). Additional pH, ion-pair and modifier effects can be incorporated. Retention times are reproducible between runs provided that the column is equilibrated to the starting solvent mixture. Methanol:water gradients effect the separation of the major adrenal sterids. Dioxane is a better choice for the separation of polar adrenal steroids and acetonitrile is preferred for resolving testicular steroids. Peak shape, resolution and reproducibility can be improved by maintaining the column at a fixed temperature above ambient, e.g. at 45–60°C that may need optimising for the required separation (Dolan, 2002). At these temperatures, the eluant viscosity is reduced (Burgess, 1978). If working at ambient temperature, it is advisable to have a room with well-controlled temperature to achieve reproducible retention times or use a jacketed column with temperature control. Temperature gradients have been tested with C8 and C18 columns (Dolan et al., 2000a, b). The difficulties in choosing the appropriate column packing for a particular separation have been eased to some extent by using three and four solvents in a mobile phase system. Systematic, statistical procedures for solvent optimisation have been developed (Hara and Hayashi, 1977; D'Agostino et al., 1985; Wei et al., 1990). Column packings may not be consistent and chromatographic conditions may have to be adjusted (Dolan et al., 2002). Derks and Drayer

(1978b) reported the separation of very polar 6α– and 6β-hydroxylated metabolites of cortisol by isocratic elution from a silica column with water:chloroform:methanol.

An LC separation of a complex mixture containing 14 androgenic anabolic steroids (natural and synthetic) for anabolic steroid screening purposes has been carried out. The optimization of the method assessed the use of binary, ternary and quaternary mobile phases containing acetonitrile, methanol or tetrahydrofuran as organic modifiers (Izquierdo-Hornillos and Gonzalo-Lumbreras, 2003). The effects of different reversed-phase packings, and temperature on the separation using acetonitrile as organic modifier were also studied. The optimum separation was achieved by using a water–acetonitrile (55:45, v:v) mobile phase and a Hypersil ODS (250 × 4.6 mm) 5 μm column (30°C) in about 38 min, allowing the separation of all 14 compounds tested (when danazol was excluded, 13 out of 14 were separated in 23 min). Calibration graphs were obtained using bolasterone, methyltestosterone and canrenone as internal standards. Detection limits were in the range 0.012-0.11 μg/mL (Gonzalo-Lumbreras and Izquierdo-Hornillos, 2000).

In some cases, phosphate is incorporated into the mobile phase (Shimada et al., 1979). With these systems, the buffer anion and pH exert significant effects on the separation (Shimada et al., 1986). Salts used in the eluting solvent (see section on analysis of oestrogen conjugates) may in the long term corrode the steel of the columns and tubing. Oestrogens can be effectively separated when silver nitrate is included in the mobile phase to give 2 g of silver nitrate with 60 mL methanol and 40 mL water at 0.55 mL/min (Tscherne and Capitano, 1977). To prevent metallic silver building up on the column, a water:methanol (50:50, v/v) mobile phase is used each evening to flush excess silver nitrate from the system. Even so, a small build-up can occur which requires a rinse with dilute nitric acid or replacement of the tubing when back-pressure rises.

Addition of cyclodextrins to the mobile phase has been claimed to improve the HPLC separation of a variety of steroids, from C21 corticosteroids (Shimada and Nonaka, 1991) to bile acids (Shimada et al., 1990) and oestrogens (Lamparczyk et al., 1994). There is an increasing literature on this subject, particularly from Shimada's laboratory but it is not entirely clear how this improvement is effected. There are a variety of different cyclodextrins and not all confer the same improvement in resolution. It is often necessary to try a number of the polymers before discovering the best for the particular separation. Addition of other compounds to the mobile phase have also provided advantages such as the use of micellar chromatography which can be achieved by the addition of sodium dodecyl sulphate (SDS) (Izquierdo-Hornillos and Gonzalo-Lumbreras, 2003; Izquierdo-Hornillos et al., 2005). Use of micellar chromatography and a two-column system has allowed the measurement of serum cortisol (Nozaki et al., 1991) and urinary free cortisol (Nozaki et al., 1992) by direct injection of urine onto the first column, washing off protein and elution of the concentrated analyte onto the second analytical column. Cetyl trimethyl ammonium bromide has been used to improve the separation of betamethasone and dexamethasone (Pena-Gracia-Brioles et al., 2004).

Ion-pair chromatography is preferred for chromatography of steroid conjugates and bile acid products. Andreolini et al. (1987) have shown excellent separations of

oestrogens on RP-18 packings by eluting with a gradient of acetonitrile/methanol and phosphate buffer containing cetyltrimethylammonium bromide. Gradient elution is usually necessary to elute a series of steroids and bile acids after extraction from biological fluids. Gradient elution reduces analysis times and depending upon the gradient shape can optimise separation and improve peak symmetry. Non-linear, stepped and linear gradients have been used, largely dictated by the available facilities for programming the pumps. Flow and temperature programming can also be used.

The separation of bile acids by HPLC can be achieved using normal-phase or reversed-phase columns. In a normal phase system, an organic acid is usually added to the mobile phase. Bile acids are eluted in the order of decreasing number of hydroxyl groups on the steroid nucleus. The chemically bonded ODS-RP column is the most widely used column for the resolution of bile acids (see Fig. 3.13). The retention of bile acids is markedly influenced by the pH of the mobile phase. The acidity of the eluent and the pK of the bile acid have to be considered (unconjugated pK = 6, glycine-conjugated pK = 4.5, taurine-conjugated pK = 1.5). With an anion-exchange column, bile acid conjugates are readily separated.

For the separation and detection of bile acids in biological specimens with an ODS column, an acidic mobile phase is used to separate glycine- and taurine-conjugated bile acids. The unconjugated bile acids are then eluted with a neutral or weakly alkaline mobile phase. For the analysis of complex mixtures of bile acids, it is preferable to fractionate according to conjugation before HPLC of each group in isocratic mode.

3.7.3 Sample Injection

Extracts are usually dissolved in the mobile phase. The addition of a suitable macromolecular matrix, e.g. polyethylene glycol to the extracting solvent prior to evaporation improves the recovery of steroids (Culbreth and Sampson, 1981) suggesting that the steroids dissolve poorly in the mobile phase alone. Injectors which use rubber septa should be avoided. At the high instrument sensitivities often used for the analysis of steroid hormones, such septa may lead to the production of spurious and irreproducible peaks in the chromatogram. These may reflect the action of injected solvents on the septum. Septumless injection valves (e.g. JADE™ injectors – see www.asapanalytical.com/pdf/inlet.pdf) are, therefore, preferred.

3.7.4 Detection

The α,β-unsaturated ketone in the A-ring of naturally occurring steroid hormones absorbs ultraviolet light with maximum around 240 nm and molar extinction coefficients of 12,000–20,000. Isolated carbonyl groups absorb with a maximum around 280 (275-285) nm and molar extinction coefficients of 17–155. The natural

oestrogens have peak absorption at 280 nm due to the aromatic A-ring. Underivatised phenolic steroids can be detected with sensitivity limits of 100–10 pg/mL. Although steroids can absorb UV below 200 nm, in practice, at this wavelength it is difficult to achieve a clear signal distinguishable from noise without a reduction in sensitivity particularly when solvent gradients are used to elute the steroids. With some gradient elution systems, it is necessary to correct for base-line variation by comparison of the response of the eluate from the analytical column with the flow of solvent alone through a reference cell. An interesting paper (Agnus et al., 1994) described a system for the measurement of non-UV absorbing steroids by the so-called "indirect" photodetection using an immobilised β-cyclodextrin column using testosterone as a probe added to the mobile phase (methanol:water, 65:35, v/v). The sensitivity of detection of pregnanolone (2 nmol) by this system was comparable to that previously reported (Agnus et al., 1991) using a C4 RP column and adding β-cyclodextrin to the stationary phase and using progesterone as the probe. Sensitivity of this method is not superb but does represent nearly an order of magnitude improvement in sensitivity over that achieved with direct detection and it may be possible to improve this further.

The detection and quantitative determination of nanogram quantities of steroids has also been realised by the use of fluorescence (Seki and Yamaguchi, 1984), refractive index (Satyaswaroop et al., 1977) and electrochemical detectors (Watanabe and Yoshizawa, 1985). C-3 and C-16 conjugated oestriol glucuronides have been converted to fluorescent derivatives and separated using column-switching between two columns (Iwata et al., 1997) and by LC-ES-MS/MS Yang et al., 2003). In some cases, it has been necessary to react the steroids in the eluate with reagents to form UV-absorbing derivatives. Post-column derivatisation methods are, however, restricted to very fast reactions limiting the scope of application (Seki and Yamaguchi, 1984). Most of the urinary steroid metabolites do not have natural absorbance in the UV region. Reactive groups have been utilised in order to make derivatives for spectrophotometric detection. HPLC has thus been used to separate individual oxo-steroids after conversion to phenylhydrazone derivatives. 3α-Hydroxysteroids can be detected using a post-column 3α-hydroxysteroid dehydrogenase reactor (Lam et al., 1988) similar to that used in bile acid analysis (see below). In general, however, the most successful methods for enhancing detection are pre-column formation of fluorescent or chemiluminescent derivatives (e.g. formation of 2-(4-carboxyl)-5,6-dimethylbenzimidazoles (Katayama and Taniguchi, 1993) for oestrogens, 9-anthroyl cyanide (Neufeld et al., 1998; Shibata et al.,1998) derivatives of glucocorticoids). Many other such derivatives have been used and these are summarised in Table 3.2. There are two good reviews summarising this approach up to 2001 (Shimada et al., 2001; Appelblad and Irgum, 2002). Increase in detection sensitivity and specificity can clearly be achieved using hyphenated LC-MS technology, which is discussed later in this chapter.

Flow-through radioactivity detectors are potentially useful for examining the products of reactions with labelled substrates (Kessler, 1983; Lundmo and Sunde, 1984). The short dwell time of the sample components in the counting chamber limits sensitivity. Several laboratories have demonstrated the variety and complexity of intermediates and products formed when radioactive steroids are incubated with

steroid metabolising tissues. Current detection limits for tritium are 10,000 dpm with flow cells incorporating scintillant (around 1% efficiency) to 1,000 dpm (50% efficiency) when the column effluent is mixed with liquid scintillant before passing through a cell.

A UV absorbance detector is relatively insensitive to bile acids and because of the variety of UV-absorbing compounds in serum there is a risk of interference. Methods based on pre- and post-column derivative formation have overcome many of these problems, although sample clean-up is still required. Bile salts in bile are present in sufficiently high concentrations for detection with a refractive index or UV detector in the range 195–215 nm. Sensitivity can be improved by the use of a fluorescence detector (Andreolini et al., 1988; Kamada et al., 1983). A highly sensitive and selective fluorescence method of indirect detection is achieved using the enzyme 3α-hydroxysteroid dehydrogenase which oxidises the bile acids after their HPLC separation. The overall reaction involves conversion of the 3α-hydroxy group on the steroid nucleus to a keto group with concomitant reduction of NAD to NADH which is detected fluorimetrically. The enzyme is expensive and in its crude form may not be specific. In order to reduce the cost of the enzyme, it can be immobilised on an aminopropyl support in a column in series with the analytical column. Detection of the compounds after the post-column reaction is not uniform; the primary bile acids yield somewhat smaller quantities of NADH than the secondary bile acids. This is influenced by the time the bile acids spend in the reactor and the affinity of the enzyme for the range of bile acids encountered in biological fluids. Quantification of a number of bile acids requires optimisation and control of flow rates as well as calibration curves to compensate for the differences in response. Over a period of time the baseline and response characteristics of the enzyme column will vary.

LC methods often show chromatographic peaks that are difficult to reproduce and can be hard to identify. Artefact peaks can arise because of contamination by the injector septum and sampling equipment leading to misinterpretation of impurities and erroneous quantification. A number of investigations may be needed to locate the source of the problems (Strasser and Varadi, 2000).

