Journal of Chromatography, 379 (1986) 91—156
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2991

REVIEW

PROFILING STEROID HORMONES AND URINARY STEROIDS

C.H.L. SHACKLETON

Children's Hospital Medical Center, Oakland, CA 94609 (U.S.A.)

(Received December 9th, 1985)

CONTENTS

0378-4347/86/$03.50

1. SCOPE OF REVIEW

I do not know when the word "profile" was first used to represent multi-component chromatographic analysis. It was probably in the mid-1960s

because, in 1968, Horning [1] discussed the use of the term describing steroid analysis and we published a paper entitled "Neutral steroid profile analysis..." in the same year [2].

According to Webster's Dictionary, a profile can be "a group of data representing quantitatively the extent to which an individual exerts traits as determined by tests and presented in the form of a graph". Although the latter part of the phrase may be superfluous, I think the definition fits what we are trying to achieve by profiling. The definition implies that a profile gives information ("traits") in addition to the numerical values obtained by individual tests. I like this definition because it is unrestrictive, allowing data to be obtained by a variety of methods. Purists might consider that it is too broad and that in current usage profiling implies the collection of all relevant data in a single chromatogram. This type of profile may be satisfactory for diagnosis of the inborn errors affecting amino acid metabolism (organic acidurias), but it is not adequate for clinical steroid assay where some of the most important steroids are present in the lowest concentration in plasma and urine and are not detectable in chromatographic profiles. There are also many examples when batch analysis of selected compounds may be more time efficient than sequential analysis by chromatography.

In the opinion of this reviewer a steroid profile can be produced by a variety of techniques with the prerequisite that all facets of the steroid hormone milieu are covered. An extreme example of a profile may be one where analysis of steroids is achieved by highly specific radioimmunoassays without chromatographic separation. Intermediate examples may be the use of radioimmunoassay after chromatographic fractionation, or specific color reactions carried out on thin-layer chromatography (TLC) plates.

Although it has not been achieved, the goal (through a chromatographer's eyes) remains a single profile where all components of interest are determined in a non-discriminatory way and which, in clinical situations, will clearly delineate patients independently of their biochemical lesion. The imposition of selectivity, e.g., a specific immunoassay or a specific gas chromatographic- mass spectrometric (GC—MS) selected ion monitoring method always leaves open the possibility that the appropriate questions are not being addressed for the patient being considered. In deference to the title of this journal, this review will concentrate on profiling techniques involving a chromatographic separation. In the main, it will relate to human biochemistry; published work on other species is discussed only if the methodology used was novel and potentially useful for human studies. I have sought in this review to be reasonably comprehensive in presentations of tabulated steroid data. It was felt that it would be useful for reference purposes to list in one place steroid concentrations in a variety of biological materials. Obviously there will be serious omissions; in particular, comparative data from only two or three publications may have been tabulated even though similar profiles had been obtained by other investigators. This was done to keep the tables lucid. For further discussion of steroid profiling the reader should consult the reviews by Sjövall and Axelson [3] (preparative techniques and GC—MS), Shackleton [4] (GC), Shackleton et al. [5] (atlas of GC profiles), Gaskell [6] (mass spectrometry) and Adlercreutz et al. [7] (preparative techniques and profiling of estrogens).

2. STEROIDS OF INTEREST

Steroids of significance in human normal and pathophysiological conditions fall into several distinct groups (Table 1) (abbreviations are given in the Appendix, section 10.2) [8]. There are the primary steroid hormones which have assigned biological functions. These or their metabolites are frequently determined for evaluation of endocrine function. There is a secondary series which I term secondary hormonal steroids. These are generally proximate to the primary hormones in the biosynthetic pathways so their measurement gives important information on possible errors in biosynthesis. Examples of these are 17α-hydroxyprogesterone (17-OHP), 21-deoxycortisol (S) and 18-hydroxycorticosterone. Then there are steroid hormone precursors which are

TABLE 1

STEROIDS OF INTEREST

Primary hormonal steroids:	Typical metabolites:
Cortisol	Mainly glucuronides:
Aldosterone	Tetrahydrocortisone
Progesterone	Tetrahydrocortisol, 5α-tetrahydrocortisol
Testosterone	α- and β-cortolone, α- and β-cortol
Dihydrotestosterone	Cortoic acids
Estradiol	Tetrahydroaldosterone
	Pregnanediol
	Androsterone, etiocholanolone
	Estrone
	Estriol
	Unconjugated:
	Cortisol
	20-Dihydrocortisol
	6β-Hydroxycortisol
Secondary hormonal steroids:	Typical metabolites:
17α-Hydroxyprogesterone	Mainly glucuronides:
21-Deoxycortisol (S)	17α-Hydroxypregnanolone
Corticosterone (B)	Pregnanetriol
Deoxycorticosterone (DOC)	Pregnanetriolone
18-Hydroxy-DOC	Tetrahydro-substance S
18-Hydroxy-B	Tetrahydro-A
18-Hydroxycortisol	Tetrahydro-B
Androstenedione	5α-Tetrahydro-B
Dehydroepiandrosterone (DHA)	Tetrahydro-DOC
	18-Hydroxytetrahydro-DOC
	18-Hydroxytetrahydro-A
	Androsterone
	Etiocholanolone
	16α-Hydroxy-DHA
	5-Androstene-3β,16α,17β-triol
Hormonal precursors:	Typical metabolites:
DHA sulfate (DHAS)	16α-Hydroxy-DHAS
Pregnenolone sulfate	Androstenetriol sulfate
17α-Hydroxypregnenolone sulfate	Pregnenediol sulfate
	16α-Hydroxypregnenolone sulfate
	Pregnenetriol sulfate
	Androstenediol disulfate
	21-Hydroxypregnenolone disulfate

directly secreted into the circulation by the adrenal glands and gonads. These are primarily 3β-hydroxy-5-ene steroid sulfates. 3β-Hydroxy-5-ene steroids are particularly important during pregnancy when large amounts are secreted into the umbilical circulation by the fetus. Several of these latter compounds are also routinely measured during clincial investigation. Finally there are the terminal metabolites of all the aforementioned steroids which are excreted in urine or bile either free or as conjugates. The form of conjugate is generally associated with the structure of the aglycone. Steroids with the 3-oxo-4-ene structure are mainly excreted unconjugated or as 21-sulfates. 3α-Hydroxy-5α- and 3α-hydroxy-5β-steroids are excreted as 3-glucuronides and 3β-hydroxy-5-ene steroids are excreted as monosulfates or disulfates. Estrogens are mainly excreted as glucuronides. These divisions are not clearcut; for example, while 3α-hydroxysteroids are mainly glucuronide conjugated, significant amounts are also sulfated.

Monosulfates, monoglucuronides and disulfates are the common conjugate types and other possibilities such as mixed conjugates are extremely rare. Only estrogens have been reported excreted in this form and the best known is estriol-3-sulfate-16-glucuronide. Tikkanen [9] and Ahmed and Kellie [10] measured the excretion of this conjugate in late pregnancy urine and have found that it represents no more than 14% of total estriol excreted.

3. ISOLATION PROCEDURES

3.1. Extraction of steroids

While solvent extraction with methylene chloride or diethyl ether is still favored for the extraction of unconjugated plasma steroids, solid-phase extraction has become the method of choice for recovery of steroid conjugates and free steroids from aqueous solution. Current methods date back to the paper of Bradlow in 1968 describing the use of columns containing Amberlite XAD-2 neutral polystyrene resin [11]. This material absorbed the steroids and steroid conjugates, which could later be recovered by elution with methanol. Recoveries were generally very high although there were always some problems regarding the extraction of steroid disulfates and the properties of the material seemed to change differentially over the years, necessitating changes in the technique [12].

The advent of bonded phase silica supports for high-performance liquid chromatography (HPLC) has spurned a whole new field of solid-phase extraction techniques. When placed in small columns, C_{18}-substituted silica effectively retains steroids and steroid conjugates when water-based solutions are passed through. As with Amberlite XAD-2, the free and conjugated steroids can be recovered by elution with methanol. The first commercial disposable column cartridges were marketed by Waters Assoc. (Milford, MA, U.S.A.) under the name Sep-Pak and early showed great promise for steroid extraction [13]. Since then other companies have produced similar products and now a great variety of different bonded phase materials are available in cartridge form. Also a simple vacuum apparatus has been developed that permits multiple simultaneous sample processing. Detailed methodologies for the use of C_{18} cartridges

in the extraction of conjugated neutral steroids from plasma and other fluids were described by Axelson and Sahlberg [14] and Vanluchene et al. [15]. According to the former workers the main requirement for plasma analysis is prior incubation at 64°C to reduce protein binding.

The use of Sep-Pak cartridges for the extraction of estrogens and estrogen conjugates has been described by Heikkinen et al. [16]. Prior to extraction the urine was buffered to pH 3 to suppress the ionization of highly polar conjugates. The recovery of labelled estrone, estriol-16-glucuronide and estriol-3-sulfate-16-glucuronide added to urine was always between 95 and 99%. For plasma the extraction method was modified slightly to reduce the effects of protein binding. Plasma was incubated for 30 min with 100 nmol of norgestrel (to compete with estradiol for SHBG binding sites), diluted with 3 ml of water, 2 ml of acetate buffer (pH 5) and 10 ml of 0.5 mol/l trimethylammonium sulfate solution. This was extracted with a cartridge in the conventional fashion. Recoveries of estrone, estradiol, estrone sulfate, estriol-10-glucuronide and estriol-3-sulfate-10-glucuronide added to plasma were between 93 and 98%.

Plasma steroid conjugates are frequently recovered following precipitation of plasma proteins with about 20 volumes of acetone—ethanol (1:1) [17]. Sonication (15 min) should be employed during this process and a period at −20°C following sonication aids precipitation. After centrifugation the supernatant is decanted and dried by rotary evaporation. This type of procedure results in complete extraction of all lipids. Methanol—chloroform (1:1) and hexane—isopropanol (3:2) are also frequently employed for total extraction of lipid material from plasma and tissue [18,19]. All of these methods rely on a combination of a polar solvent and a less polar solvent to obtain optimal recoveries as polar solvents alone are often inefficient since they do not disrupt lipid membranes.

In contrast, O'Hare and Nice [20] favor homogenization in ethanol for the extraction of unconjugated steroids from adrenal tissue as it extracted all steroids with high efficiency while minimizing the extraction of some lipids. Remaining lipids could be efficiently removed on a Partisil 10 ODS HPLC minicolumn.

Ruokonen and Vihko [21] use solvent extraction for the recovery of steroids and steroid sulfates from tissue (principally testis). Pieces of tissue were dropped into liquid nitrogen in plastic bags and were then transferred into a PTFE capsule containing a tungsten carbide ball. This was vibrated for 30 min using a microdismembrator until the tissue was pulverized to a fine powder, which was placed in an extraction tube with water. Free steroids were recovered by diethyl ether—ethyl acetate extraction. The aqueous phase from the above extractions was dried under nitrogen and absolute ethanol added. Protein precipitates were spun down and the supernatant was dried and solvolysed in 3 ml of acidified ethyl acetate to recover steroid sulfates.

3.2. Group separation of steroid conjugates

One of the earliest methods for specifically isolating steroid monosulfates and disulfates from conjugate extracts was the Sephadex LH-20 technique, employing salinated methanol—chloroform (1:1) [22]. Free steroids and

steroid glucuronides are eluted together and early from these columns but monosulfates and disulfates form distinct fractions with no overlapping compounds. This method has been widely used since the late 1960s and remains today the easiest method for the group fractionation of steroid sulfates [23].

Sjövall's group were not satisfied with this initial method as it failed to resolve free steroids, steroid acids and steroid glucuronides. They subsequently developed a series of lipophilic ion-exchange Sephadex derivatives from the original LH-20, the first really successful derivative being DEAP-LH-20 (diethylaminohydroxypropyl-LH-20). With the use of this support Setchell et al. [24] were able to separate neutral (free) steroids from steroid acids, glucuronides, monosulfates and disulfates and this method has been used for analysing human adult urinary steroids. A further improvement in methodology was achieved with the introduction of a stronger anion exchanger, TEAP-LH-20 (triethylaminohydroxypropyl-LH-20) [25]. This material was found to give a purer fraction of unconjugated steroids, as organic acids were more efficiently absorbed. Further, phenolic steroids could be separately isolated. Glucuronides of neutral steroids and A-ring glucuronides of phenolic steroids can be eluted prior to estrogen glucuronides with a free phenolic hydroxy group. The disadvantage of TEAP-LH-20 rests in its commercial unavailability, in contrast to DEAP-LH-20, which is sold by Packard (Downers Grove, IL, U.S.A.) as DEAP-Lipidex.

The separation of estrogen conjugates from biological materials presents particular problems partly owing to their low levels compared with conjugates of neutral steroids. Fotsis et al. [26] have published extensively on the anion-exchange separation of estrogen conjugates, building on previous studies of Hähnel [27]. In their method the acetate form of DEAE-Sephadex A-25 gel in methanol was packed in 13 × 0.5 cm I.D. columns. Following sample application, seven groups of steroids were eluted in the following order: uncon-

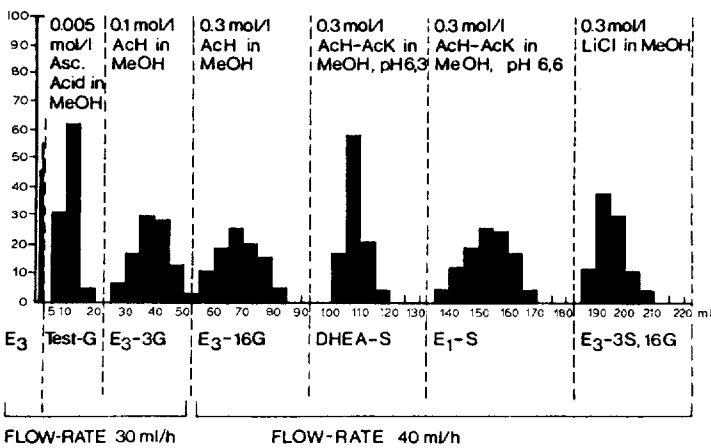

Fig. 1. Separation of phenolic and neutral steroid conjugates on DEAE-Sephadex A-25. Elution of the following radioactive standards: estriol (E_3), testosterone glucuronide (Test-G), estriol-3-glucuronide (E_3-3G), estriol-16-glucuronide (E_3-16G), dehydroepiandrostene sulfate (DHEA-S), estrone-3-sulfate (E_1-S) and estriol-3-sulfate-16-glucuronide (E_3-3S,16G). Abbreviations: Asc. Acid = ascorbic acid, MeOH = methanol, AcH = acetic acid, AcK = potassium acetate, LiCl = lithium chloride. From ref. 26, with permission.

jugated steroids, glucuronides of neutral steroids, estrogen A-ring glucuronides, estrogen D-ring glucuronides, monosulfates of neutral steroids, monosulfates of estrogens and mixed and double conjugates of neutral steroids or estrogens (Fig. 1).

3.3. Hydrolysis of conjugates

Quantitative enzyme hydrolysis of all urinary steroid glucuronides is difficult to achieve. There are four major β-glucuronidases in common use:

(1) Bacterial (*Escherichia coli*). Sigma (St. Louis, MO, U.S.A.) has several variously purified enzymes available. The optimal pH is 6.8—7.0.

(2) Digestive juice of the Roman snail (*Helix pomatia*): several solutions and powders are available from Sigma. These contain substantial sulfatase activity. The optimal pH is 4.5—5.0.

(3) Limpet (*Patella vulgata*). Sigma supplies lyophilized powder which has weak sulfatase activity in addition to β-glucuronidase.

(4) Beef liver: the most commonly used enzyme, Ketodase (Warner-Chilcott, Morris Plains, NJ, U.S.A.), is no longer available. Sigma beef liver β-glucuronidases B1 has similar properties (H.L. Bradlow, personal communication).

All the enzyme mixtures have been used over the years for hydrolysis and probably the best discussions on optimal conditions are those of Bradlow [28], Beale et al. [29] and Vestergaard [30]. The last reference is the most useful as it compares the efficiency of hydrolysis achieved with each type of preparation. In our studies we use the snail enzyme preparation for profile analysis as it has strong sulfatase activity allowing hydrolysis of the key 3β-hydroxy-5-ene steroid sulfates. Vestergaard [30] illustrated the importance of solvolysing steroid sulfates in addition to carrying out β-glucuronide hydrolysis. Confirming previous studies, he showed that an average of 17% of androsterone, 11% of etiocholanolone and 73% of dehydroepiandrosterone (DHA) is excreted sulfate conjugated. The digestive juices of *Helix pomatia* do not contain all the sulfatases necessary for hydrolysis of all steroid sulfates. Whereas the hydrolysis of 3α-hydroxy-5β- and 3β-hydroxy-5-ene steroid sulfates is efficient, 3α-hydroxy-5α-sulfates are not hydrolysed. In addition, the C_{21}-steroids sulfated at position 20 and C_{19}-steroids conjugated at position 17 are resistant to hydrolysis. In our studies, as we only use *Helix pomatia* enzymes, we are bound to underestimate, or ignore, the excretions of certain steroids. Important examples are androsterone sulfate, 3,17-disulfates and 3,20-disulfates.

One of the major concerns in urinary profile analysis in a clinical setting relates to the time required to report patient results. A sizeable amount of the total period required is taken by the enzyme hydrolysis, as periods of 24—72 h have frequently been used. Although optimal conditions are probably best achieved by prolonged hydrolysis, some compromises have to be made in order to produce timely results. Hydrolysis can be substantially speeded up by utilizing a higher temperature. Vestergaard [30] tabulated data on the effect of temperature in a 24 h hydrolysis of urinary steroids, and for the purpose of this review the results are summarized in Fig. 2. The interesting features are (1) that for most steroids there is little difference in the efficiency of hydrolysis at different temperatures (all steroids analysed are not graphically rep-

resented in Fig. 2); (2) that the hydrolysis of DHA sulfate is improved at higher temperatures; and (3) that the recovery of androsterone and etiocholanolone falls at higher temperatures. From the data presented by Vestergaard, the optimal temperature seemed to be between 52 and 60°C. Axelson et al. [25] suggested using *Helix pomatia* material with an incubation temperature of 62°C and a 1 h hydrolysis period; we tried this procedure but found that the recoveries of androsterone and 5α-THF glucuronide were poor compared with a 48 h hydrolysis at 40°C. We therefore opted for a 3 h hydrolysis at 55°C and found this to be a suitable compromise. In our investigations we found that the recoveries of androsterone and etiocholanolone glucuronides were about 10% lower than those achieved by a hydrolysis at 40°C and the 5α-THF recovery was about 5% lower. All other measured steroids gave identical results.

These findings illustrate the major problem with glucuronidase hydrolysis, viz., the quantitative recovery of some 3α-hydroxy-5α-steroids. The glucuronides of 11β-hydroxyandrosterone and 5α-THF are poorly hydrolysed compared with other conjugates, and more enzyme than usual, or more time, is required to achieve optimal recoveries. This is well illustrated by comparing data produced from the hydrolysis of several steroid glucuronides with increasing concentrations of β-glucuronidase (Fig. 3). This figure was drawn for this review from the tabulated data of Vestergaard [30]. Although maximum recoveries of most steroids were obtained with the lowest enzyme concentrations, 5α-THF and 11β-hydroxyandrosterone may not have even been completely hydrolysed with the maximum enzyme concentrations utilized.

Most of Vestergaard's studies were obtained by direct urine hydrolysis but he did show that solid-phase (XAD-2) extraction of steroid conjugates did improve

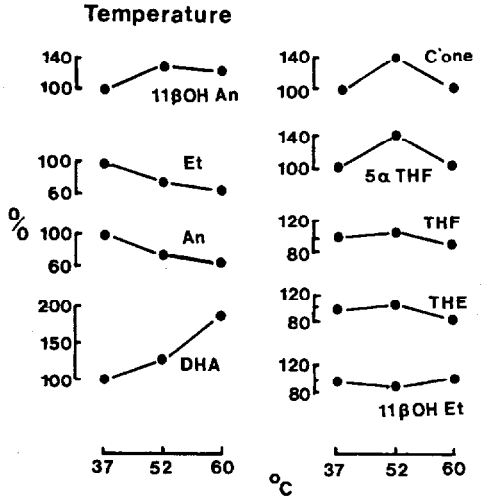

Fig. 2. Effect of increasing temperature on 24 h hydrolysis of urinary steroids with *Helix pomatia*. For the purpose of the illustration it was assumed that recovery at 37°C was 100%. The recovery of androsterone and etiocholanolone shows a significant decrease following an increase in the hydrolysis temperature to 60°C. In contrast, DHA hydrolysis is improved dramatically and most other steroids show a slight improvement at 52°C. When the temperature was increased to 70°C almost all enzyme activity was lost (not illustrated). Graph prepared from data of Vestergaard [30].

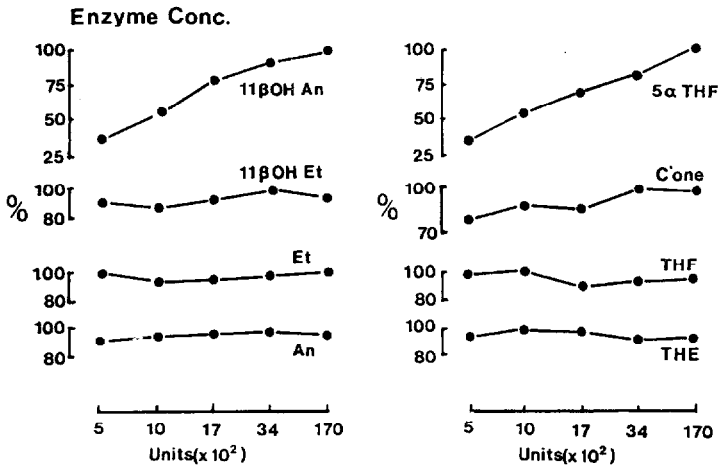

Fig. 3. Effect of enzyme concentration on hydrolysis of urinary steroid glucuronides. Most steroids are completely hydrolysed with a low β-glucuronidase (*Helix pomatia*) concentration (500 Fishman units/ml). Certain 3α-hydroxy-5α-steroid glucuronides are resistant to hydrolysis, so quantitative recovery is difficult. This is particularly true for 5α-THF and 11β-hydroxyandrosterone glucuronides. This figure was drawn from the tabulated data of Vestergaard [30]. It was assumed that the highest recovery achieved during the experiment was 100%.

the recovery of steroid glucuronides and this is also likely to be the case for C_{18} cartridge extraction. One may assume that there is some removal of β-glucuronidase inhibitors taking place. Phosphate and sulfate ions are known inhibitors of steroid sulfatase [31] and these would be effectively removed by Sep-Pak C_{18} extraction.

In our methodology the enzyme hydrolysis is conducted in 3 ml acetate buffer, pH 4.6 at 55°C for 3 h; 25 mg of Sigma type H1 sulfatase—β-glucuronidase is used. This amounts to 7500–10 000 units β-glucuronidase (2500—3300 units/ml buffer) and 625—1000 units sulfatase (108—330 units/ml buffer). While the hydrolysis of each urine sample should be considered individually we have not found serious losses of steroids when these conditions are used. When a secondary hydrolysis of a Sep-Pak extract of hydrolyzed conjugates has been carried out, little further steroid has been recovered.

The hydrolysis of estrogen conjugates enzymatically has been well reviewed by Adlercreutz et al. [7] and will not be discussed here at length. According to their experience complete hydrolysis (98—99%) of estrogen sulfates and glucuronides (including double conjugates) with no measureable destruction or conversion of estrogens can be obtained using the following conditions. Purified samples (after Sep-Pak C_{18} extraction): 0.15 *M* acetate buffer pH 4.1 with 0.1% ascorbic acid and 1000 Fishman units of β-glucuronidase (*Helix pomatia*) per ml and incubation for 16 h at 40°C; non-purified urine (diluted 1:4): 0.15 *M* acetate buffer pH 4.5 with 0.1% ascorbic acid and 2000—2300 Fishman units enzyme per ml. Hydrolysis 16 h at 40°C.

Although beef liver β-glucuronidase has the capacity to hydrolyse all estrogen glucuronides, the activity against certain substrates may be low. Bradlow (personal communication) reported that less than 40% of estriol-16-glucuronide is measured following overnight hydrolysis using the Sigma B1 preparation.

Vanluchene et al. [32] have shown that *Helix pomatia* enzymes can convert 3β-hydroxy-5-ene steroids into 3β-hydroxy-5α-steroids and 3-oxo-4-ene steroids during hydrolysis of steroid sulfates. This is most likely to occur during extended hydrolysis periods and is a cause for concern.