3.7.5 Identification

The identification of material in chromatograms is usually assumed from a homogenous peak with elution time which coincides with that of the reference compound under similar conditions. This assumption may, however, be dangerous since it is not always possible to recognise homogeneity by inspection. The use of a photodiode array detector has a considerable advantage in this context in that it allows inspection of the peak to ensure homogeneity. A good example of this is given in the vitamin D Chapter (Chapter 11) in this book. Since the detectors currently in use are not selective for distinct classes of substances, some further demonstration of specificity is required. 3-Keto-4-ene steroids can be distinguished from other possible compounds

eluted from the column by monitoring the UV absorption at further wavelengths using a photodiode array detector (Fell et al., 1983). The sample can also be analysed separately with a different column (preferably of differing polarity or selectivity) or a different gradient elution system. Should elution times in each system coincide with those of a standard, it is highly probable that each chromatogram reflects the same steroid content. These criteria have not been rigidly applied in the published work relating to steroids. Retention indices have been widely used for recording and comparing retentions, for identifications and as the basis for prediction methods (Kuronen et al., 1998). 1-[4-(2,3-Dihydroxypropoxy)phenyl]-1-alkanone standards have been used in RP-LC for steroids with photodiode array detection (Kuronen et al., 1998). In some work, identification is enforced by a second separation of quite different selectivity (orthogonal separation). This may sometimes reveal a second peak that was masked in the first separation (Pellett et al., 2006). Eluate fractions can of course be collected and subjected to GC-MS after appropriate derivatisation.

The ultimate system for on-line identification is LC-MS/MS and considerable advances have been made over the last 10 years in improving methods of linking the eluent from an LC column to MS instruments as well as improved ionisation procedures. While LC-MS is more selective, the advent of tandem LC-MS/MS, with collision cells in between MS1 and MS2 has improved selectivity even further to the extent that the extra expense of acquiring LC-MS/MS rather than simply LC-MS is fully justified. Applications of LC-MS and other types of mass spectrometry are discussed below (see Section 3.8)

3.7.6 Quantitation

The height or area of the chromatographic peak is measured manually or with the aid of an integrator and ideally the response of the analyte is compared to the response of an appropriate internal standard. The ratios of response for the analyte to the signal from the internal standard are plotted for the concentration range of interest. The concentration of an unknown amount of steroid in the sample is determined from a calibration line. There are a large number of synthetic steroids available which can be used as internal standards. Since a number of steroid-based drugs are widely used in hospital patients, the use of two very different internal standards, for example, 19-nortestosterone and 6α-methyl prednisolone, prevents erroneous results in the case of medication by either one of the steroids selected as internal standard provided they behave in a similar way to the analyte during the analytical procedure. When internal standards are not used, the extraction and injection must be carefully controlled before peak response can be reliably derived from a calibration line using injected standards. A deferred standard technique can be adopted in which a known amount of the analyte is injected in pure form some time after but during the chromatographic run of the unknown sample.

The availability of switching valves which can be operated automatically has enabled the use of multiple columns, automatically switching selected peaks

from one column to another – so-called "column switching" or "heart cutting". Schoneshofer et al. (1983) described such a system for the measurement of triamcinolone in urine and later adapted the procedure for the measurement of urinary free cortisol (Schoneshofer et al., 1985) and for 20-reduced metabolites of cortisol and cortisone in urine (Schoneshofer et al., 1986). Henion and Lee (1990) commented that despite the obvious value of such column switching techniques, they were then not widely used. Henion and Lee (1990) also reported preliminary results using column switching techniques demonstrating that the anabolic steroid dianabol could be isolated from horse urine by such techniques and subsequently analysed by on-line M/MS. As an illustration of this technique, readers are referred to the paper by McLaughlin and Henion (1990) which describes the estimation of dexamethasone in bovine liver and muscle. After extraction of the tissues, the sample was injected onto a phenyl HPLC column. The fraction containing dexamethasone was then diverted onto a SIL column which retained the steroid. The SIL column was then back-flushed (i.e. column flow was reversed) onto a third (cyclopropyl) column from which clean extracts of dexamethasone were obtained. These techniques can be of considerable use as a means of automating assays involving HPLC and in addition offer a convenient method of measuring steroids present in trace amounts, by concentrating the analyte on the first column, "heart cutting" onto the second column, and finally by back-flushing, the third and final analytical column is presented with a tight band of concentrated analyte, without non-specific material from the matrix, which has been largely removed by the first column. An automated coupled-column HPLC system has been described for the measurement of melengesterol acetate in bovine tissues (Chichila et al., 1989) and serum cortisol has been measured using pre-column de-proteinisation and on-line extraction using column-switching (Vogeser et al., 2001). A similar method was employed to measure three androgens in cell culture medium (Chang et al., 2003). Further examples in the steroid field have been described (e.g. Magnusson and Sandstrom, 2004 (*in vitro* generated hydroxyl testosterones), Watabe et al., 2006 (estradiol-17β in river water), Rauh et al., 2006 (17-hydroxyprogesterone, androstenedione and testosterone measurement in 100 μL aliquots of serum, plasma and/or saliva from neonates with and without CAH) and Cho et al., 2006 (DHEA sulphate in plasma from patients with Alzheimer's disease).

3.8 Mass Spectrometry

3.8.1 Introduction

Simplistically, a mass spectrometer consists of an "ion source", a "mass analyser", a "detector" and a "data system". Sample molecules are admitted to the "ion source" where they are vaporised and ionised; the ions are separated according to their mass-to-charge ratio (*m/z*) in the "mass analyser" and are then detected. The resulting signals are transmitted to the "data system" and a plot of ion

abundance against *m/z* corresponds to a mass spectrum. In many cases, a "separating inlet" device precedes the ion-source so that complex mixtures can be separated prior to admission to the mass spectrometer. Today, the "separating inlet" device is usually either a capillary gas chromatography (GC) column or a high-performance liquid chromatography (HPLC) column, although capillary electrophoresis or thin layer chromatography can also be interfaced with mass spectrometry.

For steroid analysis, a number of different types of ionisation methods are used to generate gas-phase ions and include; electron ionisation (EI), chemical ionisation (CI), electrospray (ES), atmospheric pressure chemical ionisation (APCI), atmospheric pressure photoionisation (APPI), and the recently introduced, desorption electrospray ionisation (DESI) technique. Other ionisation techniques used, but to a lesser extent, are: fast atom bombardment (FAB), liquid secondary ion mass spectrometry (LSIMS), matrix-assisted laser desorption/ionisation (MALDI) and desorption/ionisation on silicon (DIOS). The selection of the appropriate ionisation mode is one of the key decisions for the analyst to make, and thus, we discussed the most important ionisation modes in some detail in Chapter 2.

3.9 Liquid Chromatography–Mass Spectrometry

3.9.1 Choice of LC-MS Interface

The decision on what form of LC to couple with mass spectrometry depends on the desired application, and it may in fact be preferable to negate an LC separation step all together when, for example, screening clinical samples for metabolic errors of cholesterol metabolism (Bove et al, 2004). Conventional LC encompasses both normal-bore (3–4.6 mm i.d., 0.5-3 mL/min) and narrow-bore (1–2 mm i.d., 20–300 µL/min) columns, while capillary LC includes micro-bore (150–800 µm i.d., 2–20 µL/min) and nano-bore columns (20–100 µm i.d., 100–1,000 nL/min). The earliest generation of electrospray (ES) interface (which is in use even today) is well-matched with flow rates of the order of 5–100 µL/min, with maximum sensitivity being achieved at the lower end of this flow-rate range, and is most compatible with narrow-bore or micro-bore columns. It is possible to interface normal-bore LC columns to such ES interfaces, but with the requirement of a post-column split. Pneumatically assisted ES, sometimes called ion-spray or turbo-ionspray, has been developed to allow the direct coupling of normal-bore columns with the ES interface which is modified to receive flow rates of up to 1 mL/min. APCI interfaces are also capable of operating at this flow rate and receiving eluate from normal-bore columns.

Theoretically, a reduction in column diameter produces a higher concentration of sample in an eluting peak (Abian et al., 1999). As ES is also a concentration dependent process, this dictates that maximum sensitivity can be achieved by using miniaturised LC, and has lead to the increasing popularity of capillary LC-MS and capillary LC-tandem mass spectrometry (MS/MS) in biological mass spectrometry.

A new generation of micro-ES interfaces have been developed which perform optimally at low flow rate (<1 μL/min) and thereby provide maximum sensitivity when coupled with capillary column LC. Despite providing maximum sensitivity, capillary LC performed at low flow rate has its limitations. Although the concentration of sample in an eluting peak is dependent on the reciprocal square of column i.d., the column loading capacity and optimum injection volume also follow a similar relation (with respect to column i.d.). This creates problems in terms of sample injection and column overloading, particularly for columns of i.d. < 300 μm where optimum injection volumes are less than 1 μL (Tomer et al., 1994). The problem of low injection volume can be overcome simply by on-line sample pre-concentration on a trap column arranged in series with the analytical column. Sample pre-concentration is performed at microlitre per minute flow rates on the trap column, which is then flushed, and sample separated on the analytical column. However, the problem of column loading capacity still exists. The best solution to column overloading is to include a group separation step prior to capillary LC. This is illustrated in the work performed by Yang et al. (1997), in which a urine extract from a child with cholestatic liver disease was separated into four fractions according to acidity on an anion-exchange column. Each fraction was analysed by capillary-LC-ES-MS and MS/MS in a 1 h run, allowing the partial characterisation of over 150 bile acids and conjugated bile alcohols. The remaining drawback with capillary column LC, is one of analysis time. For example, in the study performed by Yang et al. (1997), each LC run took 60 min precluding the possibility of high throughput analysis. However, with the development of ultra high pressure liquid chromatography (UPLC – a Waters trademark, 15,000 psi, see review by Swartz, 2005) using smaller particles (around 2 μm), or alternatively monolithic columns operated at lower pressure, it is likely that the time constraint associated with capillary chromatography will be overcome (Wang G. et al., 2006; Licea-Perez et al., 2007; Touber et al., 2007; Wang and Zhang 2007).

While capillary column LC combined with micro-ES will provide the maximum sensitivity for steroid analysis, and is preferable for the profiling of conjugated steroids and bile acids isolated from biological samples, many screening and quantitative studies require high throughput as their main priority, in which case narrow-bore chromatography combined with ES or APCI is often the method of choice.

3.9.2 Derivatisation for LC-MS

The ionisation properties of unconjugated steroids and bile acids can be improved by derivatisation. A good derivatisation reaction should be simple to perform, give a high yield and generate a minimum of side products. Preferably, the reaction will be performed under mild conditions to avoid decomposition of the target analyte. Finally, the derivative should give a higher ion yield than the target analyte, and ideally fragment upon collision-induced dissociation (CID) in an informative

manner generating abundant product ions suitable for multiple reaction monitoring (MRM) studies. Derivatives have been designed with APCI (Singh et al., 2000; Higashi et al., 2002,, 2003, 2006a), ES (Shackleton et al., 1997; Griffiths et al., 2003, 2006; Higashi et al., 2005a, b, 2007c; Nishio et al., 2007; Yamashita et al., 2007a) and MALDI (Khan et al., 2006; Wang Y. et al., 2006) ion sources in mind. There is a useful review on this derivitisation topic (Higashi and Shimada, 2004).

Girard Hydrazones are useful derivatives of carbonyl groups. The Girard reagents, i.e. Girard P (GP) hydrazine, 1-(2-hydrazino-2-oxoethyl)pyridinium chloride, and Girard T (GT) hydrazine, trimethylammonium acethydrazide chloride, are quaternary ammonium salts and effectively tag the steroid with a positively charged group, thereby greatly improving the sensitivity of analysis by ES-MS and MALDI-MS (Griffiths et al., 2003, 2006). Additionally, upon CID these derivatives usually give a prominent neutral loss of 79 and/or 107 Da for GP hydrazones, and 59 and/or 87 Da for GT hydrazones, suitable for high-sensitivity MRM or neutral-loss scans.

Shackleton et al. (1997) were the first to use the GT derivative for ES-MS in their analysis of testosterone and testosterone esters. More recently, Lai et al. (2002) have used the GP derivative to enhance the ionisation of 17-hydroxyprogesterone, while Griffiths et al. (2003) demonstrated the sensitivity gains provided by the GP and GT derivatives for the analysis of a panel of oxosteroids using both ES and MALDI. Johnson (2005) has described LC-MS/MS of a serum 17-hydroxyprogesterone, cortisol and androstenedione panel following derivatisation with the Girard T reagent. To the evaporated supernatant of a diethylether/hexane (9:1, v/v, 1 mL) extract of 100 µL of serum, Johnson added 140 µL of GT reagent (10 mM in methanol containing 1% acetic acid). After 15 min at room temperature, solvent was removed under nitrogen and 150 µL of acetonitrile/water/formic acid (50:50:0.025, v/v/v) and hexane 1 mL were added. After vortexing and centrifugation, the supernatant was removed and the aqueous layer analysed by LC-ES-MS/MS. Double derivatives can be prepared by substituting trifluoroacetic acid for acetic acid, and performing the reaction at 75 °C.

Griffiths and colleagues have used GP derivatisation for oxysterol profiling in brain and blood samples (Griffiths et al., 2006; Wang Y. et al., 2006). As many oxysterols do not posses a ketone(oxo) group, the oxysterol extract was treated with cholesterol oxidase which will convert 3β-hydroxy-5-ene and 3β-hydroxy-5α-hydrogen groups to 3-oxo-4-ene and 3-oxo groups, respectively, which are suitable for GP derivatisation. Sterols were oxidised with cholesterol oxidase essentially as described by Brooks et al. (1983). Cholesterol oxidase was from either *Brevibacterium* or *Streptomyces* sp. The enzyme from *Brevibacterium*, also catalyzes the oxidation of 3β-hydroxy-5-ene and 3β-hydroxy-5α-hydrogen steroids of the C_{19} and C_{21} series (MacLachlan et al., 2000). Reference sterols or those extracted from brain or blood were dissolved in 50 µL of isopropanol, and 10 µL of cholesterol oxidase from *Brevibacterium* (1 mg/mL, 20 U/mg protein) or 2 µL of cholesterol oxidase from *Streptomyces* sp. (2 mg/mL, 44 U/mg protein) in 1 mL of buffer (50 mM KH_2PO_4, pH 7) added, the mixture was incubated at room temperature (25°C for 2–12 h when using the enzyme from *Brevibacterium* or 37°C) for 60 min when using the enzyme from *Streptomyces* sp.) and subsequently used

as the starting solution for reaction with the GP reagent. The oxidation mixture, 1 mL (~50 mM phosphate buffer, 5% isopropanol, 10 or 4 µg enzyme and sterols) was diluted with 2 mL of methanol to give a ~70% methanol solution, and 150 mg of GP hydrazine and 150 µL of glacial acetic acid were added. The mixture was left at room temperature overnight. The GP reaction mixture (3 mL, 70% methanol) after overnight incubation was directly applied to a Sep-Pak C_{18} bed (1 × 0.8 cm in a glass column) followed by 1 mL of 70% methanol and 1 mL of 35% methanol. The combined effluent (now 5 mL) was diluted with 4 mL of water. The resulting mixture (now 9 mL in 35% methanol) was again applied to the column followed by a wash with 1 mL of 17% methanol. To the combined effluent, 9 mL of water was added. The sample was then in 19 mL of about 17.5% methanol. This was again applied to the column followed by a wash with 10 mL of 10% methanol. Now, all the GP derivatives are extracted by the column. They were then eluted with two 1 mL portions of methanol followed by 1 mL of chloroform/methanol, 1:1 (v/v). The three fractions were analysed separately by ES mass spectrometry. The derivatisation protocol has been applied to mixtures of oxosteroids on the µg–ng level and is suitable for the low-level (pg) derivatisation of neutral steroids extracted from tissue (Griffiths et al., 2006; Wang Y. et al., 2006).

2-Hydrazino-1-methylpyridine provides an alternative charged derivative to the GP reagent (Higashi et al., 2005c). The derivatisation reagent requires in-house synthesis, but reacts quantitatively with oxosteroids at 60°C in 1 h. The resulting derivatives of mono-oxosteroids provide 70- to 1,600-fold higher sensitivity than the underivatised steroids in LC-ES-MS experiments. Surprisingly, the derivative appears unsuitable for dioxosteroids, providing little improvement in sensitivity for androstenedione and progesterone.

Dansyl Chloride. Estrone, estradiol and ethinylestradiol are readily derivatised with dansyl chloride. Anari et al. (2002) have shown dansyl derivatives to give an enhanced response in ES-MS experiments and Nelson et al. (2004) have used these derivatives with APCI in a method for estrone and estradiol measurement in human plasma. After adding [$^2H_4$]estrone and [$^2H_5$]estradiol to 0.5 mL of serum, steroids were extracted with 6 mL of methylene chloride. After evaporation of solvent, 50 µL of sodium hydrogen carbonate (100 mM, pH 10.5) and 50 µL of dansyl chloride (1 g/L) were added. The samples were analysed by LC-APCI-MS after heating at 60°C for 3 min.

Oximes. Liu et al. (2000) derivatised oxosteroids in 1 mL of 70% methanol with 50 mg hydroxyammonium chloride. After heating at 60°C for 3 h, the solution was concentrated to near dryness. One millilitre of 10% methanol was added and steroid oximes extracted on a C_{18} column. After a wash with 2 mL of water, oximes were eluted with 1 mL of methanol. No, or incomplete derivatisation was achieved for the 11-oxo group and oxo groups hindered by two adjacent hydroxyl groups. For those steroids, the reaction was carried out in 100 µL of pyridine at 60°C for 1 h with 10 mg of reagent as described by Thenot and Horning (1972). Liu et al., (2000) found that oxime derivatisation gave an improvement of 20-fold in ES-MS sensitivity and applied this methodology to the LC-ES-MS profiling of neurosteroids in rat brain (Liu et al., 2003b). Neurosteroids were derivatised with 2-nitro-4-trifluoromethyl

phenylhydrazine for LC-EC-APCI-MS (Higashi, 2006). In the same paper, LC-ESI-MS is used to detect steroids after reaction with 2-hydrazino-1-methylpyridine to introduce a charged moiety. For oestrogens, picolinyl derivatives have been detected with LC-ESI-MS (Yamashita et al., 2007b). The process of derivative formation may be speeded up with the assistance of microwaves (Zuo et al., 2007).

3.9.3 Applications of LC-MS/MS to Steroid Analysis

With the ever increasing maturity of LC-MS/MS instrumentation and methodology, many commercial and clinical laboratories are embracing this technology for high sensitivity, high throughput analysis of steroids.

Internal Standards. This question has been discussed earlier in this chapter (see Section 3.1), but should perhaps be reiterated here as proper use of internal standards (IS) in MS systems is fundamental for accurate quantitative measurements. Ideally, the IS should not differ in structure from the analyte, so stable-isotope analogues with a mass difference of 3 or greater Da from the analyte are desirable. The following companies supply internal standards (mainly deuteriated) and will undertake custom synthesis: Cambridge Isotope Laboratories, www.isotope.com; CDN Isotopes, www.cdnisotopes.com; Medical Isotopes, www.medicalisotopes.com; Isotec, www.sigmaaldrich.com. Internal standards should always be added to the sample before extraction.

Sample Introduction. The major bottleneck in clinical steroid analysis by MS/MS is sample extraction, so the development of automated extraction techniques is vital. Quest Diagnostics in California (www.questdiagnostics.com) are leaders in the use of an on-line extraction system based on turbulent flow principles, and through this have overcome the rate-limiting step impeding high throughput LC-MS/MS analysis. They use a Cohesive technologies (www.cohesivetech.com) TX4 multiplexing high turbulence liquid chromatography (HTLC) system for on-line extraction of steroids from 150 µL of sera previously treated with 1% trichloroacetic acid (TCA) (Dr. Nigel Clark, personal communication). The HTLC system is divided into two functions: (1) Solid-phase extraction using a large particle size (50 µm) column and high flow rate. The high flow rate causes turbulence inside the column, which ensures optimised binding of steroid to the large particles and the passage of residual protein and debris to waste. (2) Following a loading step from 96-well plates, the flow is reversed and the sample is eluted off the loading column and onto the analytical column. Typically, sequential sample injections can be made every 1–2 min.

3.9.4 Steroid Hormones

The relevance of steroid hormone assay in the clinical environment has been reviewed by Holst et al. (2004).

Steroid Hormone Profiles. Guo et al., (2004) have published a method for the profiling of nine steroids from 760 µL of serum with minimal work-up, comprising acetonitrile protein precipitation. An API-3000 tandem quadrupole instrument (Applied Biosystems) was used with APPI source in the positive-ion mode. The MRM transitions utilised are shown in Table 3.3. The lower level of sensitivity was 100 pg/mL for each steroid, but the authors suggest that the use of an API 4000 instrument would allow a tenfold improvement in sensitivity. The latest tandem quadrupole from Applied Biosystems, the API 5000, would be expected to give an even greater improvement in sensitivity (Guo et al., 2008). The one drawback of their methodology, if used commercially, is the long chromatography time of 14 min. This method has been further improved now encompassing 12 steroids, using only 200 µL of serum with a run time of 8 min (Guo et al., 2006) and has been applied to the investigation of steroid levels in pregnancy (Soldin et al., 2005) and causes of adrenal insufficiency (Holst et al., 2007).

17-Hydroxyprogesterone is the preferred analyte for the diagnosis of 21-hydroxylase deficiency (Fig. 3.14). Investigators at the Mayo Clinic measured a panel of three relevant steroids (17-hydroxyprogesterone, cortisol and androstenedione) in blood spots as a follow-up test for newborn screening (Lacey et al., 2004; Minutti et al., 2004). The mass spectrometry was carried out on an API 3000 tandem quadrupole instrument (Applied Biosystems) with a Turbo-ionspray source operating in the positive-ion mode. LC was conducted on a narrow-bore C_{18} column (50×2.1(i.d.) mm) with a methanol/water solvent system operating in gradient mode (250 µL/min). The internal standard for the three steroids analysed was [$^2H_8$]17-hydroxyprogesterone. Steroids were analysed by MRM. The vast majority of false-positive cases had 17-hydroxyprogesterone levels < 10 µg/L accompanied by a substantial cortisol peak. Samples from confirmed cases characteristically lacked cortisol but had increased 17-hydroxyprogesterone and androstenedione. The reproducibility of the assay was good with inter- and intra-assay CVs of about 20% at the lowest level of 1.9 µg/L, improving to a mean of about 5% at 50 µg/L. The authors admit that some methodological improvement is needed before the method is ready for use as a primary screen. While the analytes of interest elute in 2–4 min, a filter paper contaminant requires a 12 min run to remove. This would translate to maximum daily run of 120, without allowing time for servicing or cleaning. However, 4min runs are possible with serum so the method can already be used for high-throughput analysis by commercial laboratories.