3.3.1. Steroid sulfates

Most steroid sulfates can be hydrolysed enzymatically, although this is probably not the method of choice if only these compounds are of interest in a particular situation. Solvolysis is preferable as it only hydrolyses sulfates and does not discriminate against steroid disulfates. Current solvolysis methods are adaptations of the method described by Burstein and Lieberman [33]. Solvolysis on a dried sample can be achieved by addition of ethyl acetate saturated with 4 *M* sulfuric acid. We use 3 ml of ethyl acetate and add 10 μl of 4 *M* sulfuric acid to it. Alternatively, if the steroid sulfates are in water-based solution, they can be extracted into ethyl acetate following adjustment to pH 1 and saturation with ammonium sulfate. Solvolysis itself is most readily achieved by incubation at 40°C for 1 h or more. After completion, traces of acid from the ethyl acetate should be removed by washing with sodium hydroxide (1 *M*) or sodium hydrogen carbonate (8%) and water.

4. PROFILING PLASMA STEROIDS

4.1. Column liquid chromatography

Column liquid chromatography (LC) has long been used to remove non-steroidal impurities and separate steroids according to polarity. Silicic acid is one material that has been used for many years [34,35], but it is relatively active and its use can result in chemical modifications of the compounds of interest. Alumina also has been frequently used [36] but is subject to the same drawbacks. In the recent literature on clinical steroid analysis, Celite, Sephadex LH-20 or Sephadex LH-20 derivatives seem to have been used most frequently.

4.1.1. Sephadex LH-20

Sephadex LH-20 (hydroxypropyl-Sephadex) was the first lipophilic Sephadex to become commercially available and initially it was used principally in the separation of free steroids from steroid sulfates [22,23]. Many groups have since used Sephadex LH-20 columns for fractionating unconjugated steroids and solvent systems have usually been composed of chlorinated hydrocarbons with an alcohol {e.g., methylene chloride—methanol (98:2) [37] } or a hydrocarbon with an alcohol {e.g., cyclohexane—ethanol (90:20) [38] }.

When it was introduced, Sephadex LH-20 had several advantages over other media used for LC. For example, it was completely inert compared with the more "active" materials such as silicic acid and alumina then in common use. Hence there was virtually no danger of labile compounds undergoing chemical modifications during separation. The inertness also gave rise to extremely good recoveries, as no strong absorption of steroid to support took place. LH-20 gave completely reproducible separations which were not subject to the "between-column" retention volume fluctuations often obtained with other

TABLE 2

UNCONJUGATED PLASMA STEROID PROFILES PRODUCED BY RIA FOLLOWING LIQUID CHROMATOGRAPHY (ng/ml)

Steroid	HPLC*	LH-20**			Lipidex 5000			Celite			
	M	M	F	Child	M***	F***	M§	M§§	F§§	MN§§§	FN§§§
Testosterone					4.62	0.26	3.70	5.22	0.31	0.12—1.04	0.05—0.25
Androstenedione							0.91			0.17—1.42	0.11—0.09
DHT					0.65	0.19	0.41	1.30	1.11		
DHA								6.42	5.15	0.45—7.0	0.96—4.1
Progesterone	0.24	0.23	0.81	0.31	0.24	0.20	0.33			0.06—3.2	0.27—10.5
Pregnenolone	0.67				1.56	1.18	0.78			0.46—2.0	0.46—1.88
17-Hydroxyprogesterone	1.01	1.48	0.57	0.32	0.97	0.43	0.76			0.40—2.4	0.62—30.5
17-Hydroxypregnenolone	1.72									0.70—17.90	0.86—21.4
DOC	0.07	0.08	0.07	0.10							
Corticosterone (B)	3.68	4.10	8.64	1.67							
Substance S	0.38			0.57							
Cortisol	118	101.8	107.2	89.0				16.1	18.0 μg/dl		
Cortisone		17.3	13.5	13.5							
18-Hydroxy-DOC	0.10										
18-Hydroxy-B	0.35										
Aldosterone	0.14	0.14	0.24	0.31							

*Data from Schöneshöfer et al. [59]; median values.
**Data from Sippell et al. [40].
***Data from Apter et al. [45]; 22 males (M) and 11 females (F) (follicular phase).
§Data from Leinonen et al. [214]; 7 elderly prostate cancer patients.
§§Data from Forest [235]. Adults (6 males, 9 females).
§§§Data from DePeretti and Forest [236]. Male and female newborns (range).

column packings caused by a variable water content. With other absorbents, consistency of the conditioning method and storage was essential if reproducible results were to be obtained. Sephadex LH-20 also had the advantage that it could be used repeatedly with or without removal from the column. By the early 1970s Sephadex LH-20 had become widely used for the separation of steroids for clinical steroid analysis either by saturation analysis or gas chromatography [3,23,37—39].

Sephadex LH-20 columns could not be operated with a high head pressure because under these conditions the bed contracts substantially. Hence LH-20 columns were never fast to run and the separation of steroids on multiple columns was very time consuming. It was evident that some of the process would have to be automated to make the technique practical for clinical use. Sippell and co-workers [39,40] developed a technique in which six columns were eluted simultaneously. The solvent was delivered from a single reservoir by peristaltic pump to the top of each column (Fig. 4). The exit of each column was attached to a microflow meter so that the flow could be accurately adjusted. From the top of the flow meter the eluate went to a fraction collector, the multiple dispensing mouthpiece of which allowed parallel collection of eluates from six columns running simultaneously. The fraction collector was activated by a programmer unit controlled by punched tape. On this tape was encoded the message to change the tubes after the required volume of solvent for each steroid had been collected.

Using this device Sippell et al. described a method for the simultaneous radioimmunoassay (RIA) determination of eight major corticosteroid hormones and precursors in 0.8—2 ml of plasma [41]. After extraction of the unconjugated steroids from plasma to which tritiated steroids had been added, progesterone, deoxycorticosterone (DOC), 17α-hydroxyprogesterone, corticosterone, substance S, aldosterone, cortisone and cortisol were simultaneously separated using

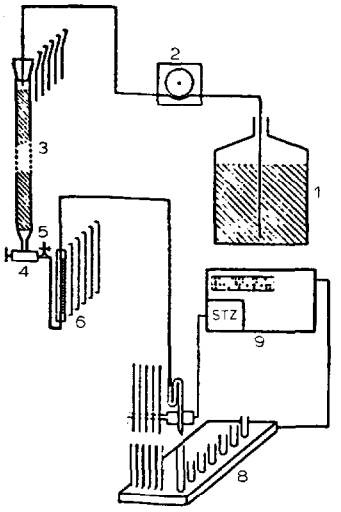

Fig. 4. The automated multiple column LH-20 chromatography system. 1, Solvent reservoir; 2, peristaltic pump; 3, columns; 4, valve; 5, bubble trap; 6, micro-flow meter; 7, siphon with photocell; 8, linear fraction collector; 9, STZ control unit and punched tape controlled programmer unit. From ref. 40, with permission.

eight mechanical Sephadex LH-20 columns in parallel. Each of the isolated steroids was quantified by radioimmunoassay after taking an aliquot for recovery evaluation. In the primary solvent system used, methylene chloride—methanol (98:2), progesterone, DOC and 17α-hydroxyprogesterone were not adequately separated and were collected as a single fraction. These were then resolved using a second system consisting of heptane—chloroform (1:1) plus 0.25% of ethanol and water to saturation. The recovery of all steroids through the extraction process and one or two Sephadex LH-20 columns was 52—68%. Although several steroids overlapped in the chromatography, the low cross-reactivity of the appropriate antisera resulted in little reduction in specificity. The lower limits of sensitivity ranged from 4.7 pg (aldosterone) to 11.0 pg (cortisone) per assay tube. The coefficient of variation within and between complete assays were as follows: progesterone, 10.8 and 14.3%; DOC, 11.2 and 14.4%, 17-hydroxyprogesterone, 6.9 and 12.3%; corticosterone, 7.7 and 16.3%; substance S, 10.2 and 13.3%; aldosterone, 10.7 and 13.3%; and cortisone, 14.5 and 16.3%, respectively. As many steps in the multi-steroid assay procedure were simplified and mechanised, during five working days technicians can measure eight different steroids in 48 samples, which yields a total of 384 individual steroid values per week. Results obtained by this procedure for men, women, prepubertal children, umbilical cord blood and amniotic fluid are given in Tables 2—4 and also later in Table 14.

TABLE 3

UNCONJUGATED STEROID CONCENTRATIONS IN PLASMA OR SERUM OBTAINED AT THE END OF PREGNANCY (ng/ml)

Steroid	40 weeks*	40 weeks**	37—41 weeks***	35—40 weeks§
Testosterone	0.8			
Androstenedione	2.7			
DHT	0.1			
DHA	4.2		4.5	30.8
Δ^5AD	0.05			
Progesterone	150	120	110	127
Pregnenolone	7.5		5.0	
16α-Hydroxyprogesterone	24			
17α-Hydroxyprogesterone	9	11.0		16.0
20α-Dihydroprogesterone	25			53.7
17-Hydroxypregnenolone	2			
DOC		2.93		
Corticosterone		36.3		
Cortisone		60.5		40.3
Cortisol		548	257	258.8

*Data from Buster et al. [52,53]. Celite chromatography and RIA.

**Data from Sippell et al. [228]. Samples collected during delivery, $n=12$. Sephadex LH-20 chromatography and RIA.

***Data from Laatikainen et al. [74]. Samples (19) collected prior to labor. Sephadex LH-20 chromatography and RIA.

§Data from Axelson and Sjövall [71]. Pooled plasma analysed by TEAP-LH-20 chromatography and GC—MS. In addition to the steroids listed, many saturated steroids were quantified.

TABLE 4

UNCONJUGATED STEROIDS PRESENT IN UMBILICAL CORD BLOOD (ng/ml)

Steroid	Umbilical vein*	Umbilical vein**	Mixed cord blood***	
			M	F
Testosterone			39.4	29.2
Androstenedione			86.7	92.6
DHA		5.0		
Pregnenolone		9.3		
Progesterone	271	201		
17-Hydroxyprogesterone	32.9		20.4	25.8
DOC	6.25			
Corticosterone	10.5			
11-Dehydrocorticosterone	2.11			
Cortisol	69.5	78.7		
Cortisone	138			

*Umbilical vein: spontaneous delivery (n=12). Sippell et al. [228]. Sephadex LH-20 chromatography and RIA.

**Umbilical vein: Caesarean section after spontaneous labor (n=14). Laatikainen et al. [74]. Sephadex LH-20 chromatography and RIA.

***Mixed cord blood (mainly venous) (n=25). Males and females. Forest and Cathiard [229].

4.1.2. Lipophilic Sephadex (Lipidex)

Sephadex derivatives with long alkyl residues were prepared in the late 1960s by Sjövall's group at the Karolinska Institute [42,43] and one material, hydroxyalkyl-Sephadex LH-20 is marketed by Packard as Lipidex 5000. Lipophilic Sephadex derivatives are stable hydrophobic materials that can be used in both normal- and reversed-phase systems. Normal-phase solvent systems usually consist of a hydrocarbon and a low percentage of a chlorinated hydrocarbon. Purification of non-polar steroids is often carried out by reversed-phase chromatography. A mixture of methanol—water—chloroform (9:1:2) or 72% aqueous methanol gives rapid elution of steroid hormones whereas cholesterol and other neutral lipids are retained on the column [44]. Sjövall and Axelson published useful tabulated data on the relative elution volumes of many C_{19} and C_{21} steroids in various solvent systems [3].

The major disadvantage with liquid-gel chromatographic systems is that the flow-rates must be low because of slow diffusion in the gel phase. In addition, high pressures cannot be applied because the bed volumes contract. The swelling and contraction of the gel beds according to the solvent system used tends to preclude the use of solvent polarity gradients and constant phases are commonly used. This frequently means that more than one column needs to be run to separate the full complement of plasma steroids. Particular advantages of lipophilic gels are inertness and low bleed, giving high yields and low contamination. They have high sample capacities and can often be used repeatedly without repacking.

Apter et al. [45] described a method for the profiling of the gonadal steroids pregnenolone, progesterone, 17α-hydroxyprogesterone, testosterone and 5α-dihydrotestosterone (DHT) in 1—2 ml of serum from men or women. Using

microcolumns of Lipidex 5000 and light petroleum–chloroform (97:3) as the solvent they resolved these five steroids into four fractions, with pregnenolone and 5α-dihydrotestosterone eluting together (Fig. 5). By use of appropriate antibodies these two steroids were also determined separately. The chromatography system (Fig. 6) consisted of a 5-l flask, fifteen 50-ml pipettes and a corresponding number of chromatography columns. Pipettes (50 ml) were used as solvent reservoirs because by use of their graduations it was possible to follow the volume of the solvent eluted through the columns. Consequently, collection tubes without a volume scaling could be used for fraction collection.

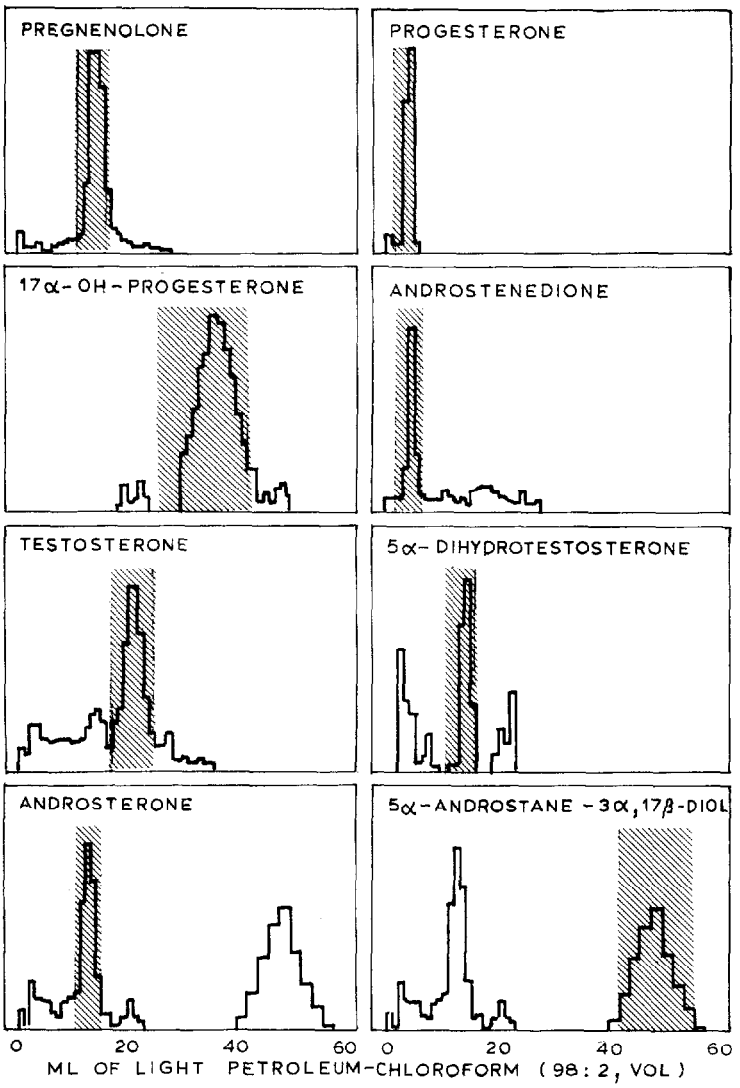

Fig. 5. Lipidex 5000 chromatographic profiles of unconjugated steroids measured by their respective antisera. Between 0 and 40 ml 1-ml fractions were collected and between 40 and 60 ml 2-ml fractions were collected. The shaded areas indicate the fraction volumes collected for the measurement of individual steroids. The same antiserum was used for the two compounds at the bottom of the figure. From ref. 45, with permission.

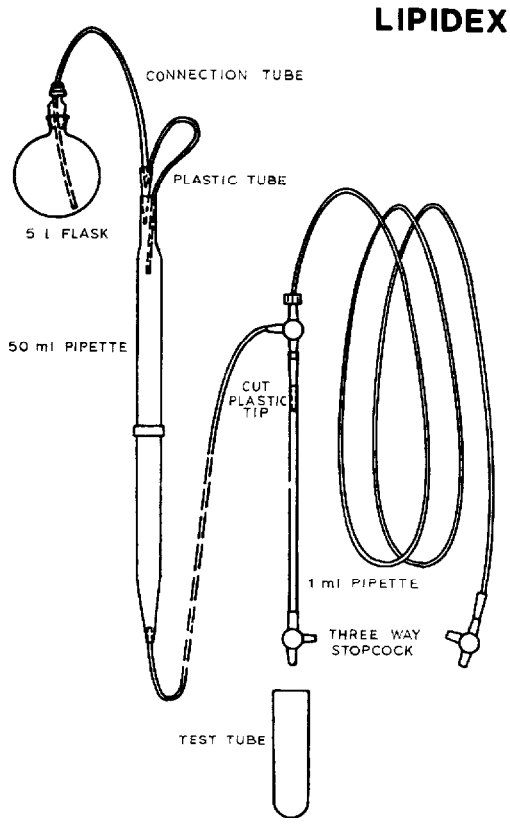

Fig. 6. Chromatography of plasma unconjugated steroids on Lipidex 5000. The chromatography system consists of a 5-l solvent container, up to fifteen 50-ml pipettes (individual column reservoirs) and chromatography columns (1-ml pipettes). As each column reservoir is a graduated pipette it is possible to monitor the volume of solvent eluted, so non-graduated disposable test-tubes could be used for fraction collection. The generation of air bubbles in this type of chromatography system is unavoidable but they are removed into a blind-end loop. If the air was not removed, air-locking occurred. From ref. 45, with permission.

Initially, problems were caused by air bubbles forming in the tubes and air-locking the columns. This problem was solved by connecting the top of the column to a blind end cap (100 cm) of plastic tubing. The columns were 1-ml pipettes which, when filled with 0.5 g of Lipidex 5000, gave a bed height of 32 cm.

The precision of the method was investigated by repeated analyses of the five steroids from 1 ml of human serum. The intra-assay coefficient of variation was 5—9% and the inter-assay coefficient of variation, calculated from results accumulated over 4 months, was 10—15%. Concentrations of the five steroids in serum from young women and men are given in Table 2 and were similar to those found by other workers using different methodologies. In terms of practicality one technician can analyse all the five steroids from two series of fifteen unknown samples together with appropriate controls in a five day working period. Once prepared the columns can be used without repacking for several months provided they are washed for 8 h between subsequent runs.

A later publication from the group described the adaptation of the method to quantify two further steroids (androsterone and androstenedione) [46]. To achieve this the solvent system was made slightly less polar [light petroleum—chloroform (98:2)]. In addition, recovery monitoring was included by adding radiolabelled testosterone to the serum sample prior to extraction. Results were presented for the concentration of steroids in peripheral and spermatic vein plasma. The methodology has also been used for profiling steroids in human testis [47] and epididymis [48]. Through a further change in the solvent system to light petroleum—chloroform (65:35) it was possible to use the method for measuring estrone and estradiol in the testis and spermatic and peripheral venous blood of elderly men [49].

4.1.3. Celite chromatography

Chromatography on Celite columns has been used for many years for profiling unconjugated steroids and a universal chromatographic system for separation of steroid hormones and their metabolites has been published by Siiteri [50]. The stationary phase for this partition system was ethylene glycol and steroids were recovered according to increasing polarity by elution with isooctane to which was added increasing percentages of ethyl acetate.

Microcolumns of Celite (about 800 mg) have been used to fractionate plasma and amniotic fluid steroids prior to radioimmunoassay [51—54]. Buster et al. [52,53] have used the technique to determine the following steroids in maternal blood collected during the third trimester: estradiol, estriol, DHA, DHA-sulfate, androstenedione, Δ^5-AD, testosterone, DHT, progesterone, 16α-hydroxyprogesterone, 17α-hydroxyprogesterone, 20α-dihydroprogesterone, pregnenolone, pregnenolone sulfate and 17-hydroxypregnenolone (Table 3).

4.2. High-performance liquid chromatography

4.2.1. Unconjugated steroids

The literature on the HPLC of steroids up to 1981 has been well reviewed by O'Hare and Nice [20]. In particular they have carried out extensive studies on the reversed-phase separation of corticosteroids using C_{18} columns. In their review they tabulated the properties of many commercial columns, which include the order of elution of many steroids using three solvent systems, methanol—water, acetonitrile—water and dioxane—water. These authors required a system for studying steroid synthesis in adrenal and testicular cells and tissue and one of the requirements was the efficient recovery of the 18-hydroxylated steroids (18-hydroxy-DOC and 18-hydroxycorticosterone). It was found that these could only be successfully chromatographed on fully end-capped C_{18} columns.

A recent paper by Capp and Simonian [55] details the separation of 25 steroids using a propanol—water solvent system and a fully end-capped C_{18} column. They compared the retention times of steroids using this system with the more common methanol—water system. They found that propanol—water was superior as it allowed resolution of almost all Δ^4- and Δ^5-steroids likely to be encountered in tissue extracts. They used the technique to separate the products of [^{3}H]pregnenolone metabolites in cultured fetal adrenal cells [56].

Monitoring of the pregnenolone conversion had to be carried out by radioactive monitoring as 3β-hydroxy-5-ene steroids are poorly detected by UV absorption. Previous studies by Simonian and co-workers have addressed the steroidogenic function of cultured bovine adrenocortical cells through HPLC profile analysis of steroid intermediates [57,58].

The use of HPLC for the systematic analysis of a multitude of serum and urinary corticosteroids has been primarily the result of methodological development by Schöneshöfer and co-workers [59–64]. They believed that the customary chromatographic methods used for steroid profiling based on semi-automated multi-column separations were time consuming and tedious. Examples of such methods were those using the Sephadex derivatives previously described (see above). It was evident that even if the separation step could be replaced by HPLC, the final assays would have to be carried out by radioimmunoassay for sensitivity reasons (with the possible exception of cortisol). The HPLC method developed by Schöneshöfer employed a normal-phase separation using a silica column bonded with a polar stationary phase (DIOL) and *n*-hexane–isopropanol as the solvent system. A stepwise linear gradient elution was used. Fig. 7 illustrates the separation achieved for thirteen reference steroids and the gradient profile applied. The Δ^5-steroids illustrated were radiolabelled to allow for radioactivity detection as they are poorly detected by UV monitors. The lower chromatogram represents the UV trace of a diethyl ether extract of serum, but with the exception of cortisol the peaks

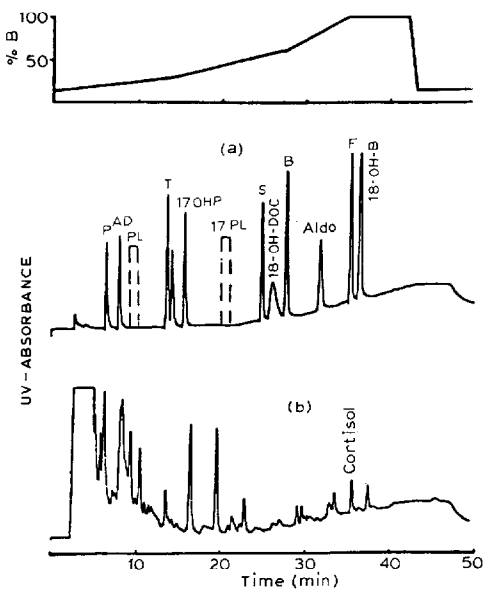

Fig. 7. HPLC of unconjugated plasma steroids. Chromatogram of (a) a mixture of steroid standards and (b) an ether extract of a normal serum sample. The amount of the individual standards was 500 ng. Steroids not detectable by UV absorption were located by ^{3}H radioactivity measurements on the eluted fractions (dotted lines). The upper part represents the gradient profile applied. Steroids: P, progesterone; AD, androstenedione; PL, pregnenolone; T, testosterone; 17PL, 17-hydroxypregnenolone; 17OHP, 17-hydroxyprogesterone; 18-OH-DOC, 18-hydroxydeoxycorticosterone; 18-OH-B, 18-hydroxycorticosterone. From ref. 59, with permission.

seen do not represent steroidal constituents. The recovery of steroids in the HPLC fractions varied from 43% (18-hydroxy-DOC) to 69% (substance S). The inter-assay precision (coefficient of variation) varied between 4.3% (cortisol) and 9.7% (18-hydroxy-DOC). The inter-assay variation was between 5.3% (cortisol and 20.0% (DOC). The serum concentrations of each steroid were estimated in a series of eighteen normal males and the results are given in Table 2. The practicality of the method is dependent on the automatic injection, programming and fraction collection, as HPLC would not be an efficient method if it depended on manual operation. By running the automatic equipment overnight, processing 36 samples to purified steroid fractions takes two working days including half a day for extraction. The authors consider that in comparison with purification techniques applied hitherto, automatic HPLC provides the following advantages: (1) the resolving power of HPLC approaches the quality of gas chromatography so the volumes of steroid fractions are much lower than those obtained by the multi-column Sephadex methods; and (2) the gradient technique provides for the separation of compounds that cover a wide range of polarity within a single chromatographic run. Sephadex LH-20 derived systems only work successfully when the solvent system remains constant during each analysis.

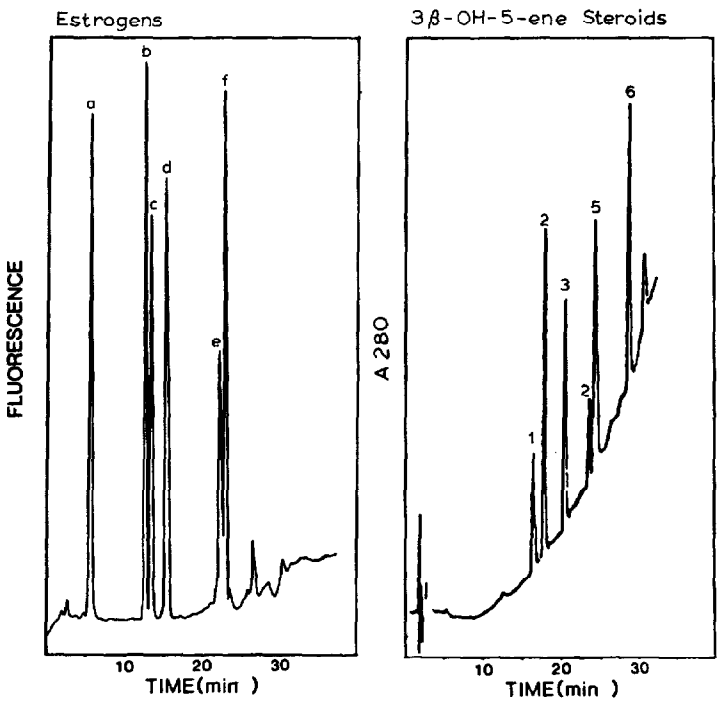

Fig. 8. HPLC separation of unconjugated and sulfated steroids. The separations were performed on a Spherisorb ODS column with a flow-rate of 1 ml/min with a linear gradient of methanol (2%/min) from 30% to 100% in 20 m*M* ammonium sulfate. Estrogens: (a) estriol-3-sulfate; (b) estrone-3-sulfate; (c) estradiol-3-sulfate; (d) estriol; (e) estrone; (f) estradiol. Detection by fluorescence with 214 nm excitation and 340 nm emission cut-off. 3β-Hydroxy-5-ene steroids: (1) DHA-sulfate; (2) 17-hydroxypregnenolone sulfate; (3) pregnenolone sulfate; (4) 17-hydroxypregnenolone; (5) DHA; (6) pregnenolone. Detection by UV absorbance at 280 nm. From ref. 65, with permission.

4.2.2. Steroid sulfates

Simonian and Capp [65] described the separation of steroid-3-sulfates and unconjugated steroids by reversed-phase HPLC. The 3β-hydroxy-5-ene steroids DHA, 17α-hydroxypregnenolone and pregnenolone and their corresponding sulfates were resolved using a linear gradient of methanol in 20 m*M* ammonium sulfate. Good separations were also achieved for estrogens and estrogen conjugates (Fig. 8). Ammonium sulfate was essential for good separations of the steroid sulfates as without its presence in the mobile phase the sulfates had decreased retention times and poor peak shapes. The increase in retention time of the steroid sulfate esters with ammonium sulfate is presumably due to ion pairing of the ionized steroid sulfate and the cation of the mobile phase. These investigators used the technique for profiling the steroid sulfates and unconjugated steroids in cell cultures of human fetal adrenal cortex [56].

4.3. Gas chromatography and gas chromatography—mass spectrometry

4.3.1. Plasma unconjugated steroids

The principal disadvantage of gas chromatography over liquid chromatographic techniques for steroid profiling relates to the necessity to protect thermally labile compounds from the high temperatures required for volatilization and separation. Even the thermally stable compounds (C_{18}, C_{19} and simple C_{21} steroids) cannot be successfully profiled, as their adsorption to the multitude of active sites on capillary columns causes peak widening, with a consequent loss of column resolution.

Innumerable methods have been published on steroid derivatization in the last 25 years and they need not be reviewed here. However, there was a rapid rationalization in the late mid-1960s and one derivative, methyloxime-trimethylsilyl (MO-TMS) ether, became the choice for steroid profiling [66,67]. The success of this derivative was the ease by which both carbonyl groups and hydroxyl groups could be protected.

Most other derivatives in common use are also "oxime-silyl" ether, differing from methyloxime-trimethylsilyl ethers by altering the oxime (e.g., benzyloxime) or silyl ether (e.g., *tert.*-butyl dimethylsilyl ether, TBDMS) [3, 6, 68—70].

In addition to the inconvenience of preparation, the necessity for derivative formation can result in a decrease in sensitivity and chromatographic complexity. Two forms of methyloxime derivatives exist for each carbonyl function, so eight different epimers could be formed from a compound with three such functions. If each of these was separable by gas chromatography, and each was formed in equal amounts, it will be appreciated that a particular steroid will give eight peaks of one eighth the intensity of that had only one derivative been formed. Fortunately, such a serious situation never exists and the maximum number of peaks usually seen for a compound is three or four. Many steroids produce only one peak. Other epimers are either not formed or not separated. However, even two or three gas chromatographic peaks formed from a particular steroid reduce the sensitivity and increase the complexity of the chromatogram.

Even though there are the disadvantages described, unconjugated steroids in plasma have been studied by GC—MS techniques. Axelson and Sjövall [71, 72] developed the following method, which was subsequently used for profiling

steroids in pregnancy plasma. After extraction by Amberlite XAD-2 (or latterly by C_{18} cartridges), the steroids were purified by filtration through a 0.5-g column of sulfoethyl-Sephadex LH-20 in methanol, and by chromatography on a 0.5-g column of TEAP-LH-20. The latter column was used in a reversed-phase system that separated neutral steroids as a group from less polar lipids, and yielded phenolic steroids in a separate fraction. The neutral steroids were converted into MO-TMS ethers, which were analysed by GC—MS on capillary columns. The identification of a steroid was based on the retention time, the complete mass spectrum and partial mass spectra obtained from fragment ion current (FIC) chromatograms for characteristic ions given by the steroid derivatives. Quantitative analyses were based on the determination of peak areas in specific FIC chromatograms. An internal standard was added to the sample and to a mixture of reference steroids. Following GC—MS analysis, peak areas were calculated in the FIC chromatograms using the two to ten most intense or diagnostically significant *m/z* values for each compound. The area of a peak given by the internal standard served to normalize the results so that areas given by known amounts of reference steroids and unknown amounts of steroids in the sample could be compared. The recoveries of added ^{3}H-labeled neutral steroids and estrogens were about 85%. When the method was applied to the analysis of unconjugated steroids in plasma from pregnant women, 27 steroids in the concentration range 1—300 ng/ml could be identified. The thirteen major pregnane derivatives were quantified and the coefficients of variation were 5—13% when five analyses of the same sample were performed.

The method described showed that a multitude of steroids could be simultaneously detected by GC—MS. The technique, however, did have a degree of

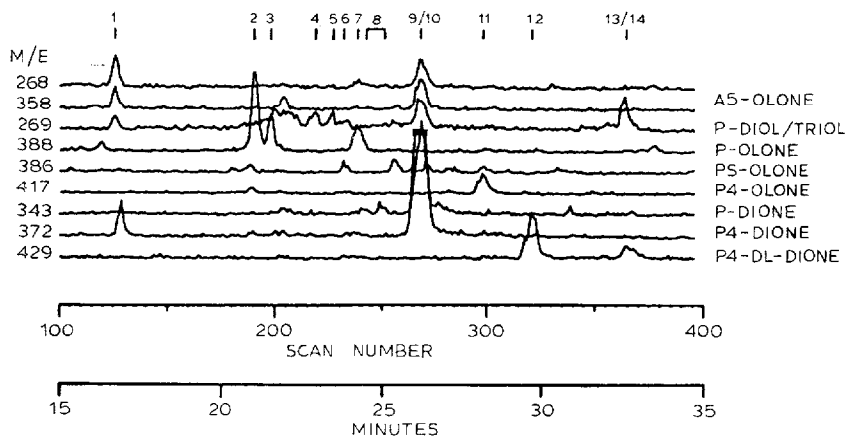

Fig. 9. GC—MS analysis of unconjugated neutral steroids in plasma. Fragment ion current (FIC) chromatograms from an analysis of MO-TMS derivatives of unconjugated neutral steroids; *m/z* values typical for the derivatives of progesterone, some of its potential metabolites and DHA were selected. Peaks: 1 = DHA; 2 = 5α-pregnan-3α-ol-20-one; 3 = 5β-pregnan-3α-ol-20-one; 4 = 5α-pregnan-3α,20α-ol; 5 = 5β-pregnan-3α,20α-ol; 6 = pregnenolone; 7 = 5α-pregnan-3β-ol-20-one; 8 = 5α-pregnane-3,20-dione; 9 = progesterone; 10 = 5α-pregnan-3β,20α-ol; 11 = 20α-dihydroprogesterone; 12 = 17α-hydroxyprogesterone; 13 = 16α-hydroxyprogesterone; 14 = 5α-pregnane-3α,20α,21-triol. From ref. 72, with permission.

selectivity as the analyst had the choice of which compounds to determine. Although GC—MS has been used many times for the analysis of plasma, tissue and urinary steroids, analysts have never tried to mimic the techniques used in RIA steroid profiling, i.e., the quantification of the "required" plasma steroids of diagnostic value in investigation of the pathophysiology of endocrine gland functions. For example, FIC chromatograms have not been produced for identifying and quantifying such plasma steroids as testosterone, DHT, androstenedione, DOC, corticosterone, 18-hydroxy-DOC, 18-hydroxy-corticosterone and aldosterone. This is not surprizing as the plasma levels of these compounds are either at the limits of sensitivity of repetitive scanning methodology or the compounds have structural features rendering them difficult to determine by gas chromatography (e.g., aldosterone).

In a more developed form, repetitive scanning GC—MS would be the ideal method for hormonal profiling as it can combine very high specificity with low selectivity (i.e., a prior knowledge of the identity of components is not necessary). However, current difficulties in the production of measurable peaks for trace but important steroid hormones renders it an unsuitable technique at present. Selected ion monitoring GC—MS has sufficient sensitivity but, like immunoassay, the analytes must be pre-selected.

4.3.2. Plasma steroid conjugates

The most comprehensive method for analysing plasma steroid conjugates is that of Axelson and Sahlberg [73]. Plasma (2 ml) is diluted to 4 ml with 0.5 *M* aqueous triethylamine sulfate and heated at 60°C for 5 min, then extracted with a Sep-Pak C_{18} cartridge. Following a water wash, the steroids are recovered by elution with 8 ml of methanol. The separation of sample fractions containing unconjugated neutral and phenolic steroids, glucuronides, monosulfates and disulfates was achieved by chromatography on TEAP-LH-20. The conjugate moiety was hydrolysed by enzymatic or solvolytic procedures and the released steroids were separated into a neutral and phenolic fraction by

TABLE 5

CONJUGATES OF STEROIDS IN PLASMA FROM A WOMAN IN THE 34TH WEEK OF PREGNANCY (ng/ml)

Data from Axelson and Sahlberg [73].

Steroid	Glucuronide	Monosulfate	Disulfate
DHA		420	
5-Androstene-3β,17α-diol			149
5-Androstene-3β,17β-diol		420	78
5α-Pregnan-3α-ol-20-one	69	755	
5β-Pregnan-3α-ol-20-one	261	265	
5α-Pregnane-3α,20α-diol	156	779	283
5β-Pregnane-3α,20α-diol	1287	398	78
Pregnenolone		58	
5α-Pregnan-3β-ol-20-one		193	
5α-Pregnane-3β,20α-diol		320	529
5-Pregnene-3β,20α-diol		81	129
Estrone	5	87	
Estriol	39	24	30

TABLE 6

MAJOR 3β-HYDROXY-5-ENE STEROID SULFATES IN UMBILICAL CORD BLOOD (ng/ml, PLASMA OR SERUM)

Steroid	Study*			
	A	B	C	D
DHA	1100	1330	980	1200
16α-Hydroxy-DHA	2550			2510
16-Oxoandrostenediol			3450	780
Androstenetriol			250	290
Pregnenolone	690	1090	1040	1210
16α-Hydroxypregnenolone			1060	1040
17α-Hydroxypregnenolone			640	1050
Δ^5PT				480
Δ^5PD**				1390
Δ^5AD (17α)**				2280
Δ^5AD (17β)**				1390
21-Hydroxypregnenolone**				990
P^5-3β,20α,21-triol**				250

*(A) Data from Laatikainen et al. [74]. Venous plasma from 19 spontaneous labor Caesarean births. Sephadex LH-20 conjugate separation, solvolysis, RIA. (B) Data from DePeretti and Mappus [80] and DePeretti and Forest [79]. Direct RIA on venous plasma (n=27 for pregnenolone sulfate; n=74 for DHAS). (C) Reynolds [225]. Umbilical vein. (D) Shackleton [223]. Mixed umbilical plasma, mostly vein (n=12). Sephadex LH-20 conjugate separation, solvolysis, GC.

**These steroids mostly disulfated.

TEAP-LH-20. The MO-TMS and TMS derivatives were analysed by capillary GC—MS. The identities and concentrations of steroids in one individual (a woman in the 34th week of pregnancy) are listed in Table 5. Data on plasma steroids in non-pregnant individuals were not given.

Other reports of steroid sulfate concentrations in pregnancy plasma have appeared, e.g., refs. 74—77. The concentrations of steroid sulfates in umbilical cord blood as determined by RIA or gas chromatography are given in Table 6. Laatikainen and Vihko [78] reported the concentrations of steroid sulfates in non-pregnant women on the 10th and 22nd days of the menstrual cycle. Surprisingly, quantitative data on steroid sulfates in men obtained by GC and GC—MS techniques are rare [23, 81]. Plasma cholesterol sulfate and DHA-sulfate have been measured in men as an aid to the diagnosis of recessive X-linked ichthyosis [82].

5. PROFILING URINARY STEROIDS

5.1. Column liquid chromatography

Whereas LC in the early years was always used for steroid metabolite separation, it never really gained a wide following for profile analysis. This contrasts with the situation for steroid hormone analysis, where even today adsorption and partition column chromatographic techniques are pre-eminent.

Vestergaard and co-workers spent many years developing column chromatographic methods suitable for semi-automated, routine measurements of

metabolites of C_{19} steroids and cortisol metabolites [83,84] and, within certain limitations, they succeeded. In brief, steroid conjugates were hydrolysed enzymatically and by solvolysis. They were fractionated into three groups (17-oxosteroids, corticosteroid metabolites and cortolones and cortols) by chromatography on silica gel columns. The cortols and cortolones were oxidized to 17-oxosteroids by sodium periodate and both original and cortol/cortolone-derived 17-oxosteroids were fractionated by long (2.4 m) PTFE capillary alumina columns. The corticosteroid metabolite fractions (THE, THF and 5α-THF) were chromatographed on 3.3 m PTFE columns containing silica gel. 17-Oxosteroids were determined spectrophotometrically by the Zimmerman reaction; the blue tetrazolium reaction was used for the α-ketolic cortisol metabolites. Up to 25 columns could be eluted simultaneously and almost all of the chromatographic and detection procedure was automated. Raw data from the spectrophotometers were processed by computer and chromatograms were printed out (Fig. 10). Data on the specificity, accuracy, precision and sensitivity of the method have been presented [84] and appear satisfactory for a semi-routine multi-component method. The urinary excretions of many of the measured metabolites are given in Table 7.

Although great improvements in the practicality of the method were introduced over the years, it still remains highly complex. Vast numbers of tubes and large amounts of solvents are required. In 1978 the originators of the method estimated the cost to be $50 per analysis. The method as described has severe limitations, the most important being the limited number of metabolites analysed. There are really only three major advantages in urinary steroid analysis over plasma analysis: (a) it non-invasive, (b) it can be non-selective and (c) an approximation of total 24 h production can be obtained. If one makes it a selective technique, as in Vestergaard and co-workers' method, then one of the great advantages is lost. Important metabolites, such as pregnanetriol, and

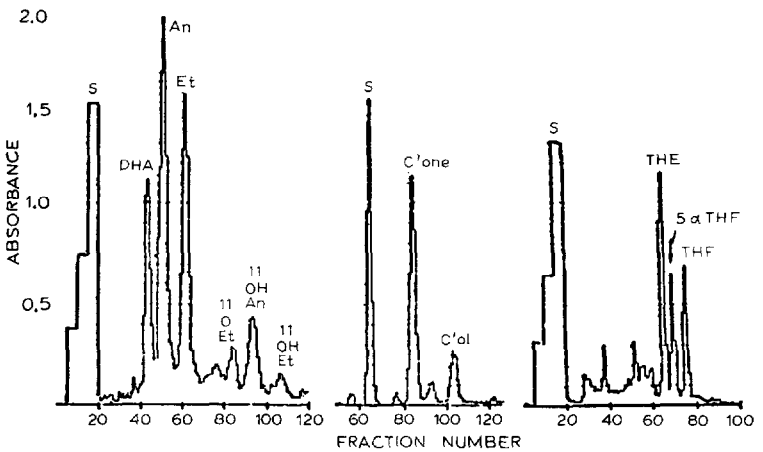

Fig. 10. Semi-automated column chromatography of hydrolysed urinary steroids. Steroids were separated on long (2.4 m or 3.3 m) PTFE capillary columns of aluminium oxide or silica. THE, THF and 5α-THF were measured by the blue tetrazolium reaction and the native 17-oxosteroids by the Zimmerman reaction. The cortols (C'ol) and cortolones (C'one) were determined as 17-oxosteroids following periodic acid oxidation. Standards (non-chromatographed) for colorimetric calibration were analysed before the urinary steroids. Absorbance data were processed by computer and reported by printer/plotter as graphs and tabulated data. From ref. 83, with permission.

TABLE 7

EXCRETIONS OF SOME OF THE MAJOR CONJUGATED STEROID METABOLITES AS DETERMINED BY DIFFERENT PROFILING TECHNIQUES (μg PER 24 h)

This table should not be considered a complete list of reported values. Several other workers have profiled the same steroids by similar methodologies.

Steroid	(A)* Column		(B)* TLC		(C)* Paper		(D)* Paper M+F	(E)* GC M	(F)* GC		(G)* GC–MS	(H)* LC GC–MS	(I)* GC	
	M	F	M	F	M	F			M	F			M	F
An	3880	1960	2600	2200				3031	9882	3270	2660	3860	2430	1847
Et	3040	2270	2500	2400				2420	6444	3050	2530	4930	3018	2471
DHA	1360	1290	1200	900					1188	462	484	910	2243	512
11-Oxo-An	570	350									225	850	515	343
11-Oxo-Et	760	500	1100	1100				547						
11β-Hydroxy-An	1160	630	1400	1000				1086	1988	626	786	2480	1031	675
11β-Hydroxy-Et	630	420	1300	1000				472	558	94	448	800	300	283
PT							800	1043	1926	1069	3570	1240	555	362
Δ^5PT							200					460	522	421
THS					62	47	20							
THA					158	154	90		90	43	32		553	526
THB					210	184	100	160	72	50	154	230	253	245
5α-THB					230	142	240	362	90	50	269	420	440	504
THE	3870	2480	2300	2100	2970	2340	2300	3516	6714	2569	2060	1700	3107	3113
THF	1800	1240	1200	1100	1100	810	1200	1397	2916	1358	1130	2500	1956	1552
5α-THF	1370	650	900	500	480	250	700	1043	1818	698	698	1100	1254	625
α-Cortol	720	1480						288	254	132	220	610	194	235
β-Cortol								1128	864	399	428	1220	600	506
β-Cortolone	2220	340									759			
α-Cortolone								1117	1206	562	485	1800	524	479
Ratio THF/5α-THF:														
M	1.31		1.33		2.29			1.33	1.60		1.61		1.55	
M+F							1.71							
F		2.0		2.20		3.24				1.94		2.27		2.48

*(A) Vestergaard and Sayegh [83]; 33 men and 14 premenopausal women (mg per 24 h). (B) Shackleton et al. [2]. (C) Cost and Vegter [90]; 9 adult males and 7 females (mg per 24 h). (D) Nielsen et al. [91]; 40 normal adults. (E) Setchell et al. [24]; GC following DEAP-Sephadex separation of conjugates; 12 male adults (mg per 24 h). (F) Pfaffenberger and Horning [125]; original results on 31 females and 21 males reported per gram of creatinine. Corrected to 24-h excretions assuming males excrete 1.8 g and females 1.17 g of creatinine (data from Documenta Geigy). (G) Vrbanac et al. [118]; one individual only; MSSMET GC–MS quantification. (H) Axelson et al. [25]; GC–MS quantification following TEAP-LH-20 fractionation of conjugates; 24-h excretion of one female in follicular phase of cycle. (I) Shackleton (unpublished laboratory normal values); excretions determined on random samples and corrected for mean daily urine excretions of men and women reported by Documenta Geigy [237]; 17 males and 13 females, 22–50 yrs.

the metabolites of corticosterone are not determined. Although overproduction of these would undoubtedly be noticed in the chromatograms, near-normal levels cannot be measured. In spite of this severe drawback, it is difficult to be overcritical of the method as it is probably the one which has been most used under routine circumstances. The authors reported that several thousand analyses have been carried out and in several instances it has contributed particularly useful data. This is certainly true for investigations into optimal hydrolysis conditions for steroid conjugates [30] and the production of rare data on the excretion of the C-20 reduced metabolites of cortisol (the cortols and cortolone) [84].

5.2. Paper chromatography

To a considerable extent we owe paper chromatographic (PC) methods for steroid profiling to the early studies of Bush. He published a valuable book on the subject in 1961 [85] and a comprehensive review of clinical applications 2 years later [86]. The book still has particular value as it documents many ancillary techniques frequently used in steroid analysis, such as microchemical reactions.

Generally, at least two chromatograms are necessary for the determination of urinary steroid metabolites after hydrolysis. The first chromatogram is used to purify the crude mixture. This chromatography is carried out on thick and dense paper (Whatman 3 mm). By simultaneous application of dyes of known retention time it is possible to cut the chromatogram into pieces that contain steroids of a particular group. The steroids are eluted from the paper segments and are further separated on secondary chromatograms using the over-run technique. Individual components are then localized by application of a suitable staining reagent (e.g., *m*-dinitrobenzene for 17-oxosteroids; blue tetrazolium for C_{21} α-ketol steroids). The steroids in the chromatograms can then be quantified by densitometric scanning. A good example of the use of this technique has been reported by Birchall and co-workers [87,88]. They measured many steroids in urine from newborn infants and young children. Savage et al. [89] have reported the excretion of THE, THF, 5α-THF, THA and THB by infants and children using this technique and the results obtained are little different from those obtained recently by more advanced methodologies such as gas chromatography.

A comprehensive method for profiling urinary steroids was published by Cost and Vegter [90]. After enzyme hydrolysis the steroids were extracted with chloroform and were subdivided into 11 fractions by PC. Some of these fractions were further chromatographed to allow the separation of the following components: (1) THF, THE and 5α-THF; (2) cortisol and 20β-dihydrocortisone; (3) cortisone, THS and THB; and (4) THB, 5α-THB, THA, corticosterone and 11-dehydrocorticosterone. All of the above steroids were detected and quantified by the blue tetrazolium and/or alkaline fluorescence reaction and quantitative data are presented in Table 7.