In two other publications, investigators have chosen to use derivatives in order to improve sensitivity. Whether this is needed will depend on the instrument used since sensitivity did not appear to be a problem in the Mayo studies. Lai et al. (2002) use the Girard P reagent with 6α-methylprednisolone as internal standard. They had partially automated the extraction and derivatization by employing 96-well technology and used an API 2000 tandem quadrupole instrument (Applied Biosystems) with a Turbo-ionspray source. The collision gas was nitrogen, chromatography was narrow-bore C_{18} (50 × 2 (i.d.) mm) with an acetonitrile/water (1:1) solvent at a flow rate of 50 µL/min. Each analysis took 3 min and 300 could be carried out before instrument cleaning was required. Intra-assay and inter-assay CVs were <12%. The maximum number of samples analyzed daily was 192 for one technician and one instrument, an equivalent workload to the RIA assay employed in their laboratory. Johnson (2005)

described LC-MS/MS of a serum 17-hydroxyprogesterone, cortisol and androstenedione panel following derivatisation with the Girard T reagent. He reported that preparation of the derivative resulted in a tenfold improvement in sensitivity compared to analysis of the steroids with underivatised ketones. This method was then at an initial stage of development since automation of sample handling was not addressed. Sample size was 100 μL and the linearity of response was over a 1–1,000 ng range. Johnson did address the issue of internal standards, deciding that employing a stable isotope analogue of each analyte would improve the accuracy by reducing the importance of variable instrument performance. As well as the [$^2H_8$]17-hydroxyprogesterone standard; he utilized [$^2H_3$]androstenedione and [$^2H_3$]cortisol. He also addressed an important issue which does not get enough attention, the instability of deuteriums in some currently available labelled standards. When subject to a harsh Girard T derivatisation, deuterium was lost from the [$^2H_8$]17-hydroxyprogesterone since this standard is originally prepared by proton/deuterium exchange. However, a mild derivatisation was developed that prevented loss of deuterium.

Redor-Goldman et al. (2005a, b) report a 17-hydroxyprogesterone, progesterone and androstenedione panel on 150 μL of serum. They transfer samples to 96-well plates, add labelled internal standards and 150 μL formic acid. After 30 min incubation, 70 μL is injected using HTLC (TX4, Cohesive Technologies) on a 50 × 1 mm extraction column followed by chromatography on a 75 × 3 mm analytical column. Mass spectrometry is on a Thermo Electron TSQ quantum tandem quadrupole with an APCI source operated in the positive-ion mode. The total run time is 1.5 min per analyte.

21-Deoxycortisol is the key analyte overproduced in congenital adrenal hyperplasia (CAH). This is the precursor of the urinary metabolite pregnanetriolone, which has long been considered a hallmark analyte for confirming CAH in infancy (Fig. 3.14).

Cristoni et al. (2004a) report the analysis of 21-deoxycortisol by ES-MS and APCI using a Thermo Finnigan LCQ ion-trap instrument operated in the positive-ion mode. They found that ES afforded greater sensitivity. They addressed the problem of the possible interference of the 21-deoxycortisol isomer, 11-deoxycortisol, itself the analyte for diagnosis of 11β-hydroxylase deficiency. While the steroids share many fragmentations, some of these are specific for each steroid and can be used for MRM. In addition, these two compounds are resolved by reversed-phase chromatography (C_{18} 250 × 2.1 (i.d.) mm, 200 μL/min). Cristoni et al. (2004a) maintain that 21-deoxycortisol is a good analyte for identifying heterozygous individuals for 21-hydroxylase deficiency, and certainly their measurement of 11-deoxycortisol will in the future allow diagnosis of 11β-hydroxylase deficiency. The report is preliminary in that they did not use internal standards and their chromatography time of 9 min would have to be shortened before a truly routine analysis could be validated. A further publication from this group describes the use of surface-activated CI ion-trap mass spectrometry for the analysis of blood 21-deoxycortisol (Cristoni et al., 2004b).

Cortisol and Related Compounds (see chapter 5). The measurement of these compounds in urine is essential for the study of Cushing's disease, glucocorticoid remediable aldosteronism (GRA), apparent mineralocorticoid excess (AME) syndrome, and related conditions. Taylor et al., (2002) at the Mayo Clinic published a method for the simultaneous analysis of urinary cortisol and cortisone. Measuring both these steroids allows the diagnosis of Cushing's disease and the AME syndrome.

Table 3.3 Unconjugated steroids and steroid sulphates of clinical importance in human serum: Reference ranges (classical techniques or LC-MS), LC-MS monitored ions and MRM transitions. Modified from Shackleton (2008) with permission [*Copyright 2008 Springer*]

Steroid	Parent ions and MRM transitions	Adult range (μg/L)	Child (μg/L) [where given age in years]	Males (μg/L)	Females (μg/L)	Follicular (μg/L)	Luteal (μg/L)	Reference (MS method)
Pregnenolone	(317)[a]	0.1 – 2.3	≤0.1	0.1 – 2.0	0.1–2.3			
Progesterone	315 → 109	0.1 – 2.5	≤0.2	**≤0.3**[b]		**<0.14**[b]	**<0.31**[b]	Guo et al. (2004)
d_9 – labeled	324 → 100							Redor – Goldman et al. (2005b)
17 - OH – progesterone	331 → 97	0.5 – 5.0	<1 year, 0.15 – 4.2	**0.32 – 3.07**[b]		**<1.85**[b]	**<2.85**[b]	Guo et al. (2004)
d_8 - labeled	339 → 100		<10 years, 0.15 – 0.8					Redor – Goldman et al. (2005b)
			>10 years, 0.15 – 3.7					Minutti et al. (2004)
17 - OH – pregnenolone	(333)[a]	0.4 – 4.5	<1.0	0.4 – 4.5	0.2 – 4.0			
21 - Deoxycortisol	347 → 311							Cristoni et al. (2004a)
	347 → 293							
Desoxycorticosterone (DOC)	(331)[a]		0.01 – 0.1	0.035 – 0.11		0.015 – 0.08	0.035 – 0.13	
Corticosterone	347 → 121	0.6 – 12.9	0.8 – 20.3					
Aldosterone	361 → 325	**0.3 – 1.6**[b]	**5 – 9** years, **<0.9**[b]					Redor – Goldman et al. (2005b)
	361 → 315		**1 – 17** years, **<3.5**[b]					
	359 → 331[c]	**0.03 – 0.16**[b]						Fredline et al. (1997)
11 – Deoxycortisol	347 → 97	**0.2 – 1.3**[b]	**0.1 – 2.0**[b]					Guo et al. (2004)
d_2 – labelled	349 → 97							
Cortisone (serum, total)	361 → 163	**6 – 27**[b]	2.3 – 17.7					Kushnir et al. (2004)
	361 → 105							

Cortisone (urine free, µg/24 h)	361 → 163	**23 – 195**[b,d]				Clarke and Goldman (2005), Palermo et al. (1996)[e]
				17 – 141[b,d]	**15 – 122**[b,d]	Taylor et al. (2002)
Cortisol (serum, total)	363 → 121 363 → 97	**20 – 240**	**20 – 230**			Guo et al. (2004)
d_4 labelled	367 → 121					Kushnir et al. (2004)
Cortisol (urine free, µg/24 h)	363 → 121 363 → 97	**3.0 – 55**	1 – 4 years, **0.9 – 8.2**			Clarke et al. (1996) Palermo et al. (1996)[e]
d_4 labelled	367 → 121		5 – 14 years, **1.0 – 45**			
				4.2 – 60	**3.0 – 43**	Taylor et al. (2002)
18 - OH – cortisol (urine, µg/24 h)	379 → 121	**43 – 515**		**43 – 295**	**51 – 515**	Clarke et al. (1996) Palermo et al (1996)[e]
DHEA	271 → 213	1.3 – 12.5		1.8 – 12.5	1.3 – 9.8	Guo et al. (2004)
DHEA sulphate	367[c]	**1,000 – 2,850**	<5 days, 100 – 2,540 <5 years, 10 – 400 10 – 17 years, 150 – 5,550	**1,800 – 3,450**	**670 – 3,700**	Shackleton et al. (1990)
Testosterone (total)	289 → 109 289 → 97		<10 years, F, **≤0.35** < 10 years, M, **≤ 0.4**	**1.2 – 11.1**	**0.03 – 0.46**	Redor – Goldman et al. (2005a)
d_5 - labelled	294 → 112 294 → 99		>10 years, F, **≤0.40** >10 years, M, **0.21 – 10.0**			
Analyte d_3 - labelled	289 → 97 292 → 97					Starcevic et al. (2003)
Dihydrotestosterone (DHT, total)	291 – 255	0.05 – 0.75		0.25 – 0.75	0.05 – 0.3	Zhao et al. (2004)

(continued)

Table 3.3 (continued)

Steroid	Parent ions and MRM transitions	Adult range (μg/L)	Child (μg/L) where given age in years	Males (μg/L)	Females (μg/L)	Follicular (μg/L)	Luteal (μg/L)	Reference (MS method)
Androstenedione	287 → 97	**0.2 – 2.85**		**0.4 – 2.2**		**0.2 – 0.75**	**0.6 – 2.85**	Guo et al. (2004)
d_7 - labelled	294 → 100							Minutti et al. (2004)
								Redor – Goldman et al. (2005b)
Estriol[c]	287 → 171	<0.1		<0.1	<0.1			Guo et al. (2004)
d_2 - labelled	289 → 147							
Estrone (total)	504 → 171[f]			**0.01 – 0.06**	**0.017 – 0.20** PM[g] <0.05	0.015 – 0.25	0.015 – 0.2	Nelson et al. (2004)
Estradiol	506 → 171[f]		>10 years, 0.005 – 0.06 10 – 17 years, F, 0.005 – 0.4 10 – 17 years, M, 0.005 – 0.04	**0.01 – 0.04**	**0.015 – 0.35**			Nelson et al. (2004)
Cholesterol sulphate	465[c]	**50 – 300**						Shackleton and Reid (1989)
$^{13}C_2$ - labeled	467[c]							

[a]Bracketed number corresponds to the protonated molecule if mass spectrometry was used for quantification.
[b]Figures in bold obtained by tandem MS; others by immunoassay techniques (from Quest Diagnostics manual).
[c]Negative ion.
[d]Urine μg/24 h.
[e]Data also from GC-MS.
[f]Dansyl derivative.
[g]PM is post-menopausal.

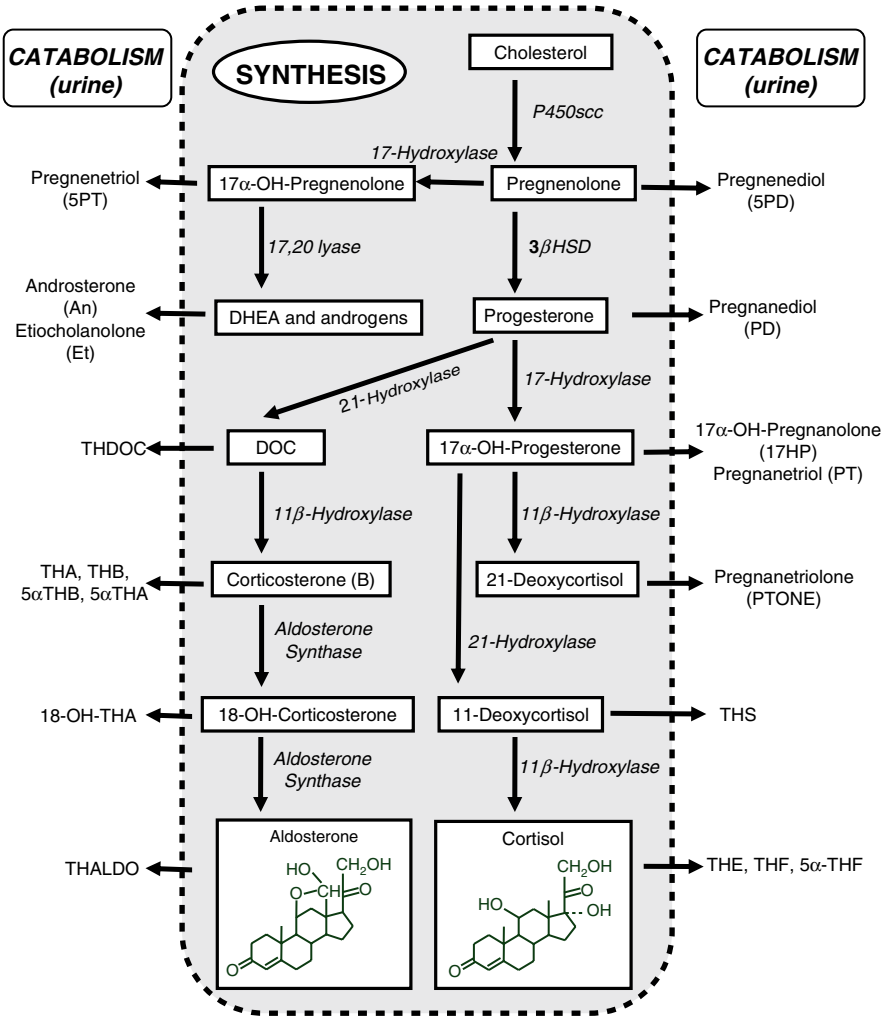

Fig. 3.14 Synthesis of adrenal steroids and major excreted metabolites. Modified with permission from Shackleton (2008)

They used [$^2H_4$]cortisol as internal standard and took a 0.5 mL urine sample. There was an extensive extraction and washing step with 4.5 mL methylene chloride. An API 2000 tandem quadrupole (Applied Biosystems) was used in the positive-ion mode with MS/MS. Chromatography was conducted on a normal-bore C_{18} column (33 × 4.6 (i.d.) mm) with C_{18} pre-column (4 × 2 mm). An isocratic methanol/water solvent system was used at a flow rate of 1 mL/min. The source was of the Turbo-ionspray type, and using a column splitter, 200 μL/min was introduced to the source. MRM was conducted in the positive-ion mode monitoring *m/z* 363 → 121 for cortisol, 367 → 121 for [$^2H_4$]cortisol and 361 → 163 for cortisone. Cortisol and

cortisone were separated and both were eluted within 2 min. Inter- and intra-assay variation for both compounds was <9% for amounts above 2 μg/dL.