Nielsen et al. [91] adapted Cost and Vegter's method to allow the quantification of further compounds. Pregnanetriol and pregnenetriol which are present in the same fraction as the corticosterone metabolites were separately

determined by the fluorescence given with trichloroacetic acid. Tetrahydroaldosterone was determined following separation on four paper chromatograms, two of which were obtained following acetylation of the steroid [92]. The eluate from the last chromatogram was quantified using the blue tetrazolium reaction. The amount of tetrahydroaldosterone triacetate was calculated from a DOC acetate calibration graph and the amount of tetrahydroaldosterone excreted per 24 h was calculated. Tritiated tetrahydroaldosterone was added to the extract after enzyme hydrolysis but before chromatographic separation, so an estimate of recovery was possible and the results could be corrected accordingly. However, no estimate could be made to detect possible incomplete hydrolysis of the tetrahydroaldosterone glucuronide. Nielsen and co-workers used paper chromatographic profiling methods for the analysis of a wide variety of steroids in normal individuals and patients with selected endocrinopathies. Selected data from these investigations are reported in Tables 7 and 8.

Bush [86] designed and built a mechanized system for staining and scanning paper chromatograms after they had been developed, called CASSANDRA (chromatogram automatic soaking, scanning and digital recording apparatus). The chromatograms were placed on a belt and were passed at a constant speed through the reagent, a drying oven and through a scanning and recording system that provides a digital integral record in addition to a record of the peaks. It was found that use of this apparatus gave rise to a great improvement in the reproducibility of the "scanning technique" compared with the manual method formerly used.

Apart from these developments in methodology suitable for routine determinations, PC was widely used until recently in research applications. Notable studies are those of Kornel and co-workers, who made exhaustive studies profiling a huge number of sulfate, glucuronide and free steroids present in urine, particularly from patients with hypertensive disease [93,94]. Klein et al. [95] quantified many conjugated corticosteroid metabolites in urine from newborns by double isotope dilution following extensive PC separations. Wortmann et al. [96] perfused liver in situ with radiolabeled cortisol and profiled the metabolites in the perfusate by radioscanning the paper chromatograms used

TABLE 8

URINARY METABOLITES OF ALDOSTERONE AND ITS PRECURSOR 18-HYDROXY-CORTICOSTERONE (μg PER 24 h, MEAN + RANGE)

Steroid	A*	B*	C*	D*
18-Hydroxy-THA glucuronide	122 97—182	43 25—82		
THAldo glucuronide	53 34—93	21 12—37	44 18—77	28
Aldosterone-18-glucuronide				7.2
Ratio 18-hydroxy-THA/THAldo	2.30	2.05		

*(A) Data of Ulick [175]; normal adults (n=9); hydrolysis, TLC, double-isotope dilution. (B) Data of Ulick [175]; children 10 days to 7.5 yrs (n=7). (C) Data of Nielsen et al. [92]; 7 adults; hydrolysis, paper chromatography and blue tetrazolium reaction. (D) Data of Gomez-Sanchez and Holland [226]; 43 white adults; hydrolysis, Celite micro-columns, RIA.

for separation. More than twenty labeled components were identified, varying in polarity from 11-oxoandrosterone to α-cortol and 6-hydroxylated metabolites. The percentage conversion into each of these metabolites was determined. By the mid-1970s the use of PC had all but ceased.

5.3. Thin-layer chromatography

Steroid profiling by TLC was relatively short-lived. With its introduction in the early 1960s it had a speed advantage over paper chromatography and could achieve many of the same goals. Lisboa had laid the groundwork for its use in profiling through exhaustive testing of a series of solvent systems with a multitude of reference steroids with 3α-hydroxy-5α- (and β-), 3-oxo-4-ene and 3β-hydroxy-5-ene structures [97–99]. Today, his tables of R_F values remain the most comprehensive available. We developed a profiling method based on TLC at this time [2,100]. Urinary extracts were spotted on three plates which, following separation, were developed with the three reagents *m*-dinitrobenzene (for individual 17-oxo-steroids), blue tetrazolium (for α-ketols, e.g., cortisol metabolites) and antimony trichloride (for 3β-hydroxy-5-ene steroids and pregnanetriol). Varying concentrations of reference steroids were spotted on the plates to allow quantification of the urine extracts by densitometric scanning. This method came as near to a true profiling method as was possible at that time, as it included most of the steroids of interest present in urine, with the exception perhaps of cortols, cortolones and the metabolites of corticosterone. The latter were visible but were at a level that could not be accurately quantified by densitometry. Quantitative data produced by this method are listed in Table 7.

The same period saw the introduction of steroid derivatives that enabled even the most polar steroids to be analysed by GC [101] and the new GC–MS technique offered the required specificity, so the TLC method soon had stiff competition. It is of interest, however, that Dr. Meta Nielsen of Glostrup Hospital in Copenhagen is still a firm believer in the utility of the TLC profiling method in spite of being a convert of some years duration to GC. For example, she believes that in a clinical setting, the rapid detection of congenital adrenal hyperplasia in the early neonatal period can best be carried out by our original TLC profiling method. Hydrolysis for 1 h, an extraction and one TLC run are all that is required to obtain a meaningful semi-quantitative result. A more complete GC analysis on the sample could then be obtained at leisure.

5.4. High-performance liquid chromatography and high-performance liquid chromatography–mass spectrometry

5.4.1. Unconjugated steroids

Schöneshöfer and Weber [60,61] developed a plasma steroid HPLC method to allow the quantification of fifteen steroids in urine. The main change was the necessity to process two urine samples, as sufficient resolution of all steroids was not obtained in one chromatographic run. In the first run androstenedione, dihydrotestosterone, testosterone, substance S, corticosterone, aldosterone, cortisol and 18-hydroxycorticosterone were simul-

TABLE 9

EXCRETION OF URINARY FREE STEROIDS DETERMINED BY HPLC AND RIA (SCHÖNESHÖFER AND WEBER [60]: MEDIAN VALUES (32 MALES)

Steroid	Median	μg per 24 h
Progesterone	0.11	
Androstenedione	2.63	
Pregnenolone	0.28	
DHT	0.17	
DHA	2.51	
Testosterone	3.89	
DOC	0.11	
17-Hydroxyprogesterone	0.34	
17-Hydroxypregnenolone	0.07	
Substance S	0.08	
18-Hydroxy-DOC	0.70	
Corticosterone (B)	0.51	
Aldosterone	0.16	
Cortisol	24.6	39.4*
Cortisone		47.5*
20α-DHF		73.5**
18-Hydroxy-B	1.96	

*Schöneshöfer et al. [63]; cortisol and cortisone measured by "on-line" HPLC; 21 normal adults.
**Data of Schöneshöfer and Weber [62]; 12 normal adults.

taneously assayed, and in the second run progesterone, pregnenolone, DHA, DOC and 18-hydroxy-DOC were determined. The mean values obtained for daily excretion by 32 normal males are given in Table 9.

Schöneshöfer and Weber [62] emphasized the importance of the chromatographic separation of urinary steroids prior to radioimmunoassay. Values of cortisol immunoreactivity in crude urine were about six times higher and values of a simple dichloromethane extract about three times higher than those obtained after HPLC. The major interfering compounds arising in organic extracts have a polarity similar to that of cortisol, which cannot easily be eliminated by simple solvent extraction procedures.

In addition to their general papers on serum and urinary steroid profiling, Schöneshöfer and co-workers have published more detailed studies on the HPLC—RIA quantification of cortisol [62], urinary 18-hydroxycorticosterone and aldosterone [63] and 20-dihydrometabolites of cortisols [64]. Their latest paper describes the virtually complete automation of cortisol and triamicimalone assays [102]. All processes from extraction to UV detection are controlled automatically. A 1-ml urine sample is concentrated and pre-purified on a reversed-phase pre-column. The organic eluate from this column passes to another pre-column and finally to an analytical column for the separation of closely related compounds. Finally, the cortisol is detected by UV absorption. Losses of cortisol throughout the procedure were negligible and external calibration was found to be adequate for quantification. Coefficients of variation were 4.1% for inter-assay variability and 2.6% for intra-assay variability. Cortisol concentrations down to 15 nmol/l can be determined. Each analysis takes about 30 min, allowing a maximum of 48 samples to be analysed every

24 h. The method as described also allows the simultaneous determination of cortisone.

Ulick et al. [103] used HPLC with C_{18} columns in an early study on the excretion of steroids by a patient with a hypertensive disorder. Using a 30% aqueous acetonitrile system they were able to separate 20α- and 20β-dihydrocortisol and 5α- and 5β-dihydrocortisol from cortisol and cortisone. Heftmann [104] also used adsorption methods for the resolution of reduced progesterone metabolites. This technique was particularly suited to the resolution of 3α- from 3β-hydroxysteroids.

5.4.2. Hydrolysed conjugates

Two aspects of urinary steroid metabolite analysis have precluded the use of HPLC as an analytical method, mainly the lack of sensitive detection methods for saturated or 3β-hydroxy-5-ene steroids and also the complexity of the mixture. Urine contains a multitude of isomeric and epimeric compounds that are difficult to resolve in single chromatograms. Of course, detection with a UV spectrometer can be achieved after the formation of UV-absorbing derivatives, but this undermines one of the major advantages of HPLC over GC. Also, derivatization modifies to some extent the polar features of the analytes which may be the basis of the separations. However, apart from these reservations, an elegant example of the future potential of HPLC for multi-component analysis has been published by Novotny et al. [105]. They used a 1 m × 0.24 mm I.D. fused-silica column packed with 3 μm bonded phase particles and a flow-rate of 1 μl/min. UV detection was achieved using a fused-silica cell with an approximate detection volume of 10 nl. Benzoyl derivatives of steroids were used and a typical separation of urinary steroids is illustrated in Fig. 11. It is immediately evident that the peak resolution is as good as with capillary gas chromatographic columns, although the chromatography takes longer. It is also clear that

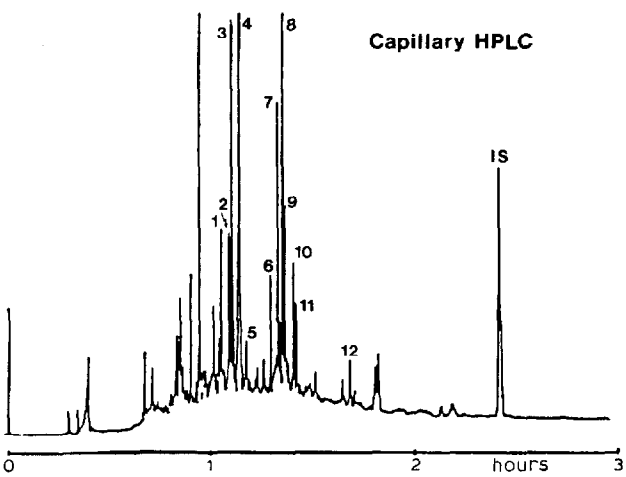

Fig. 11. Capillary HPLC: separation of steroids from urine. Stepwise gradient of acetonitrile and water. Steroids: 1 = 11β-hydroxyandrosterone; 2 = 11β-hydroxyetiocholanolone; 3 = 5α-THF; 4 = THF; 5 = THE; 6 = β-cortolone; 7 = β-cortol; 8 = α-cortolone; 9 = α-cortol; 10 = etiocholanolone; 11 = androsterone; 12 = DHA. IS = internal standard (5α-androstane-3β,17β-diol). From ref. 105, with permission.

derivatization does not impede the separation of closely related steroids, e.g., the epimeric steroid pairs androsterone—etiocholanolone and THF—5α-THF are well resolved. It is of interest that the authors did not refer to the small peak given by THE (peak 5), quantitatively the most important steroid present in urine from adults. It should be added, however, that the peak given by this steroid was small when reference compounds were analysed. This substantially different response between different metabolites is in contrast to that of a flame ionization detector in GC and would be a considerable disadvantage. It would seem likely that the variable peak size is related to the derivatization procedure.

Derks and Drayer [106] identified new 6α-hydroxylated corticosteroid metabolites in infant's urine by GC—MS following HPLC separation on silica columns with a water—chloroform—methanol eluent.

5.4.3. Steroid acids

Among the many steroid metabolites found in urine are those which contain a carboxylic acid moiety. Monder and co-workers have developed methods for profiling the steroids using GC and HPLC [107,108]. The major acids present in urine are the cortoic acids, which are structurally related to the cortols and cortolones. The GC method was based on the analysis of methyl ester trimethylsilyl ethers using a polar (Carbowax) capillary column [107]. It was found that the non-polar GC columns more useful for the resolution of neutral steroids were unsatisfactory for separating all the isomers. Reversed-phase (C_{18}) HPLC provides an excellent alternative to GC for profiling steroid acids, provided *p*-bromophenol esters are prepared [108].

Monder and Iohan [109] developed a method for the group separation of the steroid acids from neutral steroids based on the use of the weak anion exchanger polyethyleneimine (PEI) cellulose. Columns of 800 mg were used and were prepared in water. The steroid extract was applied in an aqueous alcoholic solution and further elution with 48% aqueous ethanol resulted in the recovery of neutral steroids. Steroid acids were eluted with 0.25 *M* formic acid in 72% ethanol. Lipophilic ion-exchange resins can also be used for specifically isolated steroid acids [24,25].

5.4.4. Unhydrolysed glucuronides

Thermospray HPLC—MS may at some point be used for steroid profiling and we have carried out preliminary investigations on the separation of steroid glucuronides using this technique. Such compounds give good mass spectra in the negative ion mode, similar to those produced in fast atom bombardment (FAB) MS as they are dominated by the quasimolecular ion. Promising results have been shown for the selected ion monitoring (SIM) of isomeric steroid glucuronides [110]. As yet there have been no applications of the technique to the analysis of steroid conjugates in biological systems.

5.5. Gas chromatography and gas chromatography–mass spectrometry

Modern GC profiling techniques originated at the beginning of the 1970s with Novotny and Zlatkis [111] and Völlmin [112], who successfully used

glass capillary columns for clinical steroid separations. The complexity of urinary steroid constituents really required the high resolution afforded by these columns. The early development of capillary steroid chromatography suffered a loss when the youthful Jürg Völlmin died in 1973. Of course, the introduction of capillary columns for steroid analysis was inevitable and previous developments were also critical to the development of the discipline. Examples would be the original breakthrough of Vandenheuvel et al. [113], which was the primary demonstration of steroid GC, and the production of the first true GC profile of urinary steroids in 1966 by Gardiner and Horning [101]. These workers demonstrated the GC analysis of the most complex of steroids such as the intact cortisol metabolites. A central role in these developments was played by Horning, since he and his co-workers introduced the derivatization methodologies we all use. Coincidentally, GC—MS was introduced, allowing the characterization of all GC peaks to be achieved [114].

Since Völlmin's introduction of capillary columns into clinical steroid analysis, the major advances have been gradual but critical. Some of these are as follows:

1974	Introduction of the Lipidex 5000 procedure for purifying steroid MO-TMS ethers prepared using the involatile trimethylsilylimidazole (TSIM) [72].
1976	Commercial introduction of split—splitless injection as an alternative to solid sampling.
1978	Microprocessor-controlled gas chromatographs introduced.
1979	Introduction of fused-silica columns.
1980	Introduction of C_{18} cartridges for facilitating steroid work-up procedures.
1983	Introduction of bonded phase fused-silica columns.
1984, 1985	First inexpensive capillary dedicated automated mass spectrometers; Hewlett-Packard 5970 (mass-selective detector) and Finnigan ion trap detector.

Through all these developments we are now in the position to profile steroids economically using a combination of GC and GC—MS. There is little doubt that for the foreseeable future these techniques will remain pre-eminent for the profiling of urinary steroids and the length of discussion here will reflect the importance of this methodology.

We have to define the requirements for urinary steroid analysis by GC. Does it need to be quantitative? If so, what are the tolerances for reproducibility? What are the specificity requirements? What are "acceptable" short-cuts in methodology?

A perfect quantitative profiling technique would have the following attributes: (1) accurate collection of 24-h samples; (2) quantitative extraction of all steroid conjugates from urine; (3) complete hydrolysis of all steroid conjugates; (4) quantitative recovery of freed steroids; (5) quantitative conversion of all steroids into volatile derivatives; (6) baseline GC resolution of all components of interest; (7) linearity of flame ionization detector response over a wide concentration range, e.g., 5–200 ng per component; (8) accurate response factor determinations, only possible with highly purified reference steroids; (9) reproducibility of inter- and intra-assays of individual steroids; and (10) absence of impurities.

The achievement of all these requirements is possible, although item 6 would necessitate GC—MS being used for profiling as no single GC column can resolve all steroid components of interest. However, trying to achieve all these goals would require further years of method development and constant anxiety regarding the quality of the results presented. Particular problems relate to the quantitative hydrolysis of all conjugates. Although it is generally agreed that the digestive juice of the snail *Helix pomatia* contains the best enzyme mixture for hydrolysis of urinary steroid conjugates, even this preparation does not have sulfatases capable of hydrolysing sulfates of C-17 and C-20 hydroxy groups and the hydrolysis of 3α-hydroxy-5α-steroids is inefficient. The complete hydrolysis of 5α-THF glucuronide is also particularly problematic.

We must ask ourselves what information is being sought from urinary steroid profiles and develop from this the simplest procedures for obtaining this information. I do not feel that much is to be gained by the endless testing of methods to establish quantitative recoveries of steroids and high levels of quantitative reproducibility for the chromatographic analyses. As far as I know clinical evaluation is unlikely to be affected by the difference between daily tetrahydrocortisone excretions of 2500 or 3500 μg per 24 h. Even if the methodology itself was responsible for this difference (e.g., incomplete hydrolysis), the error is far less than the difference that would be present between individuals. What is more likely to be important is the relative excretion of THE with respect to THF as this would give information on the activity of 11β-hydroxysteroid dehydrogenase*. There are clinical disorders where this pair of compounds is in imbalance owing to defects in the oxidative or reductive activities of the enzyme [115].

The ability to detect alterations in the relative amounts of a steroid converted into different metabolites or to detect enzyme inactivities through imbalance between metabolites of precursors and metabolites of products is a particular strength of the urinary steroid profile technique. In many instances steroid ratios give all the information required and complex methodologies for accurate and quantitative measurement of all urinary steroids may be superfluous. Once a simple procedure has been established by a laboratory, the method should be followed with consistency.

The strength of the urinary steroid methodology rests in its versatility. Plasma steroid profiling procedures are based first on the liquid chromatographic separation of reference steroids. When a suitable system has been developed for resolving the steroids of interest, then plasma extracts are separated and the contents of the individual fractions are quantified by use of appropriate radioimmunoassay procedures. Thus, a decision is made in advance to measure a particular compound or series of compounds based on the ability to separate the components and on the availability of the required antibodies. In contrast, a urinary steroid profile contains metabolites of all steroids excreted and visual assessment of chromatograms can often reveal a disorder whatever the lesion, and whatever steroids are present in inappropriate

*11β-Hydroxysteroid dehydrogenase is likely to be more than one enzyme. There is strong evidence suggesting that the presence of enzymes catalyses the oxidation of cortisol and the reduction of cortisone.

amounts. This feature delineates what I consider to be a true profile from a partial profile. The non-selective nature of a true profile gives information in excess of that obtained by summation of the individual bits of information obtained by measurement of individual components.

5.5.1. The requirement for GC—MS in profiling

GC alone does have limitations in profile analysis and GC—MS has an important function. Whereas GC quantification of steroids present in amounts greater than about 5% of the steroid excreted in maximum amount is relatively easy, quantification of steroids in the 1–2% range can be difficult and determinations are particularly subject to inaccuracies in baseline evaluation or the presence of impurities. Of course, these figures are approximate, as a compound present at the 1% level placed well away in the chromatogram from a major component may be readily quantified. One of the problems rests in the ability to measure these compounds in the 1–2% range. Unfortunately, these also comprise some of the most interesting components in a urine extract. Examples would be estradiol in males, children or during the follicular phase in women; cortisol, cortisone and their dihydro and 6β-hydroxy metabolites, tetrahydroaldosterone and 18-hydroxy-THA and THDOC and 18-hydroxy-THDOC.

These steroids are most conveniently analysed by specific GC—MS methods, although standard extraction and derivatization techniques can often be used. We analyse the unconjugated cortisol metabolites directly on unhydrolysed urine extracts. Although stable isotope-labeled internal standards are available (e.g., for cortisol) or could be synthesized, we have worked on the premise that unless extreme accuracy is required then inexpensive commercially available unlabeled steroids should be used. The main criterion we have adopted for choice of standards is structural and polarity equivalence to the analyte. For this reason we have chosen prednisone, 11α-cortisol and 6β-hydroxyprednisolone as standards for cortisone, cortisol and 6β-hydroxycortisol, respectively. Good internal standards for the 20-dihydrocortisol metabolites and 18-hydroxycortisol are not available, so quantification is made against stigmasterol and cholesteryl butyrate following determination of appropriate response factors through analysis of reference compounds. Although to some extent the fact that methyloxime derivatives give two peaks for each of the above compounds is a disadvantage (e.g., it reduces sensitivity), it gives an extra degree of specificity through the necessity for both peaks to be the appropriate size.

A pair of compounds of clinical importance are tetrahydroaldosterone (THAldo) and 18-hydroxy-THA. The latter steroid is the major metabolite of the aldosterone precursor 18-hydroxycorticosterone, so measuring this pair gives valuable information on imbalances in the production of aldosterone. Data on the quantification of these metabolites are given in Table 8. In earlier studies we described the analysis of each of these compounds by GC—MS SIM [116,117]. We analysed the fully derivatized open form of THAldo by SIM and determined m/z 638 (M^+) and 607 (M−31). We used as an internal standard the 3β-5α-epimer, which has a considerably longer retention time but which gives the same ions. Subsequently we changed the method slightly in order to measure the hemiacetal form, as this improves the sensitivity of the assay. As an MO-TMS

derivative this steroid has a small parent ion at m/z 609 but gives a very strong fragment at m/z 506 suitable for quantification. 18-Hydroxy-THA is conveniently measured by using 3β-5α-THE as an internal standard*. This gives the same molecular ion (m/z 609) and M−31 ion (m/z 578) as 18-hydroxy-THA and is clearly separated. Fig. 12 illustrates the quantification of THAldo and 18-hydroxy-THA against their internal standards. An alternative method of quantification uses the TMS ether alone and the internal standard 3β,5α-THAldo. The TMS ethers of both THAldo and 18-hydroxy-THA give a strong ion at M−103 (m/z 477) suitable for SIM quantification.

Some investigators have developed their methodology on the premise that GC—MS could be used for all quantitative profiling of steroids. Sjövall and coworkers analysed samples by repetitive scanning GC—MS, selected-ion current recordings for designated ions being printed out after completion of the analyses [14, 25, 71]. The designated ions were chosen for being relatively abundant and relatively specific for the compounds of interest (see Fig. 9). Quantification was achieved by prior analysis of a standard mixture, which enabled response factors to be generated for designated ions of individual components relative to ions of an internal standard. Thus, quantification of components in steroid mixtures could be achieved by relating the peak areas obtained in the selected-ion current plots to peak areas of the internal standard with employment of the appropriate response factors.

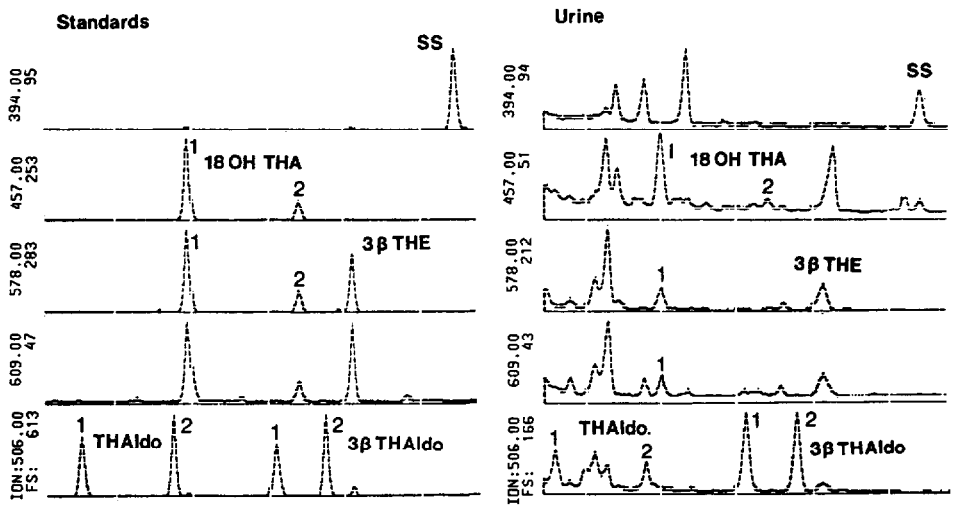

Fig. 12. Determination of tetrahydroaldosterone and 18-hydroxy-THA by SIM GC—MS. The left-hand side represents the separation of reference steroids as MO-TMS ethers. For 18-hydroxy-THA an internal standard (3β,5α-THE) was used. Both of these compounds give a molecular ion at m/z 609 and prominent M−31 ions (m/z 578). Only 18-hydroxy-THA gives an ion at m/z 457. This compound gives two peaks (1 + 2) in common with all 17-deoxycorticosteroids. For tetrahydroaldosterone the 3β,5α-epimer is used as internal standard; each compound gives two peaks. The derivative used is the hemiacetal MO-TMS derivative, which gives a strong ion at m/z 506 (M−103). SS is the common internal standard stigmasterol. The analytes are clearly seen in the urine sample (right-hand side); the amounts found were 50 μg per 24 h for THAldo and 108 μg per 24 h for 18-hydroxy-THA.

*Since submission of this manuscript the method has been improved by synthesis and use of the 3β-5α-epimer of 18-hydroxy-THA as internal standard.

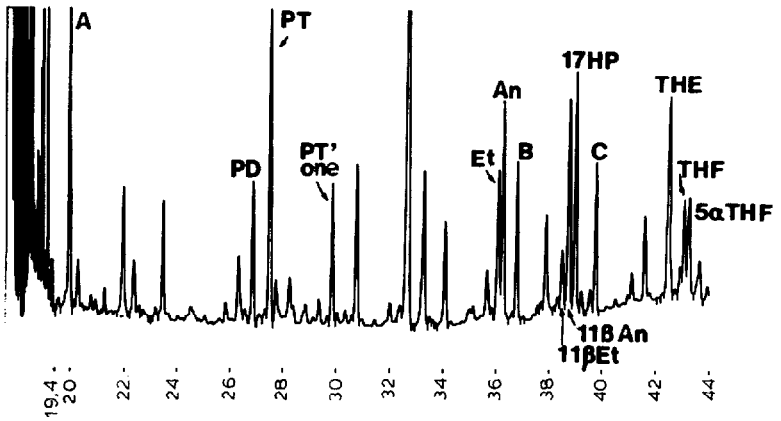

Fig. 13. Late-onset CAH: separation of urinary steroids as TMS and BO-TMS ethers. Abbreviations: PD, pregnanediol; PT, pregnanetriol; PT'one, pregnanetriolone; Et, etiocholanolone; An, androsterone; 11βEt, 11β-hydroxyetiocholanolone; 11βAn, 11β-hydroxyandrosterone; 17HP, 17α-hydroxypregnanolone; THE, tetrahydrocortisone; THF, tetrahydrocortisol; and 5α-THF, 5α-tetrahydrocortisol. A, B and C are internal standards (5α-androstane-3α,17α-diol, stigmasterol and cholesteryl butyrate, respectively). A retention time scale is shown underneath the chromatogram. The first 20 min of the chromatogram have been compressed.

This technique has been widely used by Sjövall's group [14,25,71] and latterly by Sweeley and co-workers [118,119], who incorporated a great deal of sophistication into the automation and computer programming of the procedure. Basically they expanded the capability of their earlier MSSMET technique for organic acid analysis [120] to include steroids. The technique involves accurate recording of relative retention time data (methylene units), identification of compounds by reverse library searching and quantitative analysis by the designated ion–internal standard method.

Although the methods described by Sweeley's group are extremely straightforward, sophisticated and reasonably accurate (Table 7), it is my opinion that repetitive scanning GC—MS is not necessary for the analysis of all samples. In fact, it may have a disadvantage even versus conventional GC in having a reduced dynamic range, precluding the measurement of minor components [119]. At sample concentrations that allow sufficient data points to be obtained across small GC peaks, the components present in larger concentration frequently extremely overload the column. Unless minor, but important, components can be determined with specificity (e.g., tetrahydroaldosterone) scanning GC—MS does not have significant advantages over conventional GC. No one would dispute the benefits of the MSSMET approach in the analysis of organic acids, but urinary steroids, in contrast to urinary organic acids, produce extremely reproducible patterns when subject to GC alone, even in pathological situations. It seems that under clinical conditions GC—MS is really only useful in the SIM mode for quantitative steroid analysis.

One argument for the use of repetitive scanning GC—MS in profiling the major urinary steroids relates to the individual measurement of overlapping peaks. An example is 11β-hydroxyandrosterone and 17α-hydroxypregnanolone, which inevitably co-elute on OV-1 or SE-30 columns when analysed as methyloxime-trimethylsilyl ethers. Individual measurement of these compounds is important. In our studies, 17α-hydroxypregnanolone and 11β-hydroxyandrosterone are important analytes in the diagnosis of late-onset congenital adrenal hy-

perplasia and 5α-reductase deficiency, respectively. These steroids can be readily distinguished and separately quantified by the MSSMET technique, but a simpler solution may be to alter the derivative type to one which allows the components to separate. In this instance the benzyloxime trimethylsilyl ether (first utilized by Devaux et al. [68]) is excellent and a chromatogram illustrating this separation is shown in Fig. 13.

Quantitative and semi-quantitative analyses of steroids excreted by adults have been published by a number of workers [24,25,121–126] and typical results are reported in Table 7. It is reassuring that by and large the results obtained by gas-phase methods are similar to those obtained by liquid chromatographic procedures previously described. It is particularly impressive that a key ratio (e.g., THF/5α-THF) remains relatively constant with changing methodologies and in each instance shows the same difference between males and females. All of these investigators who used GC applied slightly different methodologies, but these should not markedly affect the inter-laboratory comparison of results. However, it is probably time that some consensus is reached on methodology to allow for improved quality control.

Particular problems relate to the analysis of urinary steroids in the newborn and very young infant. Some of the major corticosteroid metabolites are different than those in adult urine and have been identified as 6α- and 1β-hydroxy metabolites of THE and the cortolones [106,127,128]. These steroids have been quantified (Table 10).

Our own method is described in the Appendix. Generally we have come to rely heavily on steroid ratios rather than accurate 24-h quantification and this has permitted some shortcuts to be employed. From receipt of a sample we expect to be able to give semi-quantitative results and appropriate ratios within 24 h. Table 11 lists many of the steroids we measure in the standard MO-TMS separation, some of the problems encountered and how they can be circumvented.

A major area which I think receives less than appropriate attention is the choice of internal standards for GC quantification. No standard is ideal, but I feel strongly that if at all possible we should have a standard that elutes before

TABLE 10

EXCRETION OF SATURATED CORTISOL METABOLITES BY NEWBORN INFANTS (FROM SHACKLETON ET AL. [128])

Steroid	μg per 10 mg creatinine (mean, $n=13$)	μg per 24 h
Tetrahydrocortisone (THE)	62.2 ±47.4	114.2
β-Cortolone	12.8 ± 6.9	22.7
6α-Hydroxy-THE	62.3 ±43.2	110.8
1β-Hydroxy-THE	12.5 ± 8.3	22.2
6α-Hydroxy-α-cortolone	8.5 ± 7.9	15.1
6α-Hydroxy-β-cortolone	31.6 ±18.0	56.2
1β-Hydroxy-β-cortolone	15.6 ±11.2	27.8
Total	207.5	269.3
% with additional hydroxyl	62.8	
% 6α-hydroxylated	49.3	
% 1β-hydroxylated	13.5	

TABLE 11

URINARY STEROIDS: SOME COMPONENTS OF THE STANDARD MO-TMS PROFILE USING AN OV-1 COLUMN

Neonates have many other steroids (particularly 3β-hydroxy-5-ene steroids) than those listed here. In very young infants it is usually preferable to analyse steroid sulfates and glucuronides separately [128,223].

Steroid	MU*	Notes
Androsterone	25.11	
Etiocholanolone	25.28	May be contaminated to a small extent with 5α (and 5β)-androstane-3α,17β-diol. May be separately measured as BO-TMS
DHA	25.75	
Epiandrosterone	25.80	
5-Androstenediol	25.85	
11-Oxo-Et 11-Oxo-An	25.95	Co-elute. Must be resolved with polar column [224]
11β-Hydroxy-An	27.00	Mixed with 17HP. These can be separately analyzed as BO-TMS ethers [160]
11β-Hydroxy-Et	27.18	
16α-Hydroxy-DHA	27.38	Gives two peaks. Usual proportion 4:3 (first second)
Pregnanediol	27.64	
Pregnanetriol	28.00	Subject to contamination
Δ^5PD	28.16	
AT	28.46	
THS	28.62	In normals usually better to determine by SIM GC—MS
PT'one	29.10	Virtually absent in normals
Δ^5PT	29.46	May co-elute with THE. Separately analyse as BO-TMS
THE	29.55	
THA	29.77	Subject to contamination
THB	30.00	
5α-THB	30.16	Co-elutes with 5α-THA. All THA and THB metabolites give small secondary peak of longer retention time
THF 5α-THF	30.24 30.39	Difficult to measure in infants <3 months of age because of presence of 6α-hydroxy-THE and other compounds; should be measured by SIM GC—MS [227]
α-Cortolone	30.51	
β-Cortolone	30.73	Co-elutes generally with β-cortolone but can be separately measured with SE-52 column [4]
18-Hydroxy-THA THAldo	30.64 30.86	Cannot be measured by GC alone except under cases of extreme elevation. Use SIM GC—MS [116]
α-Cortol	31.20	
6α-Hydroxycortolone	31.82	Neonatal steroid (many others also)
1β-Hydroxycortolone	32.00	Neonatal steroid (many others also)
Cortisol (F)	32.54	Two peaks. The first may co-elute with stigmasterol internal standard. Better to determine by SIM GC—MS
6β-Hydroxy-F	32.73	Better to determine by SIM GC—MS
20α-DHF	32.45	Two peaks. Better to determine by SIM GC—MS

*Methylene units.

the steroids of interest and a standard that elutes after. Quantification of individual components should then be carried out against each standard. The reason lies in the fact that capillary GC with splitless sampling results in discrimination against higher mass components, but it is difficult to monitor accurately the extent of this discrimination. In a particular run the peak areas may decline more rapidly with increasing retention time than in another run, perhaps carried out by a different operator. This would be immediately noticeable if "before" and "after" standards were used. We have found that very satisfactory results are obtained if the peak area of each steroid is measured against the first and second internal standard and the average taken. Our principal reason for incorporating the third internal standard cholesteryl butyrate (CB) is to keep a check on the stability of the steroid derivative. TMS ethers hydrolyse relatively easily when traces of water or acid are present, and one must always be prepared for this. Stigmasterol TMS ether hydrolyses particularly readily and so if its peak becomes markedly smaller than that of CB (which does not form a derivative) then the sample should be rederivatized.

5.5.2. Steroid excretions in pathological conditions

A compilation of the GC profiles obtained in urine from patients with diverse conditions affecting steroid synthesis and metabolism was published in 1980 [5]. It has been found that in most instances diagnosis can be achieved through measurement of appropriate relative steroid excretions. Often these represent "precursor metabolite" to "product metabolite" ratios. Many of the disorders are well defined and these will be summarized here with suitable mean ratios that can be determined by GC or SIM GC—MS. The values given should be considered to be very approximate and normal values represent adults only. A bibliography is also included, which includes in addition to GC references some references to urinary steroid assay employing other profiling techniques and some studies where diagnosis was achieved through plasma analysis.

5.5.2.1. 3β-Hydroxysteroid dehydrogenase deficiency. Noted for reduced excretion of cortisol metabolites with elevated excretions of all 3β-hydroxy-5-ene steroids [134]. This disorder is difficult to detect in the neonatal period because 3β-hydroxy-5-ene steroid levels are also high in normal infants. Considered to be a very severe disorder, mild forms probably exist that have steroid profiles only slightly different from normal [133].

Important ratios	Affected	Normal	
		M	F
DHA/THE + THF + 5α-THF	>5	0.35	0.10
Δ^5PT/THE + THF + 5α-THF	>5	0.08	0.08
Androsterone + etiocholanolone/THE + THF + 5α-THF	>5	0.86	0.66

References: 5, 129—136.

5.5.2.2. 21-Hydroxylase deficiency. This disorder manifests itself in the reduced excretion of cortisol metabolites and highly elevated excretions of

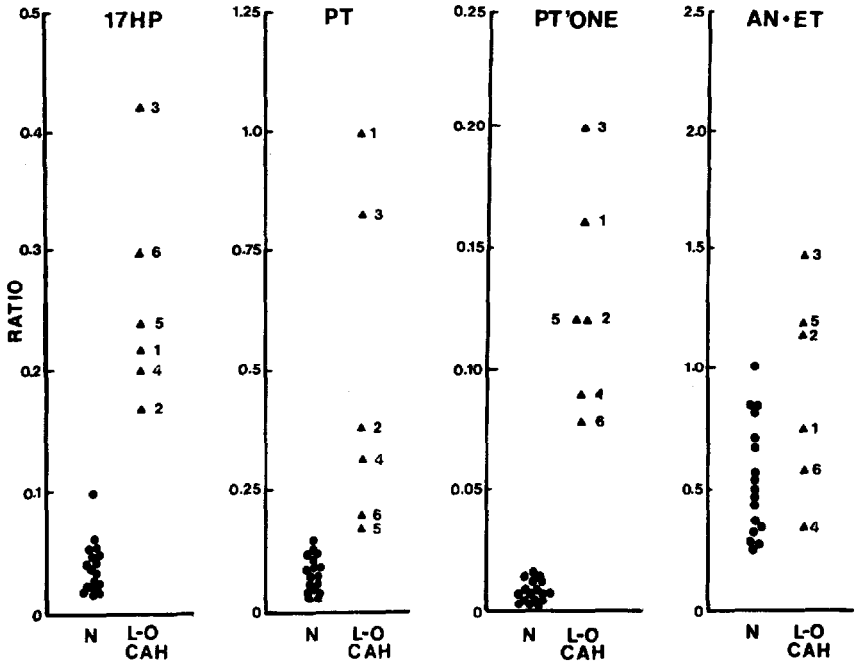

Fig. 14. Late-onset congenital adrenal hyperplasia. Precursor metabolite to product metabolite ratios. From the left, 17α-hydroxypregnanolone (17HP) to cortisol metabolites (THE + THF + 5α-THF); pregnanetriol (PT) to cortisol metabolites; pregnanetriolone (PT'one) to cortisol metabolites; and androsterone + etiocholanolone to cortisol metabolites. N, Normal individuals; L-O, late-onset CAH patients.

metabolites of the precursor 17α-hydroxyprogesterone, e.g., 17-HP, PT and PT'one.

Mild forms of the disorder (some are late-onset) have normal cortisol metabolite excretions but the ratios of the 17-hydroxyprogesterone metabolites to cortisol metabolites are elevated (Fig. 14) [138]. Diagnosis of these latter forms is most conveniently carried out by analysis of benzyloxime trimethylsilyl ether derivatives, as this allows the separate analysis of 17α-HP and 11β-hydroxyandrosterone (Fig. 13).

In the immediate neo-natal period patients with this disorder have very high 16α-hydroxypregnenolone to 16α-hydroxy DHA ratios [137,139,143] and other peculiarities. The elevated excretion of pregnanetriol and prenanetriolone may not be evident for several days [137].

Important ratios	Normals (females)	Homozygotes	Late onset
17HP/THE + THF + 5α-THF	0.02—0.10	>5	0.17—1.0
PT/THE + THF + 5α-THF	0.03—0.15	>5	0.17—2.0
PT'one/THE + THF + 5α-THF	0.02—0.014	>1	0.08—0.5

References: 5, 137—144.

5.5.2.3. 17α-Hydroxylase deficiency. Patients with a complete deficiency have almost undetectable excretions of cortisol metabolites and C_{19} steroids.

The major urinary steroids are metabolites of corticosterone (e.g., 5α-THB), a sizeable proportion of which are 21-deoxysteroids [146]. Heterozygotes can be detected by profile analysis [149]. Neonates with the disorder have elevated 16α-hydroxypregnenolone to 16α-hydroxy-DHA ratios and the presence of other corticosterone metabolites, e.g., 6α-hydroxy-THA.

Important ratios	Affected	Normal	
		M	F
5α-THB + THB/THF + 5α-THF	>20	0.19	0.33
Corticosterone metabolites/An + Et	>50	0.11	0.28

References: 5, 145—149.

5.5.2.4. 17,20-Lyase deficiency. This disorder is probably associated with 17α-hydroxylase deficiency. It manifests itself in low production of C_{19} steroids and high excretions of corticosterone metabolites. PD and PT may also be elevated, the latter suggesting an associated partial deficiency of 21-hydroxylase.

Important ratios	Affected	Normal	
		M	F
THB + 5α-THB/THF + 5α-THF	1.5—2.5	0.19	0.33
Androsterone + etiocholanolone/THE + THF + 5α-THF	<0.1	1.0	0.8
PD/PT	0.0—3.5	0.2	0.2*
PT/THE + THF + 5α-THF	0.18—0.37	0.12	0.12

*Follicular phase level of PD.
References: 5, 150—154.

5.5.2.5. 11β-Hydroxylase deficiency. Patients with a complete deficiency have almost undetectable excretions of cortisol metabolites. The major urinary steroids are androsterone, etiocholanolone, THS and hexahydro-substance S [5]. In the immediate neonatal period 3β-hydroxy-5-ene steroids and polar substance S metabolites (e.g., 6α-hydroxy-THS) are quantitatively important [155].

Important ratios	Affected	Normals
Androsterone + etiocholanolone/11β-hydroxy-An + 11β-hydroxy-Et	>10	2.46
THS/THE + THF + 5α-THF	>7	0.01

References: 5, 155—159.

5.5.2.6. 5α-Reductase deficiency. Patients with this disorder show low excretion of steroids with a 5α-hydrogen. As many steroids are present in urine as epimeric pairs (5α and 5β), the disorder can be conveniently diagnosed by the determination of selected 5α/5β ratios. This is another disorder in which benzyloxime derivatives are useful as they allow the separation of 11β-hydroxy-

androsterone from 17α-hydroxypregnanolone, thus permitting accurate determination of the 11β-hydroxyandrosterone/11β-hydroxyetiocholanolone ratio. Heterozygotes can usually be diagnosed through their low 5α/5β ratios, although occasionally normal women have relatively low ratios also.

Important ratios	Carriers	Affected	Normals	
			M	F
Etiocholanolone/androsterone	2.66	4.86	1.24	1.73
11β-Hydroxyetiocholanolone/11β-hydroxyandrosterone	0.82	2.49	0.35	0.51
THB/5α-THB	0.96	3.01	0.57	0.48
THF/5α-THF*	6.35	23.3	1.56	2.00

*These ratios were obtained following long β-glucuronidase hydrolysis (48 h). Owing to impaired recovery of 5α-THF, these ratios will be slightly higher than if hydrolysis for 3 h is used.
References: 5, 160—164.

5.5.2.7. Cortisol oxidase deficiency (11βOHSD). Most patients with this hypertensive disorder are children and diagnosis is readily achieved by noting the low excretion of THE (relative to the THFs) and elevated excretion of unconjugated cortisol metabolites (e.g., cortisol, 6β-hydroxycortisol and 20α-dihydrocortisol).

Important ratios	Affected	Normal	
		M	F
THF + 5α-THF/THE	22.9	1.03	0.70
5α-THF/THF	3.0	0.64	0.50
F, 6β-hydroxy-F, 20α-DHF/THE + THF + 5α-THF	0.1—0.3	0.03	0.03

References: 5, 165—170.

5.5.2.8. Cortisone reductase deficiency (11βOHSD). Patients with this disorder convert all their cortisol into cortisone and its reduced metabolites. This gives rise to "apparent" cortisol deficiency, ACTH stimulation and stimulation of adrenal steroid synthesis. High production of adrenal androgens causes hirsutism in females. The urine profile is characterized by very high secretion of THE and low excretion of THF, 5α-THF and the cortols.

Important ratios	Affected	Normal	
		M	F
THE/THF + 5α-THF	>20	0.97	1.43

References: 171, 172.

5.5.2.9. Hypoaldosteronism. Inactivity of the enzymes involved in conversion of corticosterone into aldosterone is a cause of hypoaldosteronism.

Elevated excretions of the metabolites of corticosterone and 18-hydroxy-corticosterone (5α-THB and 18-hydroxy-THA) is seen. In neonates 6α-hydroxy-THA is a more important corticosterone metabolite than 5α-THB.

Important analyte:
THAldo Low excretion

Important ratios	Affected	Normal
18-Hydroxy-THA/THAldo	>10	2.1
THB, 5α-THB (or 6α-hydroxy-THA)/THAldo	>50	8.0
THB, 5α-THB (or 6α-hydroxy-THA)/THE + THF + 5α-THF	>1	0.26

References: 5, 173—176.

5.5.2.10. Pseudohypoaldosteronism. Insensitivity of the renal tubule to aldosterone gives rise to apparent hypoaldosteronism. However, aldosterone secretion and tetrahydroaldosterone excretion may be highly elevated.

Important analyte	Affected	Normal
THAldo	Mean 850 μg per 24 h (8 infants, 1—33 months)	10—40 μg per 24 h

Important ratios	Affected	Normal
THAldo/18-hydroxy-THA	2.1	0.3
THAldo/THE + THF + 5α-THF	1.1	0.04

References: 5, 175—180.

5.5.2.11. Cushing's disease. Cushing's disease is associated with a high secretion of cortisol and extremely elevated excretion of cortisol metabolites. An increased ratio of THF to THE is typical, as is an increased THF/5α-THF ratio.

Important ratios	Affected	Normal	
		M	F
THE/THF	0.7	1.59	2.00
THF/5α-THF	5—10	1.55	2.48

References: 5, 181—183.

5.5.2.12. Adrenal tumors. Most patients fall into two groups, showing (1) high excretion of 11β-hydroxyandrosterone and (2) high excretion of 3β-hydroxy-5-ene steroid sulfates.

Important ratios	Affected	Normal	
		M	F
11β-Hydroxyandrosterone/THE + THF + 5α-THF	>1.0	0.14	0.12
11β-Hydroxyandrosterone/androsterone + etiocholanolone	>1.0	0.19	0.19
DHA/THE + THF + 5α-THF	>1.0	0.35	0.10
16α-Hydroxy-DHA/THE + THF + 5α-THF	High		
Δ^5PT (and other 3β-hydroxy-5-ene steroids)/THE + THF + 5α-THF	High		

References: 5, 121, 184, 185.

5.5.2.13. Placental sulfatase deficiency (PSD). Deficiency of placental sulfatase prevents conversion of 3β-hydroxy-5-ene steroids into estrogens in late pregnancy. This disorder manifests itself in a high excretion of 3β-hydroxy-5-ene steroids and a low excretion of estriol.

Important analytes	Affected	Normal
16α-Hydroxy-DHA	5—30 mg per 24 h	0.1—2 mg per 24 h
AT	2—25 mg per 24 h	0.1—1 mg per 24 h

A high ratio of these to total urinary estrogen (or urinary estriol) would be particularly diagnostic.
References: 5, 186—188.

5.5.2.14. Recessive X-linked ichthyosis (RXLI). This disorder has its origin in PSD and manifests itself by the patient developing scaly skin in early childhood. A deficiency of steroid sulfatase is the cause.

Important analyte	Affected	Normal
Plasma cholesterol sulfate	2—6 mg per 100 ml	0.1—0.5 mg per 100 ml

References: 82, 188—190.

5.6. Profile analysis of conjugates by mass spectrometry

The advent of fast atom bombardment mass spectrometry (FABMS) in 1981 suggested the possibility of the direct mass spectrometric analysis of steroid sulfates and glucuronides without hydrolysis or derivatization. Both forms of conjugates gave simple mass spectra dominated by the quasimolecular ion [190], so it seemed feasible to analyse mixtures of compounds in a single spectrum provided they differ in mass. This was the case and in the negative ion mode FAB mass spectral profiles of urinary steroids could be used for the diagnosis of some of the inborn errors affecting cortisol synthesis [191—193]. Although profiling by mass spectrometry does have some advantages, it suffers from an inability to distinguish epimers or isomers (except when tandem MS is employed [110]) and quantification presents particular problems with regard to the choice of internal standards [194]. Also the nature of the technique almost certainly precludes the analysis of the important minor components such as aldosterone metabolites.

6. PROFILING ESTROGENS

6.1. Urine

Capillary GC separations of estrogen derivatives are best achieved through the use of stationary phases more polar than those used for neutral steroids. Adlercreutz and co-workers [7,26] used OV-210 whereas Pillai and McErlane [195] achieved most success in separating human and equine estrogens with OV-225 and Silar 10C. The latter workers compared the resolution of estrogens derivatized as trimethylsilyl ethers, methyloxime trimethylsilyl ethers, *tert.*-butyl dimethylsilyl ethers and heptafluorobutyrate derivatives and concluded that the methyloxime trimethylsilyl derivative gave the most useful separation. Adlercreutz and co-workers used either TMS ethers or latterly ethyloxime ethers [26,196].