Quest Diagnostics use an LC-MS/MS panel for diagnosing cortisol-related disorders. This panel was designed to diagnose Cushing's syndrome and the hypertensive conditions, AME syndrome and GRA. The panel quantifies cortisone, cortisol, 6β-hydroxycortisol and 18-hydroxycortisol. The Quest analysis uses [$^2H_4$]cortisol as an internal standard and HTLC for on-line extraction. This panel has replaced the RIA and HPLC methods previously used by this commercial laboratory. A recent publication describes MS/MS of cortisone and cortisol in serum using APPI and similar conditions and MRM transformations to those listed above (Kushnir et al., 2004).

11-Deoxycortisol. CAH caused by 11β-hydroxylase deficiency is diagnosed by finding elevated 11-deoxycortisol. This steroid is included in the panel of Guo et al., (2004, 2006), reported above, monitoring the transitions *m/z* 347→97 for analyte and 349 → 97 for [$^2H_2$] internal standard.

Aldosterone. Fredline et al. (1997) report an MRM negative-ion ES method for aldosterone using flumethasone as internal standard. The MRM transition used was 359 → 331. The assay was linear over a 15–500 pg/mL range and the limit of quantitation 15 pg/mL. A manual extraction was used and recovery and accuracy were excellent. Quest Diagnostics use APCI in the positive-ion mode monitoring the transitions 361 → 325 and 361 → 315 (Dr. Nigel Clarke, personal communication).

Testosterone and Dihydrotestosterone (DHT). Testosterone assays have been notoriously inaccurate, particularly at the low levels found in women and children. Excess testosterone in women is often a cause of infertility, hirsutim, amenorrhoea, and obesity and accurate measurement is essential for evaluating the causes of these disorders. Accurate measurement in small sample volumes is essential in pediatrics. Starcevic et al. (2003) report the LC-MS/MS of serum testosterone using an API tandem quadrupole mass spectrometer (Applied Biosystems) in the positive-ion mode. [$^2H_3$]Testosterone was the internal standard. The run time is 1.25 min. They monitored the transition m/z 289 → 97 for analyte and 292 → 97 for internal standard. These investigators achieve excellent reproducibility and linearity. Sensitivity down to 10 pg/mL allows them to readily measure the hormone in samples from females and children. Data produced using this new method correlates perfectly with data produced by classical methodology. Quest Diagnostics have established a routine system for testosterone, which has now replaced their other methods of analysis (Clarke and Goldman, 2005; Redor-Goldman, 2005a, b). The analysis is carried out on a Thermo Finnigan TSQ Quantum Ultra tandem quadrupole operated with an APCI source in the positive-ion mode. They use two transitions 289 → 109 and 289 → 97, for analyte and 294 → 112 and 294 → 99 for [$^2H_5$] labelled internal standard. On-line extraction and short retention times allow them to assay several thousand samples per month per instrument. Higashi et al. (2005b & c) use an ES-MS/MS method for measuring testosterone and DHT with derivatization with 2-hydrazino-1-methylpyridine to improve sensitivity. Further studies using a similar approach in rat brain and serum (Higashi et al., 2006b) and prostate (Higashi et al., 2005b, 2006c) have been described.

Androstenedione has been measured as part of a CAH panel by Minutti et al. (2004), using the transition 287 → 97, and by Quest Diagnostics using HTLC and APCI (Clarke and Goldman, 2005; Redor-Goldman, 2005a, b). LC-ESI(+)-MS has been used to measure androstenedione in rat brain (Higashi et al., 2007a, c, 2008).

Estrone and Estradiol. The routine measurement of estrone and estradiol by immunoassay techniques has also given rise to the familiar problems of poor sensitivity, cross-reactivity and poor inter-method reproducibility. Most automated methods cannot measure these steroids in sera of children and men. Development of LC-MS/MS methods has also proven challenging as many investigators have found that estrone and estradiol are poor ionisers. Thus, desired sensitivity has not been achieved. The Mayo group (Nelson et al., 2004) published the LC-MS/MS quantification of estradiol and estrone using dansyl chloride derivatives. These derivatives are easily prepared, provide excellent sensitivity in positive-ion mode APCI, and produce a prominent product-ion suitable for MRM. The internal standards used were [$^2H_5$]estradiol and [$^2H_4$]estrone. Methods for measuring estrone and estradiol after derivatisation using LC-ESI(+)-MS (Nishio et al., 2007) and LC-electron capture APCI-MS (Higashi et al., 2006a) have also been described.

Using the best of modern instrumentation dansyl derivatization may not be necessary to reach sensitivity requirements for estradiol measurement. Estradiol is an analyte within the Guo et al. (2004) panel. Using an API 3000 tandem quadrupole (Applied Biosystems) with an APPI source in the positive-ion mode, they measure the *m/z* 255 → 159 transition for estradiol and *m/z* 259 → 161 for [$^2H_4$] estradiol. With the API 3000 instrument, they could not measure estradiol below 100 pg/mL, although preliminary studies showed that the API 4000 (Applied Biosystems) could achieve sensitivity of 10 pg/mL. The Applied Biosystems Company have demonstrated reproducible estradiol measurement at concentrations down to 0.1 pg 'on-column' on their API 5000 instrument with an APCI source. The linearity was excellent over the range 0.001–10 ng/mL (company demonstration data). Guo et al. (2008) describe an LC-MS/MS method for the analysis of serum estrogens in 200μL of serum without derivatisation but do not specify the source of the serum samples.

Estriol. This steroid is also part of the Guo et al., (2004) panel, monitoring the negative-ion transitions m/z 287 → 171 for estriol and m/z 289 → 147 for [$^2H_2$]estriol.

DHEA and DHEA Sulphate. Guo et al., (2004) analyzed these in the positive-ion mode with APPI using the transitions *m/z* 271 → 213 for DHEA and 273 → 213 for the [$^2H_2$] internal standard. DHEA sulphate is more suited to the measurement by negative-ion ES mass spectrometry as demonstrated in an early study by Shackleton et al. (1990a). DHEA has also been measured in human saliva using LC-ESI(+)-MS after derivatisation with 2-hydrazino-1-methylpyridine (Higashi et al., 2007d).

Neurosteroids. LC-ESI(+)-MS has been described in a number of publications from Shimada's group with (Higashi et al., 2005, 2006b, 2007a) and without (Higashi et al., 2008) prior derivatisation to improve the ESI response.

3.9.5 Steroid Metabolites and Precursors

GC-MS is the most powerful technique for steroid profile analysis, and with just a few exceptions all disorders of steroid synthesis and metabolism first had their metabolome defined by GC-MS analysis of urine. The coupling of GC to an MS system also provides unparalleled opportunity for identification of the structures of unknown steroids, especially when linked to different derivative formation prior to GC. GC-MS for steroid analysis is thus discussed in detail in Section 3.10. Here, discussion will continue on LC-MS applications. LC-MS has the potential for steroid profile analysis, however, to date; LC-MS has been mostly used for the analysis of individual metabolites or panels of metabolites as discussed above.

Early studies on bile acids, steroid sulphates and glucuronides found in urine or plasma were performed using FAB-MS by Shackleton and colleagues, amongst others (Shackleton and Straub, 1982; Shackleton, 1983; Shackleton et al. 1983; Gaskell et al., 1983; Clayton et al. 1987). Today's API techniques, in particular ES, have supplanted FAB as the method of choice for the ionisation of bile acids and steroid conjugates.

Steroid Sulphates. Acidic steroids are preferably ionised in the negative-ion mode. Pioneering work on the ES analysis of steroids was performed by Henion and colleagues in the late 1980s (Weidolf et al., 1988). They combined narrow-bore HPLC (C_{18} 100 × 1 (i.d.) mm, 40 µL/min) with negative-ion ES for the analysis of steroid sulphates. By performing selected ion monitoring (SIM) studies on standard compounds, they were able to obtain the very impressive detection limit of 10 pg on-column. When they operated their tandem quadrupole instrument in the MRM mode, which offers great gains in selectivity, they obtained an on-column detection limit of 300 pg. Shackleton and colleagues were the first to use ES-MS and ES-MS/MS for the analysis of steroid sulphates in plasma (Wong et al., 1992). Despite using off-line HPLC, they were able to achieve low nanogram detection limits. Steroid sulphates can also be analysed by ES operated in the positive-ion mode. Bowers and Sanaullah (1996) recorded positive-ion ES-MS and MS/MS spectra of a series of steroid sulphates. They achieved a detection limit of 65 pg by SIM. The MS/MS spectra were found to contain major fragment ions corresponding to $[M + H - 80]^+$, $[M + H - 98]^+$ and $[M + H - 116]^+$. 17-Keto(oxo)steroid sulphates (Higashi et al., 2007b) and DHEA (Higashi et al., 2007d) have been determined in human saliva by LC-ES-MS after extraction with RP-Strata-X cartridges. Strata columns have also been used to extract metabolites of DHEA and 4-androstenedione from EpiskinTM incubation media prior to LC-APCI-ion trap MS (Luu-The et al., 2007).

Nano-ES, without LC separation, has been used for the analysis of steroid sulphates. Chatman et al. (1999) used negative-ion nano-ES in combination with MS/MS for the analysis of steroid sulphates in plasma. By recording the product-ion spectra, they were able to identify testosterone sulphate ($[M - H]^-$, *m/z* 367) in the urine of a primate. Their results were based on the observation of MS/MS fragment ions which included *m/z* 177 which is characteristic of testosterone sulphate.

Chatman et al. (1999) also exploited precursor-ion scanning for *m/z* 97 (HSO_4^-), and were able to obtain a very impressive detection limit of 200 amol (~70 fg) for steroid sulphates added to cerebrospinal fluid. For the analysis of neutral steroids, Chatman et al. (1999) used a derivatisation method in which alcohol groups are converted to sulphate esters, and then the resulting steroid sulphates are analysed by negative-ion nano-ES. This method was also used by Sandhoff et al. (1999) for the quantification of free cholesterol in CHO (Chinese hamster ovary) cells and Golgi membranes.

Nano-ES, again without LC separation, has been used to study the fragmentation of neurosteroid sulphates (Griffiths et al., 1999) at high collision-energy (4 keV–400 eV) on a hybrid magnetic sector – TOF instrument (Micromass). Complete structural information could be obtained from 1 ng (~3 pmol) of steroid sulphate (e.g. pregnenolone sulphate), while fragment ions characteristic of the sulphate ester group, i.e. *m/z* 80 (SO_3^-) and 97 (HSO_4^-) could be obtained from 3 pg (10 fmol) of sample. In this work, the advantage of high-energy MS/MS was evident.

APPI has been used for the analysis of steroid sulphates. This was demonstrated by Guo and colleagues who interfaced a C_{18} column (330 × 3 (i.d.) mm) to an APPI source (Guo et al., 2004). With eluent flow rate of 0.5 mL/min a 20-min chromatographic run was performed going from 100% solvent A (2% methanol) to 89% B (100% methanol). The dopant used was toluene at a flow rate of 50 µL/min. In the positive-ion mode DHEA sulphate fragments in the ionisation process with loss of the sulphate ester group to give the $[M + H - H_2SO_4]^+$ ions at *m/z* 271, while free DHEA gives $[M + H - H_2O]^+$ ions also at *m/z* 271 (Table 3.3). However, the retention times on the C_{18} column for these two steroids were 7.46 and 13.99 min, respectively. By performing MRM studies (*m/z* 271 → 213 for both steroids), the sensitivity of the method was of the order of 100 pg on-column.

Steroid Sulphate Profiles. Liu et al. (2003a & b) have used capillary LC combined to low-flow-rate ES to analyse steroid sulphates from biological samples. They used a C_{18} capillary column (350 × 0.1 (i.d.) mm) preceded by a short trap column, and performed isocratic and gradient elution at flow rates of 0.2–0.3 µL/min. Liu et al. (2003a) illustrated the potential of combining low flow rate LC with MS/MS for steroid profile analysis, where numerous isomeric compounds require separation and identification, by analysing steroid sulphates in plasma. Briefly, 5 µL of plasma was diluted with 1 mL of 70% ethanol containing an isotope-labelled standard. The mixture was centrifuged to remove precipitated proteins, and the supernatant passed through a bed of Bondasil C_{18} (~30 mg) packed in a pasture pipette to remove cholesterol and non-polar lipids. The effluent was collected, dried and redissolved in 100 µL of 10% methanol, and finally an aliquot of this solution was injected onto the column. Shown in Fig. 3.15 are total ion current (TIC) and reconstructed ion chromatograms (RICs) for steroid sulphates expected to be found in plasma. Twenty microlitres of the sample corresponding to 1 µL of plasma was injected onto column. The peak at 17.76 min in the RIC of *m/z* 367 corresponds to the $[M - H]^-$ ion of DHEA sulphate, and that at 32.97 in the RIC of 395 to the $[M - H]^-$ ion of pregnenolone sulphate. Using an isotope-labelled internal standard, the concentration of these two sulphates were determined to be 4.1 and 0.22 µM (1.5 and 0.08 mg/L), respectively (cf. Table 3.3). Sulphate esters of androsterone,

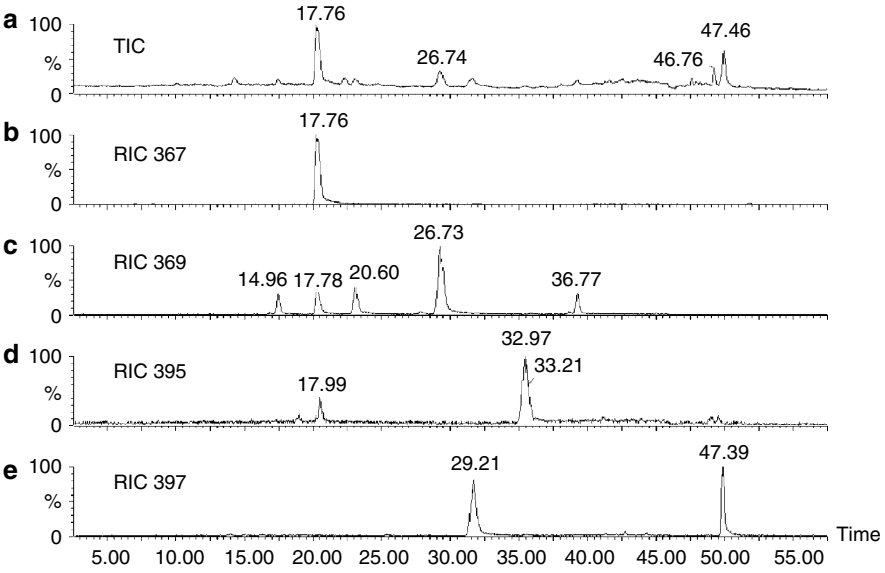

Fig. 3.15 LC-MS of steroid sulphates in human plasma. Total ion current chromatogram (TIC) and RICs obtained from an analysis of a human plasma sample. (**a**) TIC, (**b**) RIC of *m/z* 367, (**c**) RIC of *m/z* 369, (**d**) RIC of *m/z* 395, (**e**) RIC of *m/z* 397. Twenty microlitres of sample solution, corresponding to 1 μL of plasma, was injected onto the pre-column. After an initial desalting period of 20 min with solvent A (10% methanol containing 10 mM ammonium acetate), elution was performed with a gradient starting at 50% solvent B (80% methanol containing 10 mM ammonium acetate) going to 70% in 40 min, maintained at this level for 20 min and then increased to 100% solvent B in 10 min. Data acquisition was started 34 min after the start of the gradient. Time is given in minutes. Identification of the peaks is detailed in text, which also takes data from Fig. 3.16. From Liu et al., Griffiths & Sjövall (2003a) with permission)

epiandrosterone, and androstenediol all give $[M - H]^-$ ions of *m/z* 369. Shown in Fig. 3.15c is the RIC for this *m/z*, and in Fig. 3.16 are high-energy (400 eV) MS/MS spectra for the five peaks observed in the RIC. High-energy (400 eV) MS/MS spectra contain information that not only indicates the nature of the conjugating group but also its location. This is illustrated for the $[M - H]^-$ ions of *m/z* 369 in Fig. 3.15c. The predominant ion in RIC for *m/z* 369 (Fig. 3.15c) gives the MS/MS spectrum illustrated in Fig. 3.16d. The peak at *m/z* 191 indicates that the sulphate group is on the A-ring and that the C-5–C-6 bond is saturated, and comparison of this MS/MS spectrum with that of authentic androsterone sulphate indicates that this compound is androsterone sulphate. The peak at 20.60 in the RIC shown in Fig. 3.15c gives the MS/MS spectrum shown in Fig. 3.16c. The MS/MS spectra in Figs. 3.16c and d are very similar; however, the $[M - H - 80]^-$ ion at *m/z* 289 is less abundant in Fig. 3.16c. This subtle difference, and also the earlier elution of this compound, suggest it is epiandrosterone sulphate. The chromatographic peak eluting at 36.77 min is too late to be another stereoisomer of androsterone sulphate; however, the fragment ions at *m/z* 191 and 259 in its MS/MS spectrum (Fig. 3.16e) indicate that the sulphate group is on the A-ring and that the B-ring is saturated.

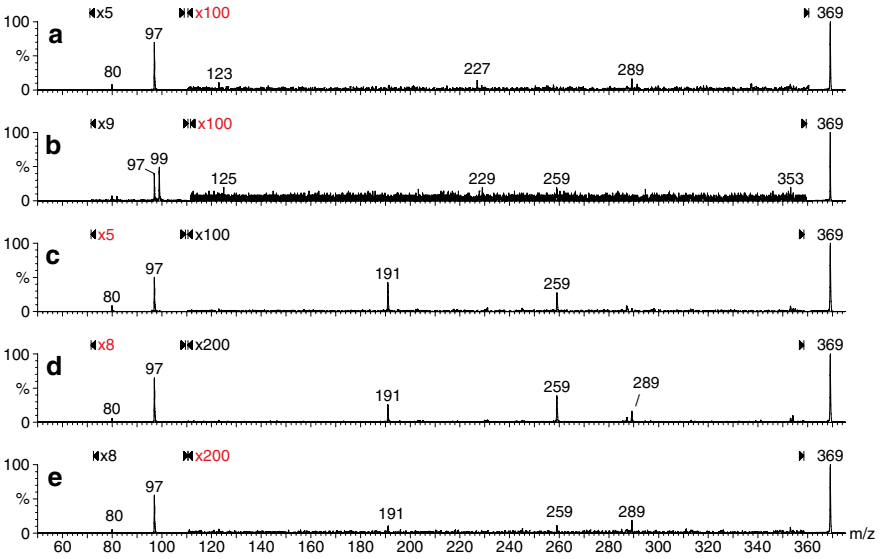

Fig. 3.16 MS/MS of steroid sulphates from human plasma. High-energy (400 eV) CID spectra of ions of *m/z* 369 from the human plasma sample in Fig. 3.15. The spectra were recorded on an AutoSpec-OATOF hybrid magnetic sector – OATOF instrument. The CID spectra of the five individual peaks in the RIC of *m/z* 369 in section c are shown in the order of their retention times (**a**-**e**). The spectra are magnified by a factor of 5, 8 or 9 between *m/z* 70 and 110 and by factors of 100 (**a**-**c**) and 200 (**d**&**e**) between *m/z* 110 and 358. Methane was used as the collision gas at a pressure which attenuated the precursor ion beam by 75%. Chromatographic conditions are given in Fig. 3.14. From Liu et al. (2003a), with permission.

The peak at 14.96 in the RIC gave the MS/MS spectrum in Fig. 3.16a. The absence of a fragment ion at *m/z* at 191, but the presence of one at *m/z* 227 indicate that this steroid has the same AB-ring structure as in DHEA sulphate, and this compound is probably androst-5-ene-3β,17β-diol-3-sulphate. Finally, the peak at 17.78 min corresponds to the ^{34} S-isotopic peak of DHEA sulphate. Liu et al. (2003a, b) investigated the sensitivity of their low-flow-rate chromatographic system, and established an on-column detection limit of 3 pg (7.5 fmol) for steroid sulphates in spectra recorded over a small *m/z* range (360–416); this was improved to 0.2 pg (500 amol) by performing SIM and 0.1 pg by monitoring the $[M - H]^- \rightarrow 97$ ($[HSO_4]^-$) transition in an MRM experiment. The above data clearly demonstrate the advantages of performing low-flow-rate LC-MS and LC-MS/MS when small quantities of biological samples are available, and the levels of endogenous metabolites are low. 17-Keto(oxo) steroid sulphates have also been measured in human plasma by LC-ES-MS (Higashi et al. 2007b).

Steroid Glucuronides can be analysed by negative- and positive-ion ES (Bowers and Sanaullah, 1996; Borts and Bowers, 2000; Kuuranne et al., 2000). When positive-ion ES spectra are recorded with an ammonium acetate buffer, steroid 17-*O*-glucuronides with a 3-oxo-4-ene structure tend to give $[M + H]^+$ and [M +

$NH_4]^+$ ions, while 3-*O*-glucuronides tend to give just $[M + NH_4]^+$ ions. This can be explained by the high gas-phase basicity of the 3-oxo-4-ene structure favouring protonation at the C-3 ketone group. Low-energy MS/MS spectra of the $[M + H]^+$ or $[M + NH_4]^+$ ions show $[M + H - 176]^+$, $[M + H - 194]^+$ and $[M + H - 212]^+$ fragment ions. This series of fragment ions is analogous to the series of major fragment ions observed in the spectra of steroid sulphate $[M + H]^+$ ions. Interestingly, 17-*O*-glucuronides with a 3-oxo-4-ene structure give steroid-skeleton fragment ions at *m/z* 97 and 109 which confirms that protonation occurs at the C-3 ketone group. In a study of anabolic steroid glucuronides aimed at identifying the most promising ionisation method for subsequent MS/MS studies, Kuuranne et al. (2000) concluded that the fragment-ion patterns generated by $[M + H]^+$ and $[M + NH_4]^+$ ions are more structurally informative than those generated from $[M - H]^-$ ions, and that positive-ion ES-MS/MS is the most promising method for further development of LC-MS/MS based methods for anabolic steroid glucuronide analysis.