Adlercreutz and co-workers have published extensively on the GC and GC—MS profiling of urinary estrogens. Important references are cited in a review [7]. As for neutral steroids, two basic methodologies can be employed: (1) quantification of total excretion of individual metabolites or (2) separate measurement of all components in each conjugate fraction.

Their method for total estrogen analysis is illustrated in Fig. 15. The first steps of this procedure have been described in Section 3.2. DEAE-Sephadex A-25 (acetate) chromatography is included to remove organic acids which would later interfere in the gas chromatography. In this column estrogens are eluted gradually because they cannot exchange with the acetate of the anion exchanger while organic acids are retained. The steroid mixture is then fractionated on Sephadex LH-20 (5 × 0.5 cm I.D. columns). Using a solvent system of 6% methanol in toluene containing ascorbic acid as anti-autoxidant the first 10 ml of eluate contains estrogens of polarity range from estrone to 16-epi-

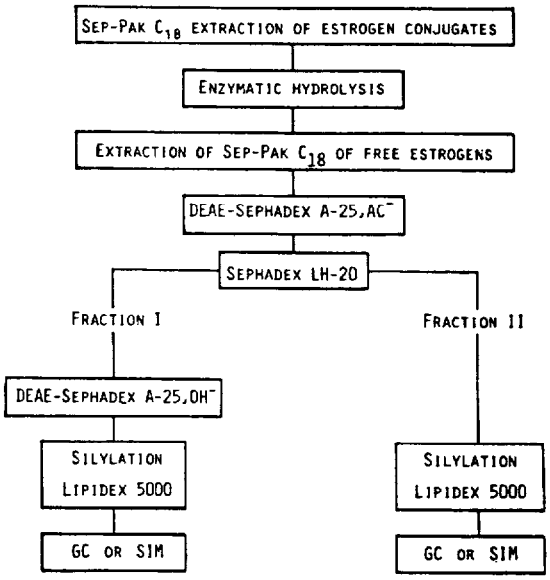

Fig. 15. Method for analysis of total urinary estrogens. From ref. 7, with permission.

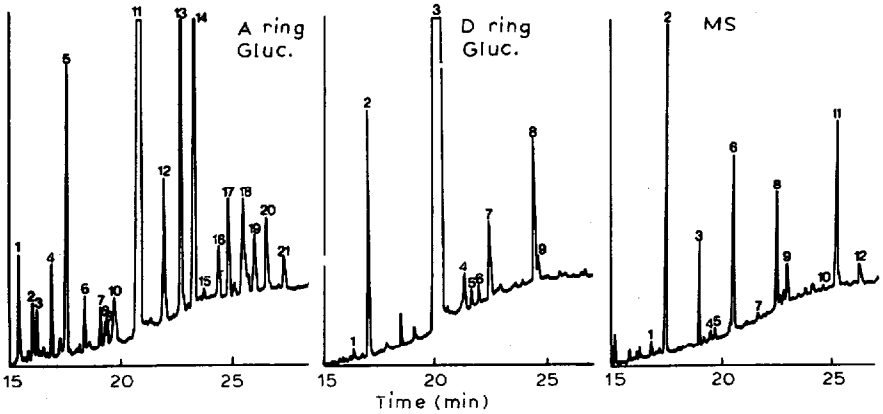

Fig. 16. Estrogens in pregnancy urine. The estrogens present in three conjugate fractions (A and D ring glucuronides and monosulfates) were analysed on an OV-210 capillary column as TMS ethers. The numbering system differs for each panel, the key to this system being given in Table 12. The conjugate fractions were separated by the DEAE-Sephadex anion-exchange method of Fotsis et al. [26]. From ref. 26, with permission.

estriol; thereafter the solvent system is changed to 30% methanol in toluene and a second 10 ml of eluate contains steroids of polarity from estriol to 15-hydroxyestriol. Prior to GC analysis the first fraction has to be subjected to anion-exchange chromatography to remove the neutral steroids, which would interfere with the chromatography.

The comprehensive analysis of urinary estrogens is achieved by separation prior to hydrolysis of the unconjugated steroids from the various forms of conjugates. This method was described in Section 3.2. Many of the steroids excreted in pregnancy urine have been quantified using this technique (Fig. 16) and the values obtained for one individual are listed in Table 12.

6.2. Plasma

Some of the earliest clinical investigations using SIM GC—MS were those of Adlercreutz et al. [197] on estrogens. Studies from their laboratory have continued and the latest paper on the subject is that of Fotsis et al. [196]. In this study, ion-exchange methodology has been employed with final detection and quantification by SIM. Particular care was taken to protect labile estrogens by derivatization and through the continual presence of antioxidants during the work-up procedure. In fact, ethoximation was the first step and was carried out before Sep-Pak extraction. This was undertaken to protect the D-ring α-ketolic estrogens. They suggested that their current technique is the first practical method covering essentially all estrogens of biological interest. Some of the SIM results (less polar estrogen fraction) are illustrated in Fig. 17. For several steroids internal standards that were labeled with a stable isotope in the derivative moiety were added to aid quantification. The mean excretions of the compounds measured in one woman are given in Table 13.

Axelson and Sjövall [71] described the separation of unconjugated plasma estrogens from unconjugated neutral steroids by TEAP-LH-20 chromatography

TABLE 12

ESTROGENS IN THREE CONJUGATE FRACTIONS OF ONE SAMPLE OF LATE PREGNANCY URINE (μg PER 24 h)

From Fotsis et al. [26].

No.*	A-ring glucuronide	μg per 24 h	No.*	D-ring glucuronide	μg per 24 h	No.*	Monosulfates	μg per 24 h
1	11-Dehydroestradiol-17α	374.2	1	Estradiol-17β	52.0	1	Estradiol-17β	51.3
2	Estradiol-17β	245.2	2	Cholestane (I.S.)		2	Cholestane (I.S.)	
3	Cholestane (I.S.)**		3	Estriol	20847.0	3	2-Methoxyestradiol-17β	46.8
4	2-Hydroxyestradiol-17β	149.8	4	2-Hydroxyestriol	209.3	4	Estriol	696.8
5	15α-Hydroxyestradiol-17β	141.3	5	4-Hydroxyestriol	253.0	5	16-Epiestriol	40.5
6	Estriol	6880.3	6	16α-Hydroxyestrone	740.9	6	Estrone	494.8
7	16-Epiestriol	788.1	7	15α-Hydroxyestriol	946.6	7	16α-Epiestriol	40.5
8	Estrone	2069.6				8	2-Hydroxyestrone	72.5
9	16α-Hydroxyestrone	2486.6				9	16-Oxoestradiol-17β	1000.5
10	2-Methoxyestriol	60.9				10	2-Methoxyestrone	186.1
11	16β-Hydroxyestrone	297.4						
12	2-Hydroxyestrone	522.6						
13	16-Oxoestradiol-17β	822.5						
14	2-Methoxyestrone	549.1						
15	15α-Hydroxyestrone	344.1						

*The numbering system refers to the peaks marked in Fig. 16. Numbers not listed are of unidentified estrogens.
**I.S. = internal standard.

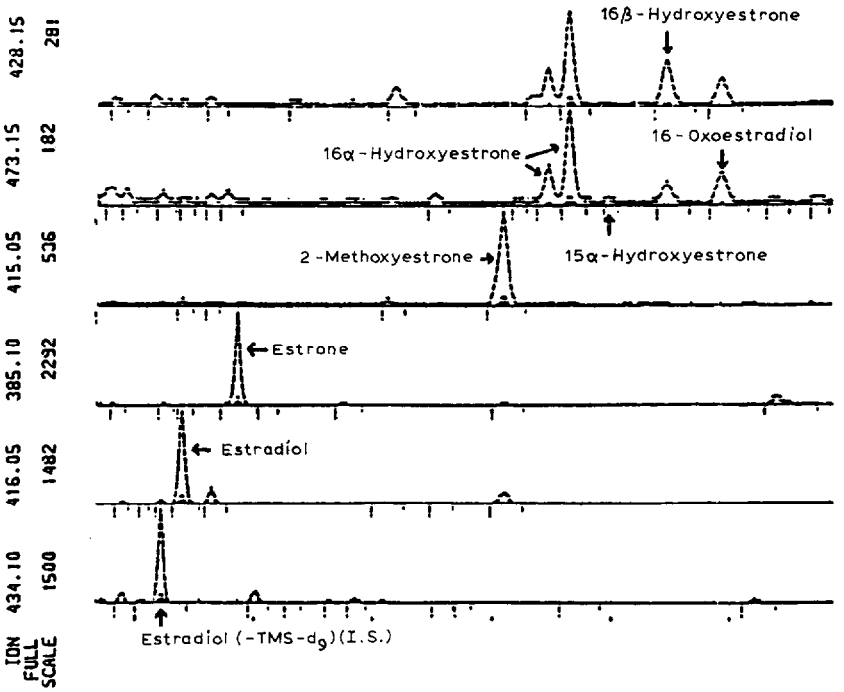

Fig. 17. Analysis of urinary estrogens (as TMS and EO-TMS derivatives) of a non-pregnant female by SIM GC—MS. The fraction analysed contains the less polar estrogens. Estradiol derivatized with labeled silylating reagent was used as internal standard. From ref. 196, with permission.

TABLE 13

EXCRETION OF ESTROGENS IN THE URINE OF A NORMALLY CYCLING WOMAN IN THE FOLLICULAR AND LUTEAL PHASES OF THE MENSTRUAL CYCLE (μg PER 24 h)

From Fotsis et al. [196].

Steroid	Days of cycle	
	5—7	19—20
Estradiol	3.5	10.6
Estrone	5.9	21.1
2-Methoxyestrone	1.8	8.7
16α-Hydroxyestrone	1.5	7.9
15α-Hydroxyestrone	0.4	0.6
16β-Hydroxyestrone	0.9	3.5
16-Oxoestradiol	1.1	3.5
2-Hydroxyestrone	13.9	50.0
4-Hydroxyestrone	2.8	4.5
2-Hydroxyestradiol	1.0	6.3
Estriol	1.5	13.6
16-Epiestriol	0.5	0.7
17-Epiestriol	0.8	7.4

and subsequent analysis by GC and GC—MS. They published a capillary chromatogram obtained by pregnancy plasma analysis that was extremely clean and contained derivatives of estrone (11.1 ng/ml), estradiol (25.8 ng/ml), estriol (16.8 ng/ml), 16α-hydroxyestrone (ca. 1 ng/ml), 16-ketoestradiol (ca. 2 ng/ml) and 2-methoxyestrone (<0.2 ng/ml).

7. PROFILING OTHER MEDIA

7.1. Amniotic fluid

7.1.1. Unconjugated steroids

Sippell et al. [198] carried out the most comprehensive analysis of hormonal steroids in amniotic fluid using the Sephadex LH-20 method (Section 4.1.1.). They analysed 70 samples and found the concentrations listed in Table 14. Values obtained at different periods of gestation were averaged. The concentrations of most steroids increased rapidly to about the 36th week and decreased thereafter.

Forest et al. [199] measured fourteen steroid hormones and precursors in mid-pregnancy amniotic fluid (Table 13). These include most steroids of the Δ^5 and Δ^4 pathways leading from pregnenolone to cortisol, testosterone and estradiol. Separation was achieved either by Sephadex LH-20 chromatography (for cortisone and cortisol) using the method of Sippell et al. [40] or by Celite chromatography. Quantification of the steroids was achieved by specific radioimmunoassay. One interesting finding was that in congenital adrenal hyperplasia (CAH, 21-hydroxylase defect), the levels of 17α-hydroxyprogesterone and androstenedione were considerably elevated. Hence the method could be used for antenatal diagnosis of the condition.

TABLE 14

UNCONJUGATED NEUTRAL STEROIDS IN AMNIOTIC FLUID (ng/ml)

Steroid	14—16* (M + F)	14—21** (M)	14—21** (F)	36—38* (M + F)	Term* (M + F)
Testosterone		0.23	0.05		
Androstenedione		0.66	0.39		
Δ^5AD		0.60	1.01		
DHA		0.21	0.27		
Pregnenolone		1.54	1.87		
Progesterone	14.70	46.4	47.20	32.40	19.20
17-Hydroxyprogesterone	1.63	0.99	1.21	3.80	1.60
17-Hydroxyprogesterone		1.16	1.57		
DOC	0.44			3.50	0.51
Corticosterone	1.49			4.60	2.35
Substance S	0.51			6.00	1.14
Cortisol	5.96	4.66	4.97		
Cortisone		15.00	17.37		
Aldosterone	0.04			0.53	0.27

*14—16 week pregnancies, from Sippell et al. [198]. Sephadex LH-20 chromatography and RIA.

**14—21 week pregnancies; M, $n = 35$; F, $n = 28$. From Forest et al. [199]. Celite chromatography and RIA.

Pang et al. [200] measured most of the same steroids in mid-term amniotic fluid from 77 normal pregnancies and 8 pregnancies at risk for CAH. In agreement with the data of Forest et al. [199], elevated 17-hydroxyprogesterone and androstenedione were seen in all samples associated with fetuses subsequently shown to have CAH. Other steroids measured were either elevated or in the upper normal range in affected pregnancies.

7.1.2. Steroid sulfates and glucuronides

The sulfated steroids present in amniotic fluid were studied many years ago

TABLE 15

STEROID SULFATES AND GLUCURONIDES IN TERM AMNIOTIC FLUID (μg/l)

Steroid	Conjugate*	Study**					
		A	B	C	D	E	F
DHA	MS	11					
5-Androstene-3β,17α-diol	DS			236	224		
5-Androstene-3β,17β-diol	DS			56	42		
5-Androstene-3β,16α,17β-triol	MS			38			
16α-Hydroxy-DHA	MS, DS	600	408	46	48	1020	146
16-Oxoandrostenediol	MS, DS	214	189	62	6	1100	46
16β-Hydroxy-DHA	DS		193	5	88		
16α-Hydroxypregnenolone	MS		120				46
21-Hydroxypregnenolone	DS		198	122			
5α-Pregnane-3α,20α-diol	G	164		36			263
5-Pregnene-3β,20α-diol	G, DS			29	22		
5α-Pregnane-3β,20α-diol	G, DS			49	38		
3α,21-Dihydroxy-5α-pregnan-20-one	DS			18			
5-Pregnene-3β,17α,20α-triol (Δ^5PT)	MS, DS			36	26		
Pregnanetriol (PT)	G						45
5-Pregnene-3β,20α,21-triol	MS, DS			12			
Estrone						2.3—4.0	
Estradiol						0.8—1.9	
Estriol		674				885—1470	163
2-Methoxyestrone						0.1—0.3	
16α-Hydroxyestrone						18—46	
16β-Hydroxyestrone						31	
16-Oxo-estradiol						16—53	
16-Epiestriol						5—17	
17-Epiestriol						1.6—4.2	
15α-Hydroxyestrone						7	
15α-Hydroxyestriol						6	

*Major conjugates (MS, monosulfate; DS, disulfate; G, glucuronide).

**(A) Schindler and Ratanasopa [209]. *Helix pomatia* hydrolysis with TLC and paper chromatographic fractionation. GC determination. Mean (n=29). (B) Mitchell and Shackleton [208]. *Helix pomatia* hydrolysis and solvolysis. TLC with colour reaction. Mean (n=28). (C) Jänne and Vihko [203]. Conjugate separation, solvolysis and measurement of disulfates only by GC. Pooled amniotic fluid. (D) Luukainen et al. [201]. Conjugate separation, solvolysis and measurement of disulfates by GC. Pooled amniotic fluid. (E) Siegel et al. [204]. Gel filtration (Sephadex G-25). *Helix pomatia* hydrolysis. Purification by silicic acid and paper chromatography GC determination. Pooled amniotic fluid. (F) Peltonen et al. [207]. XAD-7 total steroid extraction, then free steroid extraction followed by specific β-glucuronidase hydrolysis and extraction. Finally *Helix pomatia* hydrolysis. Silicic gel purification and capillary GC quantification. 20 samples.

following fractionation into monosulfates and disulfates by Sephadex LH-20 chromatography [201–204]. Profiling was carried out by packed column GC and GC—MS. Compared with other body fluids amniotic fluid is of interest because steroid disulfates predominate. Luukainen et al. [201] and Jänne and Vihko [203] found that only monosulfated 16α-hydroxy-DHA was detectable (3.9 μg per 100 ml), while a whole series of disulfates could be quantified (Table 15). Homoki et al. [205] used capillary column chromatography to analyse free steroid sulfates and steroid glucuronides in amniotic fluid collected between the 15th and 17th weeks of gestation. More than 40 steroids were detected and the results were compared with those obtained by other workers. The latter study was carried out to provide a database for the possible use of mid-term amniotic fluid in the diagnosis of enzyme disorders. Peltonen and co-workers [206,207] also published extensive data on the sulfates and glucuronides present in amniotic fluid at term. Mitchell and Shackleton [208] and Schindler and Ratanasopa [209] published data on the excretion of steroid conjugates in amniotic fluid measured by other profiling techniques.

7.2. Breast cyst fluid

Vanluchene et al. [210] applied capillary GC profiling techniques to the measurement of steroid sulfates in breast cyst fluid. The endogenous androgen sulfates of the cyst fluids were different from those in blood, which suggested intracystic metabolism of blood-borne precursors. In particular, greater amounts of 5α-reduced steroids were found in breast cysts; one in particular, 5α-androstane-3α,17β-diol, has a concentration 2000 times greater than in blood. A gas chromatogram of steroid sulfates obtained by the investigators is shown in Fig. 17 and clearly shows an inverse relationship of DHA and epiandrosterone compared with blood. The cleanliness of the chromatograms is impressive considering that no conjugate fractionation was carried out. Free steroids and steroid conjugates were extracted from the breast cyst fluids by Sep-Pak cartridges. The dried extract was partitioned between a water phase and ethyl acetate to allow the recovery of free steroids. Steroid sulfates were obtained by extracting the aqueous phase at pH 1 with ethyl acetate. Solvolysis was achieved by incubating the ethyl acetate at 37°C for 16 h. Results of the quantitative analysis of the steroid sulfates are given in Table 16.

7.3. Tissue steroids, testis and adrenal

Ruokonen and co-workers carried out detailed studies on the endogenous and secreted steroids of the human testis [211,212]. The main purpose of these investigations was to investigate the role of steroid sulfates in steroidogenesis in this organ. Unconjugated steroids were extracted from tissue homogenates and fractionated by the Lipidex 5000 procedure (see above). Steroid sulfates were solvolysed with acidified ethyl acetate and were also fractionated by Lipidex chromatography. Individual steroids were quantified by specific immunoassays. Table 17 lists the mean concentrations of steroids and steroid sulfates in twelve samples of testis tissue from patients with prostatic cancer.

In an earlier study, Ruokonen reported the testicular concentrations of three Δ^{16}-steroids [212]. The purpose of the investigation was to assess the role of the human testis in the production of these steroids. The steroids were measured using gas chromatography and were identified by combined GC—MS. The testicular samples were obtained from three cadavers aged 24—41 years. The concentrations of the unconjugated and sulfated steroids are listed in Table 17.

Further studies on the concentrations of testosterone and some of its precursors and metabolites in human epidymis and testis were reported by Hammond et al. [213] and Leinonen et al. [214].

Huhtaniemi [215] studied steroidogenesis and its trophic regulation by human fetal adrenal and testis. These organs were obtained from fetuses obtained following pregnancy termination at mid-pregnancy. The endogenous steroids were extracted from organ homogenates by acetone—ethanol precipitation and were subjected to conjugate fractionation by Sephadex LH-20

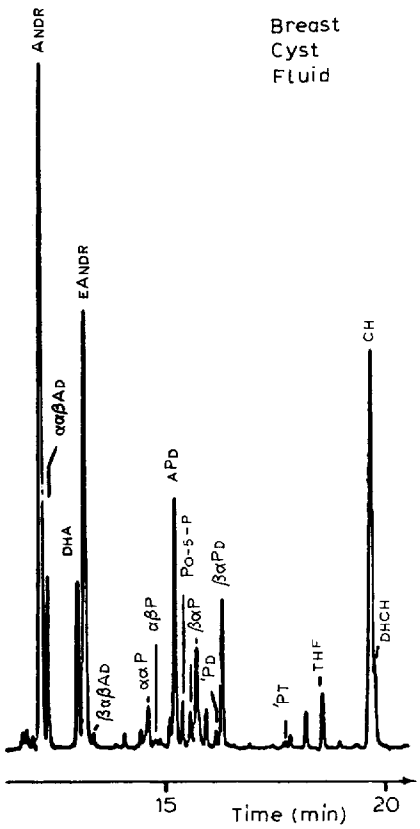

Fig. 18. Breast cyst fluid: GC separation of MO-TMS and TMS derivatives of steroid sulfates. The amount injected corresponds to 1 μl of cyst fluid. Abbreviations: Andr., androsterone; $\alpha\alpha\beta$AD, 5α-androstane-3α,17β-diol; Etio, etiocholanolone; eAndr, epiandrosterone; $\beta\alpha$AD, 5α-androstane-3β,17β-diol; Ad, 5-androstene-3β,17β-diol; $\alpha\alpha$P, 3α-hydroxy-5α-pregnan-20-one; $\alpha\beta$P, 3α-hydroxy-5β-pregnan-20-one; $\beta\alpha$P, 3β-hydroxy-5α-pregnan-20-one; αPD, 5α-prenane-3α,20α-diol; Pd, 5β-pregnane-3α,30α-diol; $\beta\alpha$Pd, 5α-pregnane-3β,20α-diol; 5P, pregnenolone, Pd, 5-pregnene-3β,20α-diol; Pt, 5-pregnene-3β,17α,20α-triol; Ch, cholesterol; DHCH, 3α-hydroxy-5β-cholestane. From ref. 210, with permission.

chromatography. Analysis of steroids was carried out by GC and GC–MS. The concentrations of steroids found in these organs are reported in Table 18. It was of particular interest that no 3-oxo-4-ene steroids could be found in adrenal tissue at this stage of gestation, suggesting that the formation of sulfated 3β-hydroxy-5-ene steroids quantitatively plays a dominant role in fetal adrenal steroidogenesis.

TABLE 16

CONCENTRATIONS OF STEROID SULFATES IN BREAST CYST FLUID (BCF) AND IN SERUM (μg/ml)

From Vanluchene et al. [210].

Steroid	BCF (mean, $n=16$)*	Serum
Androsterone	32.4	0.39
Epiandrosterone	22.1	0.16
5α-Androstane-3α,17β-diol	17.7	<0.08
5α-Androstane-3β,17β-diol	2.0	<0.06
DHA	19.0	1.1
5-Androstene-3β,17β-diol (Δ^5 AD)	0.8	0.07
3α-Hydroxy-5α-pregnan-20-one	2.8	<0.06
3α-Hydroxy-5β-pregnan-20-one	0.6	<0.06
3β-Hydroxy-5α-pregnan-20-one	3.5	<0.06
5α-Pregnane-3α,20α-diol	5.4	<0.06
5β-Pregnane-3α,20α-diol	1.5	<0.06
5α-Pregnane-3β,20α-diol	3.5	<0.06
Pregnenolone	5.6	0.07
5-Pregnene-3β,20α-diol (Δ^5PD)	2 1	0.14
5-Pregnene-3β,17α,20α-triol (Δ^5PT)	7.3	<0.06

*Eight patients in follicular phase, eight in luteal phase.

TABLE 17

MEAN CONCENTRATIONS OF ENDOGENOUS STEROIDS AND STEROID SULFATES IN SAMPLES OF HUMAN TESTICULAR TISSUE (ng/g WET WEIGHT)

Steroid	Concentration
Pregnenolone	744 ± 177*
Progesterone	60 ± 14
17-Hydroxyprogesterone	342 ± 30
DHA	130 ± 21
Testosterone	837 ± 81
Pregnenolone sulfate	723 ± 303
DHA sulfate	639 ± 216
Δ^5AD sulfate	266 ± 94
Testosterone sulfate	244 ± 103
5α-Androst-16-en-3α-ol	6–10**
5α-Androst-16-en-3α-ol sulfate	5–6
5,16-Androstadien-3β-ol	1066–180
5,16-Androstadien-3β-ol sulfate	80–185
5α-Androst-16-en-3β-ol	161–371
5α-Androst-16-en-3β-ol sulfate	38–174

*Data from Ruokonen and Vihko [211]. Mean ± range ($n=12$).
**Data from Ruokonen [212]. Range ($n=4$).