Oxysterols are formed in the first step of cholesterol metabolism, and have traditionally been analysed by GC-MS. However, LC-MS methods are now gaining popularity. To achieve maximum sensitivity, Griffiths and colleague use a derivatisation strategy which also involves an enzymatic conversion of 3β-hydroxy-5-ene or 3β-hydroxy-5α-hydrogen sterols to 3-oxo-4-ene or 3-oxo sterols (Higashi et al., 2005a), respectively, then treatment with the GP reagent to give GP hydrazones (Griffiths et al., 2006; Khan et al., 2006; Wang et al., 2006). Using this methodology, Griffiths and colleagues have profiled the oxysterol content of rodent brain and human blood. They used a micro-bore (150 × 0.18 (i.d.) mm) C_{18} column with a methanol/water gradient and confirmed 24S-hydroxycholesterol to be the major oxysterol in rat brain. Other oxysterols identified in rodent brain include 24,25-, 24,27-, 25,27-, 6,24, 7α,25-, and 7α,27-dihydroxycholesterols. In addition, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al and its aldol, two molecules linked to amyloidogenesis of proteins, were characterised in rat brain (Wang et al., 2007).

Burkard et al., (2004) have used APCI in combination with reversed-phase HPLC for the analysis of oxysterols in plasma. Chromatography was performed on a C_{18} column (125 × 2 (i.d.) mm) with a methanol/acetonitrile/10 mM ammonium acetate gradient run at 250 µL/min. The run time was 35 min, and 24S-hydroxycholesterol separated from 27-hydroxycholesterol. In the absence of protonated molecules, SIM was performed on the $[M + H - H_2O]^+$ ions at *m/z* 385. The quantification limit for plasma samples of 0.5 mL was 40 µg/L for 24S-hydroxycholesterol and 25 µg/L for 27-hydroxycholesterol, and the levels of these oxysterols in healthy volunteers were 64 ± 14 and 120 ± 30 µg/L, respectively.

Oxysterols can also be formed by cholesterol ozonolysis. Pulfer et al. (2004) separated cholesterol ozonolysis products on a C_{18} column (250 × 4.6 mm) using an acetonitrile/water/methanol 1 mM ammonium acetate gradient (1 mL/min), with a 40 min run time. Eluent was split to ES-MS (50–100 µL/min) and either a scintillation detector or fraction collector. Using negative-ion ES $[M + CH_3CO_2]^-$, adducts of oxysterols were observed while in the positive-ion mode $[M + NH_4]^+$ ions were detected. By collecting fractions from the LC column and performing MS/MS and GC-MS on these, the major cholesterol ozonolysis product was identified as 5-hydroperoxy-B-homo-6-oxa-choles-

tane-3β,7-diol or 7-hydroperoxy-B-homo-6-oxa-cholestane-3β,5-diol. Pulfer and Murphy (2004) used this methodology further to identify the major cholesterol-derived product from the treatment of lung surfactant with ozone, as 5β,6β-epoxycholesterol. 5β,6β-Epoxycholesterol was found to be metabolised by lung epithelial cells to small amounts of the expected metabolite, cholestan-3β,5α,6β-triol (Sevanian et al., 1991) and more abundant levels of an unexpected metabolite cholestan-6-oxo-3β,5α-diol. Other workers have used negative-ion ES-MS analysis of 2,4-dinitrophenylhydrazone derivatives of cholesterol ozonolysis products. By performing LC separations on a C_{18} column using acetonitrile/water/methanol (75:20:5), Wentworth et al. (2003) have observed 3β-hydroxy-5-oxo-5,6-secocholestan-6-al in arterial plaques.

3.10 Gas Chromatography–Mass Spectrometry

Despite major advances in capillary liquid chromatography and API sources, GC-MS is still the preferred method for steroid metabolite profile and structural analysis. The metabolic transformations of steroid hormones and their precursors are summarised in Fig. 3.17. About 5–10% of steroids are excreted as free compounds. 3β-Hydroxy-5-ene steroids are almost exclusively excreted as sulphate esters, while ring-A reduced steroids are excreted as glucuronides. Profiling of steroid metabolites is usually conducted on urine samples, although similar methodology can be used on serum and amniotic fluid. As an example, the measurement of pregnanolones in third trimester pregnancy plasma has recently been described using GC-MS of *O*-methyl-oxime-trimethylsilyl ether derivatives as described below (Hill et al., 2007) and perfluorobenzyl derivatives of *in vivo* DHEA metabolites (Labrie et al., 2007) – there are many similar applications.

3.10.1 Sample Preparation

Samples should be frozen on collection and may be shipped with an ice-pack or on dry ice. Alternatively, samples may be shipped after solid-phase extraction (SPE) on a C_{18} cartridge.

Urine (or serum) 1–2 mL is extracted on a C_{18} SPE cartridge, e.g. Sep-Pak from Waters. First the cartridge is washed by passing of 4 mL of methanol then 4 mL of water, and then loaded with 1–2 mL of urine. After washing with 4 mL of water, the steroids are eluted with 4 mL of methanol (Shackleton, 1986, 1993). The methanolic extract is dried, and 3 mL of 0.1 M acetate buffer pH 4.5 (0.2 M sodium acetate: 0.2 M acetic acid, 3:2, v/v) added. After adding 10 mg of sulphatase (type H1 from Sigma), and 12 μL β-glucuronidase/aryl sulphatase (Roche Diagnostics), hydrolysis proceeds for 3 h at 55°C. Steroids are re-extracted on a C_{18} cartridge. Some steroid sulphates are not hydrolyzed enzymatically, e.g. sulphates of C-17 (in C_{19} steroids) and of C-20 hydroxyls. This is significant in one disorder, oxidoreductase deficiency (ORD), as prominent excretion products are pregnenediol

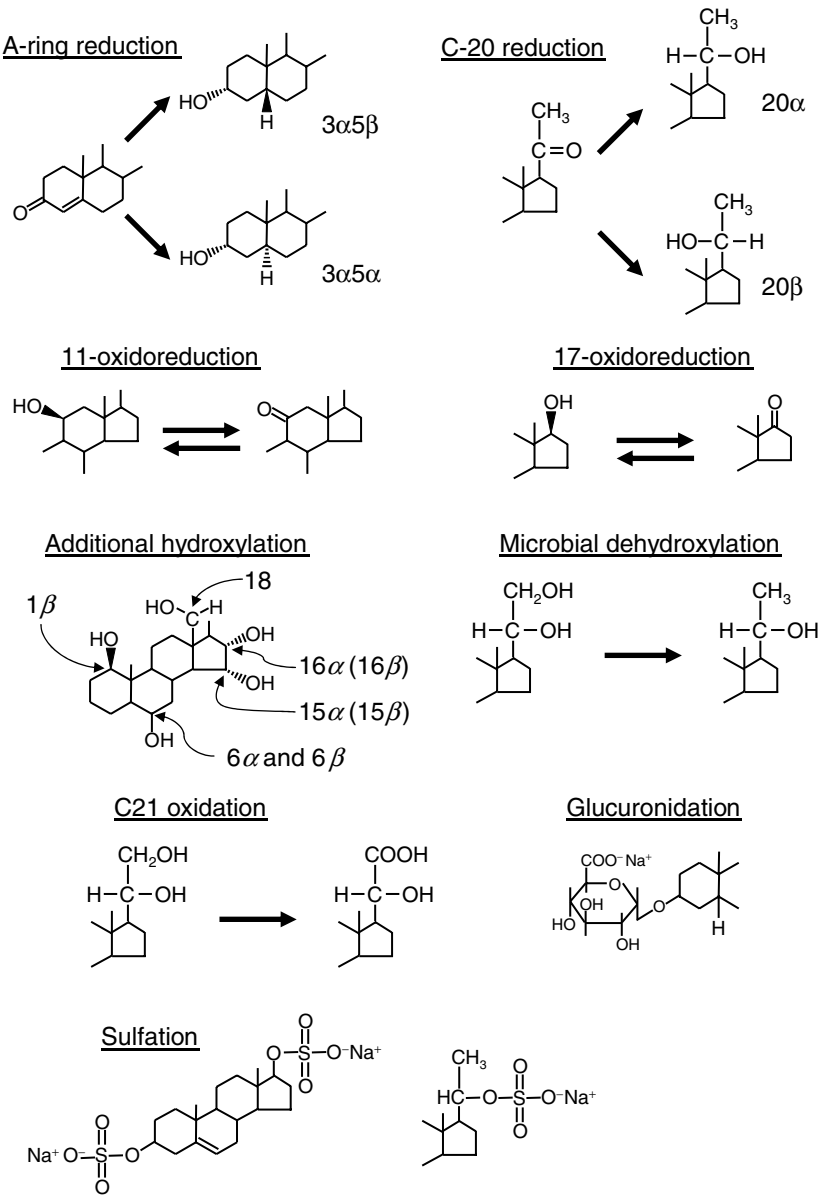

Fig. 3.17 Summary of the major metabolic transformations of hormonal steroids and precursors. (from Shackleton, 2008, with permission) [*Copyright 2008 Springer*]

disulphate in children with the disorder and epiallopregnanediol disulphate in mothers carrying an ORD fetus. It so happens that these steroids undergo desulphation and dehydration during derivatisation and can be quantified as $\Delta^{17\text{-}20}$ pregnandienol and pregnenol, respectively (Griffiths et al., 2005).

After extraction, and de-conjugation, steroids are derivatised. To the methanolic eluate from the C_{18} cartridge, methoxyamine hydrochloride (100 µL, 2% in pyridine) is added and derivatisation allowed to proceed for 60 min at 55°C. Pyridine is blown off, and 50 µL of trimethylsilylimidazole (TMSI) added. Silylation proceeds for 16 h at 100°C. The reaction can be performed for 4 h by increasing the temperature to 120°C. TMSI is involatile and must be removed prior to GC-MS. This is usually achieved by chromatography on Lipidex columns (Shackleton, 1986), but a more efficient method has recently been devised by Dr. Norman Taylor (personal communication). One millilitre of cyclohexane is added to the reaction tube while still hot. TMSI is removed by adding 500 µL of water, vortexing, centrifugation and discarding the bottom layer. The tube is vortexed after addition of a further 500 µL of water, and the top layer is transferred to an injection vial taking care not to transfer any of the aqueous layer.

3.10.2 Apparatus and Scanning

Non-polar capillary columns (DB1 type) are almost universally used for steroid separations. Mass analysis is often performed on a single-stage quadrupole with an EI source, although magnetic-sector instruments can be used for high-resolution work. Ion-trap instruments provide an excellent alternative for profile analysis. The ability of these instruments to perform MS^n can be exploited to increase the amount of structural information, or to reduce background signal giving an improvement in detection limit.

Separation of derivatised steroids can be achieved on DB-1 cross-linked methyl silicone columns, 15 m × 0.25 (i.d.) mm, film thickness 0.25 µm (J & W Scientific), using helium carrier gas at constant pressure. A 1–2 µL aliquot of the final derivatised extract is injected onto the system in the split-less mode, and the GC temperature ramped as follows: initial 50°C, held for 3 min, increased to 230°C at 30°C/min, thereafter increased to 285°C at 2°C/min. The injector and transfer line are kept at 260°C and 280°C, respectively. The *m/z* range scanned is 90–650 or 1,000.

Mass spectra of methyloxime (MO)-trimethylsilane (TMS) ether derivatives of steroids typically show the following fragments: $[M - 31]^+$(loss of CH_3O from the methyloxime), sequential losses of 90 Da (trimethylsilanol), loss of the primary TMS group $[M - 103]^+$and combinations thereof (see Chapter 2 and Griffiths et al., 2005). Often, the choice of ion for SIM or in an extracted ion chromatogram (EIC) (also called reconstructed ion chromatogram, RIC), is one of the above fragments. It should be noted that methyloximes give *syn*- and *anti*-forms which may or may not be resolved by GC. If they are resolved, for quantitative measurements it is preferable to determine the area of both peaks.

Scanning. Ion traps are usually operated in the scanning mode and EIC drawn for the ions of interest. For true profile analysis, quadrupole instruments are also operated in this mode; however, to gain additional sensitivity SIM is performed. Shackleton (2008) has developed profile analysis based on SIM of selected analytes, trying to ensure that components of every steroid class of interest is included. Table 3.4 shows a list of steroids used for adult profiles, with ions monitored, amount of steroid in calibration mixture and excretion ranges.

3.10.3 Quantification, Internal and External Standards

Quantification of all steroids can be performed by relating the intensity of specified ions to that of the internal standard stigmasterol ($[M - 90]^+$, *m/z* 394). Instrument calibration is achieved by running an external standard daily.

Capillary GC tends to discriminate against higher mass long retention time components. The degree of discrimination of individual components throughout a run is easily determined by including internal standards which elute before and after the steroids of interest. Almost all normal urine steroids elute in a window defined by 5α-androstane-3β-ol and cholesteryl butyrate internal standards. Quantification is performed against stigmasterol, but in an acceptable run the other two standards should give almost identical quantitative values. If this is not the case, calibration using the external standard should be repeated.

Internal Standards The three principle internal standards used are 5α-androstane-3β-ol, stigmasterol and cholesteryl butyrate. Stigmasterol is used for quantification. 5α-androstane-3β-ol and cholesteryl butyrate bracket the urinary steroids of interest and are used for monitoring column performance and temperature-dependent discrimination.

External (Calibration) Standards. Table 3.4 lists steroids included in the external calibration standard used in Shackleton's laboratory (Shackleton, 2008). The table also gives information on the steroids routinely measured, the ions chosen, and the expected adult ranges, and typical retention times.

Quality Assurance (QA). In Shackleton's laboratory, a QA sample is run with every batch of samples and the results should not differ daily by more than 15%. For profile analysis, quantification for each analyte may be required against a five point standard curve. In practice, a series of calibrants with identical amounts of internal standard, but increasing amounts of reference analyte, typically covering a 100-fold dynamic range are prepared. The QA sample is a urine pool prepared by mixing equal proportions of 24 h samples from men and women.

3.11 Summary

Prior to 1980, GC-MS was the only mass spectrometric technique for steroid analysis, and profile analysis was important but seldom practiced. Almost all assays were carried out by RIA and related techniques. Now, mass spectrometric steroid analysis is common but has divided into two categories; steroid profile analysis which is still preferentially carried out by GC-MS, and analysis of discrete analytes, or small panels of analytes, for which LC-MS/MS is becoming the only viable technique. These techniques should be viewed as complementary, rather than competing. Clearly, what is not viable economically is the use of GC-MS for the analysis of discrete analytes; protracted work-up procedures and long GC-MS runs make the technique overly expensive. The great advantage GC-MS has is in chromatographic

resolution, and unless a multitude of analytes are determined there is little point in using this approach. For an unknown disorder or steroid mixture, there is no technique more superior. Characterization of a steroid by GC-MS fragmentation is much more readily achieved because of the large data base of documented fragmentations.

The present and future of routine clinical steroid analysis is LC-MS/MS. This is a "new technology" and the methods used still lack consistency. Some groups favour derivatisation to improve sensitivity, and others find it is not required. This may be largely a factor of the age of the instrument and form of ionisation. Several different ionisation sources are in use; ES, APCI, APPI, to name a few. Investigators in the field have differing opinions on their relative sensitivity for steroid analysis. The biggest need, however, is for reliable extraction and work-up methodologies. The HTLC system, as used in analysis mentioned above (Section 3.9.3), appears good for on-line operation, but other systems no doubt will evolve. Another concern is the continuing use of non-labelled internal standards in analyses of some compounds. This is inherently a problem as ion-suppression effects, with any source type are less easy to control when an internal standard of different structure is used. This is in addition to having less control over steroid recovery. The use of labelled steroids has its own possible pitfalls. Some of the labelled steroids currently used have deuteriums in less-stable positions. Analysts must be rigorous in establishing that their methodologies do not result in proton–deuterium exchange. Tai and Welch and their colleagues have published a series of papers in the period 2004–2007, describing candidate reference methods using isotope dilution LC-MS/MS – in the main, validating their methodology by comparison with certified reference material from EC Institute of Reference Material and Measurement (formerly BCR, Brussels) or the Australian National Measurement Institute (Pymble, Australia) All the methods used ESI(+) and the steroids studied were not derivitised, except for oestradiol where dansyl derivatives were formed prior to LC-MS/MS (Tai and Welch, 2005). Tri-deuteriated internal standards were used except for a tetra-deuteriated standard for urine 19-nor-androsterone (Tai et al., 2006a) and a di-^{13}C label for serum progesterone (Tai et al., 2006b). Simple solvent extraction with ethyl acetate after Sep-Pak C18 extraction was used for serum cortisol extraction (Tai and Welch, 2004) and hexane replaced ethyl acetate for serum extraction of the less-polar testosterone (Tai et al., 2007).

GC-MS may eventually be replaced by LC-MS/MS for profile analysis, but this may be still some way off; however, the advantage provided by LC-MS/MS for conjugate analysis is indisputable. Further, the development of LC-MS/MS data bases, such as provided by the LIPID MAPS consortium (http://www.lipidmaps.org/about/index.html) will allow easier interpretation of MS/MS spectra. Yet, at the time of writing, GC-MS is still the favoured technique for studying known rare disorders and describing the metabolome of new ones. GC-MS (or GC-MS-MS) is, however, widely used even today (e.g. Arroyo et al., 2007; Biddle et al., 2007; Courant et al., 2007; Gambelunghe et al., 2007; Hajkovba et al., 2007; Hill et al., 2007; Hsing et al., 2007; Knust et al., 2007; Li et al., 2007; Martello et al., 2007; Meffre et al., 2007; Noppe et al., 2007; Stopforth et al., 2007a, b; Zuo et al., 2007) and still retains a role in the investigation of structural aspects of steroids under investigation (see Chapter

Table 3.4 The comprehensive urine steroid GC/MS profile. From Shackleton, 2008, with permission [*Copyright 2008 Springer*]

Component	Retention time	Specific ion Monitored	Calibration mixture amount (μg)	Excretion Males μg/24 h(n = 17)	Excretion Females μg/24 h(n = 17)
5α-Androstan-3β-ol (IS)	11.28	333	2.5	–	–
3β5βTHAldo (IS)	21.34	506	0	–	–
5P-3β,17,21-ol-20-one (IS)	21.03	594	0.5	–	–
Stigmasterol (IS)	25.27	394	2.5	–	–
Underiv.	24.50	412	0		
Cholesteryl butyrate (IS)	28.77	368	2.5	–	–
Androsterone (An)	13.09	270	2.5	798–4,705	373–3,414
Etiocholanolone (Et)	13.23	270	2.5	689–3,252	450–2,900
11–Oxo-Et	14.28	269	2.5	79–1,026	57–916
11β-OH-An	15.21	268	2.5	500–1,733	191–854
11β-OH-Et	15.38	268	2.5	18–1,034	14–687
5α-Androstan-3α,17β-diol	13.20	331	0.5	48–578	15–147
DHEA	13.83	268	2.5	5–1,476	20–1,139
5-Androstene-3β,17β-diol (5AD)	14.03	239	2.5	45–954	28–201
16α-OH-DHEA	15.68[a]	266	2.5	40–796	35–655
5-Androstene-3β,16α,17β-triol (5AT)	17.31	432	2.5	42–710	40–540
5,17-Pregnadien-3β-ol (5PD)	12.91[b]	372	0.5	10–50	10–50
5-Pregnene-3β,20α-diol (5PD)	17.17	372	0.5	10–150	10–150
5-Pregnene-3β,17α,20α-triol (5PT)	19.49	433	0.5	28–1,062	44-342
Estriol	17.90	504	0.5	1–16	2–32
17-OH-Pregnanolone (17HP)	15.19	476	2.5	41–728	32–657
Pregnanetriol (PT)	16.63	435	2.5	186–1,505	87–1,311
Pregnanetriolone (PTONE)	18.48	449	0.5	4–37	1–77
THS[c]	17.54	564	0.5	10–109	17–117
THDOC[c]	17.19	476	0.5	2–38	1–157
THA[c]	19.70	490	2.5	104–554	76–596

5αTHA[c]	20.33	490	2.5	52–277	38–298
THB[c]	19.96	564	2.5	32–238	26–262
5αTHB[c]	20.27	564	2.5	135–588	49–447
(18-OH-THA)	21.37	457,578	0	45–184	25–207
THAldo	21.12	506	0.2	10–58	6–63
Cortisone (E)	24.39[a]	531	0.5	92–366	49–215
Cortisol (F)	25.73[a]	605	0.5	35–168	25–115
THE[c]	19.38	578	2.5	1365–5,788	727–3,815
THF[c]	20.39	562	2.5	942–2,800	458–1,907
5αTHF[c]	20.61	562	2.5	796–2,456	142–1,589
(18-Oxo-THF)	20.40	594,420	0	1–10	1–10
α-Cortolone	20.99	449	2.5	449–2,044	457–1,564
β-Cortolone	21.60	449	2.5	231–1,534	216–814
β-Cortol	21.46	343	2.5	196–880	124–690
α-Cortol	22.38	343	2.5	96–509	122–365
6β-OH-F	26.53	513	0.5	122–487	53–416

[a]These steroids give two peaks both of which are integrated.

[b]This steroid is an artifact of 5-pregnene-3β,20α-diol disulphate.

[c]Abbreviations not given in table: THS, tetrahydrosubstance S, (3α,17α,21-trihydroxy-5β-pregnane-20-one); THDOC, tetrahydrodeoxycorticosterone, (3α,21-dihydroxy-5β-pregnane-20-one); THA, tetrahydro-11-dehydrocorticosterone (3α,21-dihydroxy-5β-pregnane-11,20-dione); 5αTHA, (3α,21-dihydroxy-5α-pregnane-11,20-dione); THB, tetrahydrocorticosterone (3α,11β,21-trihydroxy-5β-pregnane-20-one); 5αTHB, 5α-tetrahydrocorticosterone (3α,11β,21-trihydroxy-5α-pregnane-20-one); THE, tetrahydrocortisone (3α,17α,21-trihydroxy-5β-pregnane-11,20-dione); THF, tetrahydrocortisol (3α,11β,17α,21-tetrahydroxy-5β-pregnane-20-one); 5αTHF (3α,11β,17α,21-tetrahydroxy-5α-pregnane-20-one).

11) as the usual method of ionisation (EI+) provides detailed structural information not always available from ionisation methods used in LC-MS. There is a recent review on steroid hormone analysis by LC-MS/MS (Soldin and Soldin 2009).

The most important feature of new MS technologies for steroid measurement is the impact this will have on patient care. More cost effective analyses will encourage more detailed evaluation of patients with, for example, endocrine disorders, and the sensitivity and accuracy of measurements will make diagnosis more definitive and monitoring of the effectiveness of therapeutic manoeuvres more reliable.

Mass spectrometry will continue to be a major method for identification and structure determination of steroids with known and unknown functions. Mass spectrometry will be very important for the analysis of chemical fractions with biological activities and for detection of ligands to orphan receptors (by classical mass spectrometry combined with detection of biological activity using cells with reporter gene constructs). There are already examples of ES mass spectrometry of receptor–ligand complexes, and the future may give us methods for structural definition of ligands bound to their receptors. Metabolic studies with multiply and specifically labelled compounds will become increasingly important. Increased sensitivity and resolution can be achieved with Fourier transform mass spectrometry. One may hope to detect new ligands by screening of biological extracts mixed with receptor populations, and elucidate complex metabolic pathways in cells by specific isotope labelling. The future for mass spectrometry in the era beyond the proteome is bright.

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