TABLE 18

ENDOGENOUS NEUTRAL STEROIDS (μg PER 100 g WET WEIGHT) IN HUMAN FETAL ADRENAL AND TESTIS

From Huhtaniemi [215]. Acetone—ethanol extraction, solvolysis, GC quantitation.

Steroid	Fetal adrenal	Fetal testes
Unconjugated:		
Pregnenolone	180	180
17-Hydroxypregnenolone	130	—
Testosterone	—	170
Androstenedione	—	18
Monosulfates:		
DHA	130	45
16α-Hydroxy-DHA	63	66
Pregnenolone	500	24
17-Hydroxypregnenolone	130	—
Δ^5PD	—	22
Disulfates:		
Δ^5AD (17α)	—	3
Δ^5AD (17β)	—	2

An excellent, although now becoming dated, review of the extraction and HPLC of steroids and adrenal and testicular cells and tissue was published by O'Hare and Nice [20]. It is particularly valuable as it discusses the principles of HPLC at length and presents detailed methods for the quantitative extraction of steroids. The steroids discussed include the most difficult to analyse, e.g., 18-hydroxylated corticosteroids and aldosterone.

7.4. Bile and feces

Almost all the steroid profiling of these materials has been carried out by the Swedish and Finnish groups using GC and GC—MS [216—221]. Most of the data were obtained in the late 1960s before the advent of capillary GC in steroid profiling. The studies illustrated the little appreciated fact that substantial amounts of steroids were eliminated by biliary excretion, particularly during pregnancy [219] and in the newborn period [220,221].

8. GENERAL DISCUSSION

Naturally, there is no single profiling technique available for measuring the full complement of steroids of normal and pathophysiological interest. For the routine batch analysis of non-polar unconjugated steroids mainly of gonadal origin, Lipidex and Celite chromatography with RIA quantification are probably the methods of choice. For more polar steroids Sephadex LH-20 remains supreme. Under normal-phase conditions Lipidex retains polar steroids excessively and Celite with ethylene glycol as the stationary phase is prone to excessive bleeding when polar eluting solvents are used. Although HPLC (with RIA quantification) can carry out the analytical functions of the above procedures with greatly improved resolution, it has the disadvantage that batch processing is not possible. However, with an autosampler and automatic frac-

tion collection the instruments can be run on a 24 h basis. Further drawbacks of HPLC are the initial cost of the instrument and the possibility of malfunction, important considerations in a clinical setting. Results produced by the above profiling techniques are widely used by endocrinologists in their routine investigations.

Whereas the GC—MS literature is replete with references on methods suitable for the measurement of unconjugated plasma steroids, the technique is not used on a routine basis. However, it is pre-eminent in research situations for profiling unselected steroids in blood, other body fluids and tissues.

For conjugated steroids, GC and GC—MS are the methods of choice and are now commonly used in profiling. For most components GC alone is sufficient but in a clinical situation secondary analysis by GC—MS must be utilized for assaying the minor but important hormone metabolites such as tetrahydroaldosterone. The development of GC—MS has finally reached a point where the technique can be routinely used in clinical laboratories. Two companies (Hewlett-Packard and Finnigan) now produce instruments for around $50 000 that are capable of providing the sensitivity required for measuring steroid hormones and their metabolites. These instruments can be rapidly calibrated and easily used by novices. Through these advances, GC—MS may yet become the technique of choice even for the measurement of circulating levels of hormonal steroids.

The adaption of gas-phase methods for assessing steroid hormone homeostatis has been a painfully slow process. To some extent the techniques have been retarded by technical and reliability problems and through the competition afforded by the coincident development of RIA. However, clinical use of GC profiling is here to stay and in future years we shall see its widespread adoption as a parallel technique to RIA. I believe that the major growth area will be in the accurate detection of some of the mild defects affecting steroid synthesis and metabolism. One example is the detection of late-onset CAH, a disorder that frequently gives rise to female hirsutism. Detection of this disorder by RIA requires blood sampling for 17α-hydroxyprogesterone analysis before and after ACTH stimulation, a time-consuming process for patient and medical personnel. In contrast, this disorder is easily detected by measuring appropriate steroid ratios in random urine samples (see Figs. 13 and 14). We shall see more of this type of application reported as more profiles from individuals with similar disorders are catalogued and the specificity of the tests is confirmed. It is of interest that to date the only disorder affecting steroid synthesis that we have not been able to distinguish by urinary steroid profiling is 17β-hydroxysteroid dehydrogenase deficiency, but this may only be because we have yet to determine the correct analytes for producing diagnostic ratios.

Although GC and GC—MS currently have supremacy in steroid metabolite profiling, the use of HPLC (with direct detection) may increase beyond our expectations. The problems are severe but I find the profiles that Novotny and co-workers obtained in 1983 [105] by fused-silica capillary HPLC so similar to their 1970 profiles that began the capillary era in steroid GC [111]. Thermospray HPLC—MS is beginning to be used for unconjugated and conjugated steroids, and who can predict where it may lead? Once again one only

has to look at the achievements of GC—MS since the first profiles of plasma steroids were produced by Sjövall and Vihko in 1966 [222]. However, before we succumb entirely to analysis by complex instrumentation, I think we should question whether it is the optimal method for every situation. In recent discussions in Copenhagen, Meta Nielsen reminded me that we should be careful not to discard good "old" methods in our striving after modern technology. Nielsen probably has more experience than anyone in the use of all forms of chromatography (including capillary GC) in routine clinical steroid profiling, yet she still uses a rapid (2—3 h) TLC method with visual assessment of color reactions for profiling urinary steroids in infants suspected of having steroid disorders.

9. SUMMARY

This paper reviews techniques utilized in the profiling of steroids in body fluids and tissues. Methods for profiling plasma unconjugated steroids and urinary steroid metabolites are focused on. Concentrations or levels of excretion of a variety of steroids have been documented and reviewed. The importance of profiling techniques in the study of normal and pathophysiology of hormonal steroids is discussed.

10. APPENDIX

10.1. Steroid profiling methods used in the author's laboratory

10.1.1. Urine method

Extraction and hydrolysis: C_{18} cartridge primed with 5 ml of methanol and 5 ml of water. Urine (30 ml) centrifuged and extracted; cartridge washed with 5 ml of water and steroids eluted with 4 ml of methanol. Methanol extract dried, dissolved in 3 ml of 0.1 *M* acetate buffer (pH 4.6) to which are added 25 mg of Sigma type HL enzyme preparations (7500—10 000 units of β-glucuronidase, 625—1000 units of sulfatase). Hydrolysis proceeds for 3 h at 55°C. Steroids extracted by C_{18} cartridge as above. Extract stored in a 4 ml vial with a PTFE-lined cap.

10.1.2. Derivatization

10.1.2.1. Methyloxime and benzyloxime trimethylsilyl ethers. Methanol extract (500 μl) placed in a 5-ml tube with plastic screw cap and PTFE liner, then 2.5 μg of each of three standards (5α-androstane-3α,17α-diol, stigmasterol and cholesteryl butyrate, pre-mixed) added. Sample dried and 3 drops of oxime reagent added (2% methoxyamine hydrochloride or benzoxyamine hydrochloride in pyridine). Derivatization at 60°C for 1 h. Pyridine blown off and 3 drops of trimethylsilylimidazole added. Derivatization proceeds for 15 h at 100°C (overnight). Lipidex chromatography: Pasteur pipette plugged with glass-wool and two-thirds filled with Lipidex in cyclohexane. Column washed with 3 ml of cyclohexane, then 1 ml of cyclohexane [containing about 0.2% of hexamethyldisilazone (HMDS)] added to sample. Sample put on column. Eluate collected; additional 2 ml of solvent added to column and collected. Cyclohexane eluate concentrated to 200 μl, 2 μl injected into gas chromatograph (equivalent to 25 ng of each internal standard).

10.1.3. Gas chromatographic conditions

Column: Foxboro-Analabs, GB-1 fused-silica column (25 m × 0.25 mm I.D.). Injection temperature, 50°C; initial time, 3 min. Splitless injection with split opening at 3 min. Rapid temperature increase (30°C/min) to 210°C, thereafter at 3°C/min to 325°C.

10.1.4. Integration of peaks

Peak areas are measured relative to the androstanediol and stigmasterol internal standards for those compounds eluting between them, and relative to SS and CB internal standards for the later eluting steroids. Daily, the integrator (Shimadzu GP3) is calibrated (to allow for differing response factors) by analysis of mixtures of most steroids quantified.

Quantification is achieved by incorporation of factors into the integrator based on the aliquot of a 24 h sample analysed. In most instances, when only spot urines are analysed, the factor is calculated assuming a daily excretion of 1500 ml for an adult.

10.1.5. Standard mixtures for integrator calibration

101.1.5.1. For MO-TMS ether (in order of elution). AD (internal standard), An, Et, DHA, Δ^5AD, 11-oxo-Et, 11β-hydroxy-An, 11β-hydroxy-Et, PD, PT, THS, PT'one, THE, THB, 5α-THB THF, 5α-THF, α-cortolone, β-cortolone, α-cortol, SS and CB.

10.1.5.2. For BO-TMS ether (in order of elution). AD (internal standard), AD (3α,5β,17β), Δ^5AD (17α and 17β), PD, PT, Δ^5PD, Δ^5PT, PT'one, α-cortolone, β-cortolone, α-cortol, Et, AN, stigmasterol, 11-oxo-Et, 11β-hydroxy-Et, 11β-hydroxy-An, 17HP, CB, THE.

10.1.6. Specialized GC—MS SIM methods

10.1.6.1. Tetrahydroaldosterone and 18-hydroxytetrahydro-Compound A determination. Derivatization as for regular MO derivative with the following changes: (1) 250 ng of 3β,5α-THAldo and 3β,5α-THE* added as internal standards, (2) following oxime formation, 3 h silylation is sufficient. Prior to GC—MS analysis the sample is transferred into 100 μl conical derivative vials. GC—MS is carried out on an HP 5970 mass-selective detector. The following ions are monitored (Fig. 12): *m/z* 506, M−103 THAldo and 3β,5α-THAldo; *m/z* 609, M^+ and M−31 for 18-hydroxy-THA and 3β,5α-THE; *m/z* 457 (M−152) for 18-hydroxy-THA; *m/z* 394 (M−90) for stigmasterol. Peak areas of THAldo and 18-hydroxy-THA related to peak areas of internal standards. Corrected for responsive factors determined by analysis of standards alone.

10.1.6.2. THS and THDOC. Urinary THS can be measured by SIM using as reference endogenous THB and 5α-THB, which have the same M^+ (*m/z* 595) and M−31 (*m/z* 564). In contrast to THS, the latter steroids produce clean peaks by regular GC and are routinely measured by this method. The THS values are corrected by employing appropriate response factors determined against THB and 5α-THB daily. An alternative approach would be to use 3β,5α-THS as internal standard.

*3β,5α-18-Hydroxy-THA now used as internal standard.

THDOC can be determined by using 3β,5α-DOC as internal standard and monitoring *m/z* 507 and 476 ions.

10.1.6.3. Unconjugated cortisol metabolites. The 11α-hydroxy epimer of cortisol and 6β-hydroxyprednisolone added as internal standards to unhydrolysed urine samples. Following extraction by Sep-Pak the MO-TMS derivatives can be prepared directly. The following ions are monitored: *m/z* 724 (M^+) for 6β-hydroxycortisol; *m/z* 693 (M−31) for 6β-hydroxycortisol; *m/z* 722 (M^+) for 6β-hydroxyprednisolone; *m/z* 691 (M−31) for 6β-hydroxyprednisolone; *m/z* 636 (M^+) for cortisol and 11α-cortisol; *m/z* 605 (M−31) for cortisol and 11α-cortisol; *m/z* 476 (M−205) for 20α- and 20β-dihydrocortisol; and *m/z* 394 (M−90) for stigmasterol. Addition of prednisone as internal standard allows the additional determination of cortisone (*m/z* 652, 650, 631 and 629 monitored).

Semi-quantitative analysis of 18-hydroxycortisol MO-TMS ether can be achieved by SIM analysis of the base peak (*m/z* 385) in the mass spectrum relative to ions 484 and 368 of stigmasterol and cholesteryl butyrate, respectively. 18-Hydroxycortisol gives two major GC peaks eluting before and after cholesteryl butyrate. Derivatization and analysis of reference 18-hydroxycortisol must be carried out at the same time as urinary assays to minimize problems associated with variable derivatization. The steroid is measured prior to hydrolysis of urinary steroid conjugates.

10.1.7. Plasma steroid sulfate method

Plasma (1 ml) is dripped into 20 ml of acetone–ethanol (1:1) in a 30 ml centrifuge tube and sonicated for 20 min. The extract is cooled at −20°C for 30 min, centrifuged and the supernatant decanted into 100 ml round-bottomed flasks. The pellet is re-extracted with 10 ml of solvent and treated in the same way. Once dried by rotary evaporation, the extract is fractionated by chromatography on 4 g columns of Sephadex LH-20. The solvent system used is methanol—chloroform (1:1), salt saturated. The sample is applied in 2 × 2.5 ml portions and a further 25 ml is added to the column reservoir. All the initial eluate is collected and comprises the "free + glucuronide" fraction. A further 35 ml is added to the reservoir and when the eluate is collected it comprises the "monosulfate fraction". The disulfated steroids are recovered by elution of the column with 50 ml of methanol. Once the fractions have been dried by rotary evaporation they are dissolved in water for desalting by a Sep-Pak C_{18} cartridge. Half of the monosulfate and all of the disulfate fractions are solvolysed by addition of 3 ml of ethyl acetate and 10 μl of 4 *M* sulfuric acid. The solvolysis is allowed to proceed for 1 h at 40°C. The three internal standards (1 μg) are added and the solvolysis mixture is washed with 1 ml of 1 *M* sodium hydroxide solution, 1 ml of water and dried with anhydrous sodium sulfate. MO-TMS ethers are prepared as described for the urine method.

10.1.7.1. Standard mixture for plasma steroid sulfates calibration. AD (internal standard), Δ^5AD (17α), DHA, epiAn, Δ^5AD (17β), 16α-hydroxy-DHA, pregnenolone, Δ^5PD, AT, 17α-hydroxypregnenolone, Δ^5PT, 21-hydroxypregnenolone, 5-pregnene-3β,20α,21-triol, cholesterol, SS (internal standard) and CB (internal standard).

10.2. Steroid abbreviations

Androsterone (An), 3α-hydroxy-5α-androstan-17-one; etiocholanolone (Et), 3α-hydroxy-5β-androstan-17-one (also 11β-hydroxy derivatives); 3β-hydroxy-5-androsten-17-one (DHA); androstenetriol (AT), 5-androstene-3β,16α,17β-triol; pregnanediol (PD), 5β-pregnane-3α,20α-diol; pregnanetriol (PT), 5β-pregnane-3α,17α,20α-triol; pregnanetriolone (PT'one), 5β-pregnane-3α,17α, 20α-triol-11-one; androstenediol (Δ^5AD), 5-androstene-3β,17β-diol; pregnenediol (Δ^5PD), 5-pregnene-3β,20α-diol; pregnenetriol (Δ^5PT), 5-pregnene-3β,17α, 20α-triol; 21-hydroxy-4-pregnene-3,20-dione and tetrahydro derivative (DOC and THDOC, respectively); substance S, 11-deoxycortisol and tetrahydro derivative (THS); compound A, 11-dehydrocorticosterone and tetrahydro derivative (THA); compound B, corticosterone and tetrahydro derivatives (THB, 5α-THB); compound E, cortisone and tetrahydro derivative (THE); compound F, cortisol and tetrahydro derivatives (THF, 5α-THF); THAldo, tetrahydroaldosterone; 18-hydroxy B, 18-hydroxycorticosterone; 18-hydroxy-compound A, 18-hydroxy-11-dehydrocorticosterone (and tetrahydro derivative, 18-hydroxy-THA); SS, stigmasterol and CB, cholesteryl butyrate.

NOTES ADDED IN PROOF

Primary aldosteronism: Conn's syndrome or glucocorticoid suppressible hyperaldosteronism (addition to page 135)

Recent studies [231—234] have shown that excessive excretion of 18-hydroxycortisol and 18-oxocortisol are specific markers for differential diagnosis of primary aldosteronism.

Important analytes (μg/24 h)	Normal	Conn's adenoma	Glucocorticoid suppressible
18-Hydroxycortisol*	117 ± 67 (*n*=32)	409—946 (*n*=4)	985 and 2476 (*n*=2)
18-Hydroxycortisol**	—	—	195, 641 and 1354 (*n*=3)
18-Oxocortisol***	3.25 ± 1.98 (*n*=22)	11—17 (*n*=3)	25—55 (*n*=4)

*Ref. 233.
**Gutkin and Shackleton (unpublished results), two sons and father.
***Ref. 234.

Addition to page 145:

Finally, the reader must be directed to the most recent publication from Sjövall's group on analysis of steroids in rat testicular tissue [230]. The precise and methodical approach of this study has provided a landmark in development of GC—MS techniques and undoubtedly the best data on tissue steroid concentrations. The experimentation described in this paper should be used as the model for all interested in sensitive steroid quantification. The methodology described embodies use of many of the advances in C_{18} cartridge and derivatized Sephadex extractions, HPLC purifications and chemical derivatization (TBDMS derivatives) which have occurred over the last 20 years. The

resulting capillary GC—MS SIM recordings for individual steroids are the "cleanest" yet produced by low-resolution GC—MS for tissue steroid components. Particular attention was also paid to the choice of internal standards for all analytes, both for quantification and recovery determination.

11. ACKNOWLEDGEMENTS

I thank my colleagues Letu Pho and Esther Roitman for the production of some of the original data used in this review. I am indebted to Marion Douglass, Kathleen Hogue Gonzalez and Roxanne Short, whose patience with me, and their perseverance made the final preparation of the manuscript possible. This work was supported through a grant from NIH (AM34400).

REFERENCES

1 E.C. Horning, Gas phase analytical methods for the study of steroid hormones and their metabolites, in K.B. Eik-Nes and E.C. Horning (Editors), Gas Phase Chromatography of Steroids, Springer, New York, 1968, pp. 1—71.
2 C.H.L. Shackleton, A.L. Charro-Salgado and F.L. Mitchell, Clin. Chim. Acta, 21 (1968) 105.
3 J. Sjövall and M. Axelson, Vitam. Horm., 39 (1982) 31.
4 C.H.L. Shackleton, Analysis of steroids, in H. Jaeger (Editor), Glass Capillary Chromatography in Clinical Medicine and Pharmacology, Marcel Dekker, New York, 1985, p. 153.
5 C.H.L. Shackleton, N.F. Taylor and J.W. Honour, An Atlas of Gas Chromatographic Profiles of Neutral Urinary Steroids, in Health and Disease, Packard-Becker, Delft, 1980.
6 S.J. Gaskell, Methods Biochem. Anal., 29 (1983) 385.
7 H. Adlercreutz, T. Fotsis and R. Heikkinen, in S. Görög (Editor), Advances in Steroid Analysis, Proc. of the Symp., Eger, Hungary, May 20—22, 1981, Elsevier, Amsterdam, 1982, pp. 3—33.
8 H.L.J. Makin (Editor), Biochemistry of Steroid Hormones, Blackwell, London, 1985.
9 M.J. Tikkanen, J. Steroid Biochem., 4 (1973) 57.
10 J. Ahmed and A.E. Kellie, J. Steroid Biochem., 3 (1972) 31.
11 H.L. Bradlow, Steroids, 11 (1968) 265.
12 H.L. Bradlow, Steroids, 30 (1977) 581.
13 C.H.L. Shackleton and J.O. Whitney, Clin. Chim. Acta, 107 (1980) 231.
14 M. Axelson and B.-L. Sahlberg, J. Steroid Biochem., 18 (1983) 313.
15 E. Vanluchene, D. Vandekerckhove, J. Jonckheere and A. De Leenheer, J. Chromatogr., 279 (1983) 573.
16 R. Heikkinen, T. Fotsis and H. Adlercreutz, Clin. Chem., 27 (1981) 1186.
17 I. Huhtaniemi, J. Steroid Biochem., 8 (1977) 491.
18 M. Axelson, G. Schumacher and J. Sjövall, J. Chromatogr. Sci., 12 (1974) 535.
19 A. Hara and N.S. Radin, Anal. Biochem., 90 (1978) 420.
20 M.J. O'Hare and E.C. Nice, Analysis of Steroid Hormones in Adrenal and Testicular Cells and Tissues, in M.P. Kautsky (Editor), Steroid Analysis by HPLC. Recent Applications, Marcel Dekker, New York, Basle, 1981, pp. 277—322.
21 A.O. Ruokonen and R.K. Vihko, J. Androl., 4 (1983) 104.
22 J. Sjövall and R. Vihko, Acta Endocrinol., 57 (1968) 247.
23 O. Jänne, R. Vihko, J. Sjövall and K. Sjövall, Clin. Chim. Acta, 23 (1969) 405.
24 K.D.R. Setchell, B. Almé, M. Axelson and J. Sjövall, J. Steroid Biochem., 7 (1976) 615.
25 M. Axelson, B.-L. Sahlberg and J. Sjövall, J. Chromatogr., 224 (1981) 355.
26 T. Fotsis, P. Jarvenpaa and H. Adlercreutz, J. Steroid Biochem., 12 (1980) 503.
27 R. Hähnel, Clin. Chim. Acta, 7 (1962) 768.

28 H.L. Bradlow, The hydrolysis of steroid conjugates, in S. Bernstein and S. Solomon (Editors), Clinical and Biological Aspects of Steroid Conjugation, Springer, Berlin, 1970, pp. 131—181.
29 R.N. Beale, D. Croft and R.F. Taylor, Steroids, 13 (1969) 429.
30 P. Vestergaard, Acta Endocrinol., 217 (1978) 96.
31 S.R. Stitch and I.D.K. Halkerston, Biochem. J., 73 (1956) 710.
32 E. Vanluchene, W. Eechaute and D. Vandekerckhove, J. Steroid Biochem., 16 (1982) 701.
33 S. Burstein and S. Lieberman, J. Biol. Chem., 233 (1958) 331.
34 C.H.L. Shackleton, J.-Å. Gustafsson and J. Sjövall, Steroids, 17 (1971) 265.
35 J.L. Peltonen, T.L. Laatikainen and A. Hesso, J. Steroid Biochem., 10 (1979) 499.
36 A.B. Kellie and A.P. Wade, Biochem. J., 66 (1957) 196.
37 B.E.P. Murphy, Nature New Biol., 232 (1971) 22.
38 K.D.R. Setchell and C.H.L. Shackleton, Clin. Chim. Acta, 47 (1973) 381.
39 W.A. Golder and W.G. Sippell, J. Chromatogr., 127 (1976) 293.
40 W.G. Sippell, P. Lehmann and G. Hollmann, J. Chromatogr., 108 (1975) 305.
41 W.G Sippell, F. Bidlingmaier, H. Becker, T. Brunig, H. Dorr, H. Hahn, W. Golder, G. Hollmann and D. Knorr, J. Steroid Biochem., 9 (1978) 63.
42 J. Ellingboe, E. Nyström and J. Sjövall, J. Lipid Res., 11 (1970) 266.
43 J. Sjövall, E. Nystrom and E. Haahti, Liquid Chromatography on Lipophilic Sephadex: Column and Detection Techniques, in J.C. Giddings and R.A. Keller (Editors), Advances in Chromatography, Marcel Dekker, New York, 1968, pp. 119—170.
44 M. Axelson, G. Schumacher, J. Sjövall, B. Gustafsson and J.O. Lindell, Acta Endocrinol., 80 (1975) 149.
45 D. Apter, O. Jänne, P. Karvonen and R. Vihko, Clin. Chem., 22 (1976) 32.
46 G.L. Hammond, A. Ruokonen, M. Kontturi, E. Koshela and R. Vihko, J. Clin. Endocrinol. Metab., 45 (1977) 16.
47 G.L. Hammond, V. Ahonen and R. Vihko, Int. J. Androl., Suppl., 2 (1978) 391.
48 P. Leinonen, G.L. Hammond and R. Vihko, J. Clin. Endocrinol. Metab., 51 (1980) 423.
49 A. Ruokonen, O. Lukkarinen and R. Vihko, J. Steroid Biochem., 14 (1981) 1357.
50 P.K. Siiteri, Methods Enzymol., 42 (1973) 485.
51 M.G. Forest, Horm. Res., 7 (1976) 260.
52 J.E. Buster, R.J. Chang, D.L. Preston, R.M. Elashoff, L.M. Cousins, G.E. Abraham, C.J. Hobel and J.R. Marshall, J. Clin. Endocrinol. Metab., 48 (1979) 133.
53 J.E. Buster, R.J. Chang, D.L. Preston, R.M. Elashoff, L.M. Cousins, G.E. Abraham, C.J. Hobel and J.R. Marshall, J. Clin. Endocrinol. Metab., 48 (1979) 139.
54 M.G. Forest, E. DePeretti, A. Lecoq, E. Cadillon, M.-T. Zabot and J.-M. Thoulon, J. Clin. Endocrinol. Metab., 51 (1980) 816.
55 M.W. Capp and M.H. Simonian, Anal. Biochem., 147 (1985) 374.
56 M.H. Simonian and M.W. Capp, J. Clin. Endocrinol. Metab., 59 (1984) 643.
57 M.H. Simonian, P.J. Hafnsby, C.R. Ill, M.J. O'Hare and G.N. Gill, Endocrinology, 105 (1979) 99.
58 M.H. Simonian, M.L. White and G.N. Gill, Endocrinology, 111 (1982) 919.
59 M. Schöneshöfer, A. Fenner and H.J. Dulce, J. Steroid Biochem., 14 (1981) 377.
60 M. Schöneshöfer and B. Weber, J. Steroid Biochem., 18 (1983) 65.
61 M. Schöneshöfer and B. Weber, J. Clin. Chem. Clin. Biochem., 21 (1983) 231.
62 M. Schöneshöfer and B. Weber, Fresenius Z. Anal. Chem., 311 (1982) 426.
63 M. Schöneshöfer, A. Kage and B. Weber, Clin. Chem., 29 (1983) 1367.
64 M. Schöneshöfer, A. Fenner, G. Altinok and H.J. Dulce, Clin. Chim. Acta, 106 (1980) 63.
65 M.H. Simonian and M.W. Capp, J. Chromatogr., 287 (1984) 97.
66 H.M. Fales and T. Luukainen, Anal. Chem., 37 (1965) 955.
67 J.-P. Thenot and E.C. Horning, Anal. Lett., 5 (1972) 801.
68 P.G. Devaux, M.G. Horning and E.D. Horning, Anal. Lett., 4 (1971) 151.
69 D.J. Harvey, J. Chromatogr., 147 (1978) 291.
70 H. Miyazaki, M. Ishibashi and M. Itoh, Bioned. Mass Spectrom., 4 (1977) 23.
71 M. Axelson and J. Sjövall, J. Steroid Biochem., 5 (1974) 733.
72 M. Axelson and J. Sjövall, J. Steroid Biochem., 8 (1977) 683.

73 M. Axelson and B.-L. Sahlberg, J. Steroid Biochem., 18 (1983) 313.
74 T. Laatikainen, J. Pelkonen, D. Apter and T. Ranta, J. Clin. Endocrinol. Metab., 50 (1980) 489.
75 J. Sjövall, K. Sjövall and R. Vihko, Steroids, 11 (1968) 703.
76 K. Sjövall, Ann. Clin. Res., 2 (1970) 393.
77 T.A. Baillie, R.A. Anderson, K. Sjövall and J. Sjövall, J. Steroid Biochem., 7 (1976) 203.
78 T. Laatikainen and R. Vihko, J. Steroid Biochem., 2 (1971) 173.
79 E. DePeretti and M.G. Forest, J. Clin. Endocrinol. Metab., 47 (1978) 572.
80 E. DePeretti and E. Mappus, J. Clin. Endocrinol. Metab., 57 (1983) 550.
81 J. Sjövall and R. Vihko, Acta Endocrinol., 57 (1968) 247.
82 E.H. Epstein, Jr., R.M. Krauss and C.H.L. Shackleton, Science, 214 (1981) 659.
83 P. Vestergaard and J.F. Sayegh, Acta Endocrinol., 217 (1978) 76.
84 P. Vestergaard, Acta Endocrinol., 217 (1978) 157.
85 I.E. Bush, The Chromatography of Steroids, Pergamon Press, Oxford, London, New York, Paris, 1961.
86 I.E. Bush, Methods Biochem. Anal., 11 (1963) 149.
87 K. Birchall, D.M. Cathro, C.C. Forsyth and F.L. Mitchell, J. Endocrinol., 27 (1963) 31.
88 D.M. Cathro, K. Birchall, F.L. Mitchell and C.C. Forsyth, J. Endocrinol., 27 (1963) 53.
89 D.C.L. Savage, C.C. Forsyth, E. McCafferty and J. Cameron, J. Endocrinol., 44 (1969) 453.
90 W.S. Cost and J.J.M. Vegter, Acta Endocrinol., 41 (1962) 571.
91 M.D. Nielsen, C. Binder and J. Starup, Acta Endocrinol., 60 (1969) 473.
92 M.D. Nielsen, J.O. Lund and O. Munck, Acta Endocrinol., 71 (1972) 498.
93 S. Miyabo and L. Kornel, J. Steroid Biochem., 5 (1974) 233.
94 L. Kornel, S. Miyabo, Z. Saito and F.-T. Wu, Res. Steroids, 5 (1973) 413.
95 G.P. Klein, S.K. Chan and C.J.P. Giroud, J. Clin. Endocrinol. Metab., 29 (1969) 1448.
96 W. Wortmann, J.C. Touchstone, P. Knapstein, G. Dick and G. Mappes, J. Clin. Endocrinol., 33 (1971) 597.
97 B.P. Lisboa, J. Chromatogr., 13 (1964) 391.
98 B.P. Lisboa, J. Chromatogr., 16 (1964) 136.
99 B.P. Lisboa, J. Chromatogr., 19 (1965) 333.
100 C.H.L. Shackleton and F.L. Mitchell, Steroids, 10 (1967) 359.
101 W.L. Gardiner and E.C. Horning, Biochim. Biophys. Acta, 115 (1966) 524.
102 M. Schöneshöfer, A. Kage and B. Weber, Clin. Chem., 29 (1983) 1367.
103 S. Ulick, L.C. Ramirez and M.I. New, J. Clin. Endocrinol. Metab., 44 (1977) 799.
104 E. Heftmann, J. Liq. Chromatogr., 2 (1979) 1137.
105 M. Novotny, M. Alasandro and M. Konishi, Anal. Chem., 55 (1983) 2375.
106 H.J.G.M. Derks and N.M. Drayer, Steroids, 31 (1978) 289.
107 C.H.L. Shackleton, E. Roitman, C. Monder and H.L. Bradlow, Steroids, 36 (1980) 289.
108 R.L. Farhi and C. Monder, Anal. Biochem., 90 (1978) 58.
109 C. Monder and F. Iohan, Anal. Biochem., 139 (1984) 237.
110 C.H.L. Shackleton, S.J. Gaskell and D.J. Liberto, Chromatographic (GC or LC) and non-chromatographic (FAB) mass spectrometric analysis for clinical steroid analysis, in H. Jaeger (Editor), Capillary Gas Chromatography Mass Spectrometry in Medicine and Pharmacology, Hüthig, Heidelberg, 1986, in press.
111 M. Novotny and A. Zlatkis, J. Chromatogr. Sci., 8 (1970) 346.
112 J.A. Völlmin, Clin. Chim. Acta, 34 (1971) 207.
113 W.J.A. VandenHeuvel, C.C. Sweeley and E.C. Horning, J. Am. Chem. Soc., 82 (1960) 3481.
114 R. Ryhage, Anal. Chem., 36 (1964) 759.
115 C. Monder and C.H.L. Shackleton, Steroids, 44 (1984) 383.
116 J.W. Honour and C.H.L. Shackleton, J. Steroid Biochem., 8 (1977) 299.
117 C.H.L. Shackleton and J.W. Honour, J. Steroid Biochem., 8 (1977) 199.
118 J.J. Vrbanac, W.E. Braselton, Jr., J.F. Holland and C.C. Sweeley, J. Chromatogr., 239 (1982) 265.
119 J.J. Vrbanac, C.C. Sweeley and J.D. Pinkston, Biomed. Mass Spectrom., 10 (1983) 155.
120 S.C. Gates and C.C. Sweeley, Clin. Chem., 24 (1978) 1663.

121 C.H.L. Shackleton and J.W. Honour, Clin. Chim. Acta, 69 (1976) 267.
122 W.J.J. Leunissen and J.H.H. Thijssen, J. Chromatogr., 146 (1978) 365.
123 K.D.R. Setchell, B. Almé, M. Axelson and J. Sjövall, J. Steroid Biochem., 7 (1976) 615.
124 G. Phillipou, R.G. Frith and S.K. James, Ann. Clin. Biochem., 17 (1980) 319.
125 C.D. Pfaffenberger and E.C. Horning, Anal. Biochem., 80 (1977) 329.
126 P. Pfeifer and G. Spiteller, J. Chromatogr., 223 (1981) 21.
127 N.F. Taylor, D.H. Curnow and C.H.L. Shackleton, Clin. Chim. Acta, 85 (1978) 495.
128 C.H.L. Shackleton, J.W. Honour and N.F. Taylor, J. Steroid Biochem., 11 (1979) 523.
129 O. Jänne, J. Steroid Biochem., 2 (1971) 33.
130 O. Jänne, Acta Endocrinol., 67 (1971) 316.
131 T. Laatikainen, J. Perheentupa, R. Vihko, I. Makino and J. Sjövall, J. Steroid Biochem., 3 (1972) 715.
132 Y. Yamaguchi, J. Chromatogr., 163 (1979) 253.
133 S. Pang, L.S. Levine, E. Stoner, J.M. Opitz, M.S. Pollack, B. Dupont and M.I. New, J. Clin. Endocrinol. Metab., 56 (1983) 808.
134 R.L. Rosenfield, B.H. Rich, J.I. Wolfsdorf, F. Cassorla, J.S. Parks, A.M. Bongiovanni, C.H. Wu and C.H.L. Shackleton, J. Clin. Endocrinol. Metab., 51 (1980) 345.
135 M. Zachmann, J.A. Völlmin, G. Murset, H.C. Curtius and A. Prader, J. Clin. Endocrinol., 30 (1970) 719.
136 M. Zachmann, M.G. Forest and E. DePeretti, Horm. Res., 11 (1979) 292.
137 C.H.L. Shackleton, Clin. Chim. Acta, 67 (1976) 287.
138 C.H.L. Shackleton, J. Irias, C. McDonald and J. Imperato-McGinley, Steroids, submitted for publication.
139 G.E. Joannou, J. Steroid Biochem., 14 (1981) 901.
140 J.-Å. Gustafsson, S. Gustafsson and P. Olin, Acta Endocrinol., 71 (1972) 353.
141 S. Pang, L.S. Levine, L.L. Cederqvist, M. Fuentes, V.M. Riccardi, J.H. Holcombe, H.M. Nitowsky, G. Sachs, C.E. Anderson, M.A. Duchon, R. Owens, I. Merkatz and M.I. New, J. Clin. Endocrinol. Metab., 51 (1980) 223.
142 E. DePeretti and M.G. Forest, Horm. Res., 16 (1982) 10.
143 C.H.L. Shackleton, F.L. Mitchell and J.W. Farquhar, Pediatrics, 49 (1972) 198.
144 H. Vierhapper, P. Nowotny, W. Waldhausl and H. Frisch, J. Steroid Biochem., 22 (1985) 363.
145 K.E. Petersen, A. Svejgaard, M.D. Nielsen and J. Dissing, Horm. Res., 16 (1982) 151.
146 J.W. Honour, J. Tourniaire, E.G. Biglieri and C.H.L. Shackleton, J. Steroid Biochem., 9 (1978) 495.
147 B.P. Lisboa, J.M. Halket, I. Ganschow and M.C. Ruiz-Gonzalez, Acta Med. Port., 1 (1979) 433.
148 H.J. Dean, C.H.L. Shackleton and J.S.D. Winter, J. Clin. Endocrinol. Metab., 59 (1984) 513.
149 M. D'Armiento, G. Reda, C. Kater, C.H.L. Shackleton and E.G. Biglieri, J. Clin. Endocrinol. Metab., 56 (1983) 697.
150 M. Zachmann, J.A. Völlmin, W. Hamilton and A. Prader, Clin. Endocrinol., 1 (1972) 369.
151 R.E. Peterson, J. Imperato-McGinley, T. Gautier and C. Shackleton, N. Engl. J. Med., 313 (1985) 1182.
152 M. Roger, R.E. Merceron, F. Girard, P. Canlorbe, L. Dehennin, P. Konopka, J. Seneze and J.E. Toublanc, Horm. Res., 16 (1982) 23.
153 M. Zachmann, E.A. Werder and A. Prader, J. Clin. Endocrinol. Metab., 55 (1982) 487.
154 E. DePeretti, M. Pradon and M.G. Forest, J. Steroid Biochem., 20 (1984) 455.
155 J.W. Honour, J.M. Anderson and C.H.L. Shackleton, Acta Endocrinol., 103 (1983) 101.
156 M. Zachmann, D. Tassinari and A. Prader, J. Clin. Endocrinol. Metab., 56 (1983) 222.
157 A. Rosler, E. Leiberman, A. Rosenmann, R. Ben-Uzilio and J. Weidenfeld, J. Clin. Endocrinol. Metab., 49 (1979) 546.
158 L.S. Levine, W. Rauh, K. Gottesdiener, D. Chow, P. Gunczler, R. Rapaport, S. Pang, B. Schneider and M.I. New, J. Clin. Endocrinol. Metab., 50 (1980) 258.
159 A. Glentøj, M.D. Nielsen and J. Starup, Acta Endocrinol., 93 (1980) 94.
160 R.E. Peterson, J. Imperato-McGinley, T. Gautier and C. Shackleton, Clin. Endocrinol., 23 (1985) 43.

161 J. Imperato-McGinley, R.E. Peterson, T. Gautier, A. Arthur and C. Shackleton, J. Clin. Endocrinol. Metab., 60 (1985) 553.
162 S. Akgun, N.H. Ertel, J. Imperato-McGinley, B.S. Sayli and C. Shackleton, Am. J. Med., in press.
163 E. Tvedegaard, V. Fredriksen, K. Ølgaard, M.D. Nielsen and J. Starup, Acta Endocrinol., 98 (1981) 267.
164 R.J.M. Corrall, K. Wakelin, J.P. O'Hare, I.A.D. O'Brien, A.A.A. Ishmail and J. Honour, Acta Endocrinol., 107 (1984) 538.
165 S. Ulick, L.S. Levine, P. Gunczler, G. Zanconato, L.C. Ramirez, W. Rauh, A. Rosler, H.L. Bradlow and M.I. New, J. Clin. Endocrinol. Metab., 49 (1979) 757.
166 C.H.L. Shackleton, J.W. Honour, M.J. Dillon, C. Chantler and R.W.A. Jones, J. Clin. Endocrinol. Metab., 50 (1980) 786.
167 C.H.L. Shackleton, J. Rodriguez, E. Arteaga, J.M. Lopez and J.S.D. Winter, Clin. Endocrinol., 22 (1985) 701.
168 J.W. Honour, M.J. Dillon, M. Levin and V. Shah, Arch. Dis. Child., 58 (1983) 1018.
169 H.I.J. Harinck, P. van Brummelen, A.P van Seters and A.J. Moolenaar, Clin. Endocrinol., 21 (1984) 505.
170 T.J.W. Fiselier, B.J. Otten, L.A.H. Monnens, J.W. Honour and P.J.J. van Munster, Horm. Res., 16 (1982) 107.
171 N.F. Taylor, W.A. Bartlett, D.J. Dawson and B.A. Enoch, J. Endocrinol., (1984).
172 G. Phillipou and B.A. Higgins, J. Steroid Biochem., 22 (1985) 435.
173 C.H.L. Shackleton, J.W. Honour, M. Dillon and P. Milla, Acta Endocrinol., 81 (1976) 762.
174 P.J. Milla, R. Trompeter, M.J. Dillon, D. Robins and C. Shackleton, Arch. Dis. Child., 52 (1977) 580.
175 S. Ulick, J. Clin. Endocrinol. Metab., 43 (1976) 92.
176 S. Ulick, Ped. Adoles. Endocrinol., 13 (1984) 145.
177 C.H.L. Shackleton and G.A.I. Snodgrass, Ann. Clin. Biochem., 11 (1974) 91.
178 S. Petersen, J. Giese, A.M. Kappelgaard, H.T. Lund, J.O. Lund, M.D. Nielsen and A.C. Thomsen, Acta Paediatr. Scand., 67 (1978) 255.
179 J.W. Honour, M.J. Dillon and C.H.L. Shackleton, J. Clin. Endocrinol. Metab., 54 (1982) 325.
180 M.J. Dillon, J.V. Leonard, J.M. Buckler, D. Ogilvie, D. Lillystone, J.W. Honour and C.H.L. Shackleton, Arch. Dis. Child., 55 (1980) 427.
181 M.D.C. Donaldson, D.B. Grant, M.J. O'Hare and C.H.L. Shackleton, Clin. Endocrinol., 14 (1981) 519.
182 G. Phillipou, Clin. Endocrinol., 16 (1982) 433.
183 M.D. Nielsen, C. Binder, E. Hasnerad and J.K. Grant, The measurement of urinary corticosteroid metabolites, in C. Binder and P.E. Hall (Editors), Cushing's Syndrome Diagnosis and Treatment, Heinemann, London, 1971, pp. 45—52.
184 J.W. Honour, D.A. Price, N.F. Taylor, H.B. Marsden and D.B. Grant, Eur. J. Pediatr., 142 (1984) 165.
185 R.-J. Begue, J.-M. Brun, J. Desgres and P. Padieu, J. Steroid Biochem., 7 (1976) 583.
186 N.F. Taylor and C.H.L. Shackleton, J. Clin. Endocrinol. Metab., 49 (1979) 78.
187 G. Phillipou, R.F. Seamark and R. Sweet, Aust. N.Z. J. Obstet. Gynaecol., 19 (1979) 233.
188 J.W. Honour, S.K. Goolamali and N.F. Taylor, Br. J. Dermatol., 112 (1985) 423.
189 F.A.J. Muskiet, G. Jansen, B.G. Wolthers, A. Marinkovic-Ilsen and P.C. van Voorst Vader, Clin. Chem., 29 (1983) 1404.
190 A. Marinkovic-Ilsen, A. van den Ende and B.G. Wolthers, Arch. Dermatol. Res., 276 (1984) 364.
191 C.H.L. Shackleton and K.M. Straub, Steroids, 40 (1982) 35.
192 C.H.L. Shackleton, Clin. Chem., 29 (1983) 246.
193 C.H.L. Shackleton, V.R. Mattox and J.W. Honour, J. Steroid Biochem., 19 (1983) 209.
194 C.H.L. Shackleton and W. Chai, Clinical applications of steroid conjugate analysis by liquid SIMS, in A.L. Burlingame (Editor), Proceedings of International Symposium on Mass Spectrometry in Health and Life Sciences, Elsevier, Amsterdam, 1985, pp. 491—504.

195 G.K. Pillai and K.M. McErlane, J. High Resolut. Chromatogr. Chromatogr. Commun., 4 (1981) 70.
196 T. Fotsis, H. Adlercreutz and C. Bannwart, J. Steroid Biochem. (submitted).
197 H. Adlercreutz, M.J. Tikkanen and D.H. Hunneman, J. Steroid Biochem., 5 (1974) 211.
198 W.G. Sippell, W. Müller-Holue, H.G. Dörr, F. Biddingmaier and D. Knorr, J. Clin. Endocrinol. Metab., 52 (1981) 385.
199 M.G. Forest, E. DePeretti, A. Lecoy, E. Cadillon, M.T. Zabot and J.M. Thoulon, J. Clin. Endocrinol. Metab., 51 (1980) 816.
200 S. Pang, L.S. Levine, L.L. Cederqvist, M. Fuentes, V.M. Riccardi, J.H. Holcombe, H.M. Nitowsky, G. Sachs, C.E. Anderson, M.A. Duchon, R. Owens, I. Merkatz and M.I. New, J. Clin. Endocrinol. Metab., 51 (1980) 223.
201 T. Luukainen, A. Siegel and R. Vihko, J. Endocrinol., 46 (1970) 391.
202 T. Luukainen, E.A. Michie and R. Vihko, J. Endocrinol., 51 (1971) 10.
203 O. Jänne and R. Vihko, J. Steroid Biochem., 1 (1970) 270.
204 A.L. Siegel, H. Adlercreutz and T. Luukainen, Ann. Med. Esp. Fenn., 47 (1969) 47.
205 J. Homoki, E. Roitman and C.H.L. Shackleton, J. Steroid Biochem., 19 (1983) 1061.
206 J.I. Peltonen and T.J. Laatikainen, J. Steroid Biochem., 6 (1975) 101.
207 J.I. Peltonen, T.J. Laatikainen and A. Hesso, J. Steroid Biochem., 10 (1979) 499.
208 F.L. Mitchell and C.H.L. Shackleton, Adv. Clin. Chem., 12 (1969) 141.
209 A.E. Schindler and V. Ratanasopa, Acta Endocrinol., 59 (1968) 239.
210 E. Vanluchene, D. Vandekerckove, J. DeBoever and P. Sandra, J. Steroid Biochem., 21 (1984) 367.
211 A.O. Ruokonen and R.K. Vihko, J. Androl., 4 (1983) 104.
212 A. Ruokonen, Biochim. Biophys. Acta, 316 (1973) 251.
213 G.L. Hammond, V. Ahonen and R. Vihko, Int. J. Androl., Suppl., 2 (1978) 391.
214 P. Leinonen, G.L. Hammond and R. Vihko, J. Clin. Endocrinol. Metab., 51 (1980) 423.
215 I. Huhtaniemi, J. Steroid Biochem., 8 (1977) 491.
216 I. Huhtaniemi, J. Endocrinol., 59 (1973) 503.
217 T. Laatikainen and R. Vihko, Eur. J. Biochem., 10 (1969) 165.
218 T. Laatikainen and R. Vihko, Eur. J. Biochem., 13 (1970) 534.
219 T. Laatikainen and O. Karjalainen, Acta Endocrinol., 69 (1972) 775.
220 J.-Å. Gustafsson, C.H.L. Shackleton and J. Sjövall, Eur. J. Biochem., 10 (1969) 302.
221 J.-Å. Gustafsson, C.H.L. Shackleton and J. Sjövall, Acta Endocrinol., 65 (1970) 18.
222 J. Sjövall and R. Vihko, Steroids, 6 (1966) 597.
223 C.H.L. Shackleton, Steroid synthesis and catabolism in the fetus and neonate, in H.L.J. Makin (Editor), Biochemistry of Steroid Hormones, Blackwell, Oxford, 1985, pp. 441–477.
224 W.J.J. Leunissen, Quantitative aspects of the determination of steroid profiles from urine by capillary gas chromatography, Thesis, Technische Hogeschool, Eindhoven, 1979, p. 110.
225 J.W. Reynolds, Perinatal Endocrinol., 8 (1976) 23.
226 C.E. Gomez-Sanchez and O.B. Holland, J. Clin. Endocrinol. Metab., 52 (1981) 214.
227 C.H.L. Shackleton, Endocrine Rev., 6 (1985) 441.
228 W.G. Sippell, H. Becker, H.T. Versmold, F. Bidlingmaier and D. Knorr, J. Clin. Endocrinol. Metab., 46 (1978) 971.
229 M. Forest and A.M. Cathiard, Pediat. Res., 12 (1978) 6.
230 S.H.G. Anderson and J. Sjövall, J. Steroid Biochem., 23 (1985) 469.
231 M.D. Chu and S. Ulick, J. Biol. Chem., 257 (1982) 2218.
232 S. Ulick, M.D. Chu and M. Land, J. Biol. Chem., 258 (1983) 5498.
233 J.E.T. Corrie, C.R.W. Edwards and P.S. Budd, Clin. Chem., 31 (1985) 849.
234 C.E. Gomez-Sanchez, M. Montgomery, A. Ganguly, O.B. Holland, E.P. Gomez-Sanchez, C.E. Grim and M.H. Weinberger, J. Clin. Endocrinol. Metab., 59 (1984) 1022.
235 M.G. Forest, J. Clin. Endocrinol. Metab., 47 (1978) 931.
236 E. De Peretti and M.G. Forest, Hor. Res., 16 (1982) 10.
237 Documenta Geigy (Scientific Tables), Ciba-Geigy, Basle, 1976.