Journal of Steroid Biochemistry & Molecular Biology 121 (2010) 481–490

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Review

Clinical steroid mass spectrometry: A 45-year history culminating in HPLC–MS/MS becoming an essential tool for patient diagnosis☆

Cedric Shackleton [a,b,*]

[a] Centre for Endocrinology, Diabetes and Metabolism (CEDAM), Institute of Biomedical Research, University of Birmingham, UK
[b] Children's Hospital Oakland Research Institute, Oakland, CA, USA

ARTICLE INFO

Article history:
Received 4 November 2009
Received in revised form 4 February 2010
Accepted 18 February 2010

Keywords:
Steroid analysis
HPLC–MS/MS
Steroid mass spectrometry

ABSTRACT

Automated rapid HPLC tandem mass spectrometry has become the method of choice for clinical steroid analysis. It is replacing immunoassay techniques in most instances because it has high sensitivity, better reproducibility, greater specificity and can be used to analyze multiple steroids simultaneously. Modern multiplex instruments can analyze thousands of samples per month so even with high instrument costs the price of individual assays can be affordable.

The mass spectrometry of steroids goes back decades; the first on-line chromatography/mass spectrometry methods for hormone analysis date to the 1960s. This paper reviews the evolution of mass spectrometric techniques applied to sterol and steroid measurement There have been three eras: (1) gas chromatography–mass spectrometry (GC/MS), (2) Fast Atom Bombardment (FAB) and (3) HPLC/MS. The first technique is only suitable for unconjugated steroids, the second for conjugated, and the third equally useful for free or conjugated.

FAB transformed biological mass spectrometry in the 1980s but in the end was an interim technique; GC/MS retains unique qualities but is unsuited to commercial routine analysis, while LC–MS/MS is rightly stealing the show and has become the dominant method for steroid analysis in endocrinology.

© 2010 Elsevier Ltd. All rights reserved.

Contents

1. Preface 481
2. GC/MS 482
3. Fast Atom Bombardment (FAB) 484
4. HPLC/MS 485
5. Tandem MS as a mainstream technique in diagnostics 485
   5.1. CAH diagnosis 485
   5.2. Improving accuracy of hormonal measurement 487
   Acknowledgements 488
   References 488

1. Preface

Mass spectrometry development has taken a century with several notable early contributors, but it was F.W. Aston of this university (originally an assistant of the famed J.J. Thompson) who in 1919 developed an instrument recognized as the forerunner of current equipment [1]. He was subsequently awarded the 1922 Nobel prize for this achievement. Surprisingly, as early as the 1930s following Urey and co-workers. [2] isolation of deuterium, sterol metabolism studies utilizing labeled precursors were being carried out by Schoenheimer and Rittenberg [3] so the combination of steroids, stable isotope labeling and mass spectrometry goes back a long way. By the 1950s systematic electron impact (EI) fragmentation studies of steroids were being carried out and the rudiments of structure determination were evolving, but it was the next decade before the facile identification of steroids from biological matrixes became possible with the combination of chromatography and mass spectrometry. The following sections describe early develop-

☆ Article from special issue on "Steroid profiling and analytics: going towards Sterome".

* Correspondence address: CHORI, 5700 Martin Luther King Way, Oakland, CA 94609, USA. Tel.: +1 510 450 7660; fax: +1 510 450 7910.
E-mail address: cshackleton@chori.org.

0960-0760/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2010.02.017

Table 1
The genesis of steroid mass spectrometry.

Year	Development
1930s	Discovery of deuterium; use in sterol metabolism studies
1950s	Electron impact/MS studies of sterols and steroids.
1960	First gas chromatography of sterols and steroids
1963	Molecular separator developed. Combination “GC/MS” invented
1964	First GC/MS publication on human sterol metabolism
1965	The first commercial GC/MS introduced, the LKB 9000
1966	Full urinary steroid profile shown; steroids of complexity range from androgens to cortisol separated in one run
1971	Quadrupole MS introduced. Glass capillary columns used for steroid GC
1972	Computerization and data systems developed
1974	“Mass fragmentography” (selected-ion-monitoring). Hormone assay with labeled internal standards
1974	GC/MS first used for steroid doping control in sports
1979	Fused silica columns introduced
1980	Solid Phase Extraction (SPE) introduced for steroid extraction
1982	Fast Atom Bombardment (FAB). First MS of steroid conjugates
1983	Finnigan triple quadrupole mass spectrometer introduced
1987	Thermospray LC/MS
1988	HP 5970 MSD bench-top GC/MS introduced
1991	ESMS and LC/ESMS introduced
1994	Isotope ratio MS introduced in sports doping control
1990s	Tandem MS developments, APCI, APPI
2002 onwards	On-line extraction, full automation. Commercialization of clinical steroid analysis by tandem MS

ments and the subsequent advances in steroid mass spectrometry which culminate in routine use of the technique in clinical diagnosis. A timeline of these developments is given in Table 1. Recent reviews of the status of mass spectrometry in clinical steroid analysis are those of Griffiths et al. [4] and Shackleton [5].

2. GC/MS

It was the demonstration of gas-chromatographic separation of sterols by Sweeley and Horning in 1960 that set the stage for the practical use of mass spectrometry in endocrine studies [6]. Within three years the two techniques were combined and the first paper on combined gas chromatography/mass spectrometry (GC/MS) of human sterols published [7]. Gas chromatography and mass spectrometry are analytical techniques that operate at opposite pressure extremes; packed-column gas chromatography uses large gas volumes at high pressure and mass spectrometry is conducted in a vacuum. Although work had been carried out on combining these instruments in the 1950s, the key to producing a successful GC/MS was the development by Ragner Ryhage and colleagues of the Karolinska Institute in Stockholm of the molecular separator, a device that removed the carrier gas while allowing the analytes to pass into the mass spectrometer [8]. Electron impact was the ionization technique of choice and when used to fragment trimethylsilyl derivatives of sterols and steroids gave excellent and structurally informative mass spectra. One of the enduring advantages of this technique is the consistency of spectra obtained by GC/MS over the last 45 years. Fig. 1 shows a GC/MS chromatogram and sterol spectrum published by Eneroth et al. in 1964 [7]. True, the chromatographic peaks would be a somewhat sharper if the analysis was carried out today, but the β-sitosterol spectrum would be identical. The late 1960s saw the commercialization of GC/MS and its use for analyzing sterols, steroids and bile acids in natural fluids and matrices. There is a huge literature of steroid GC/MS data from this era, much from the laboratories of Professor Jan Sjövall

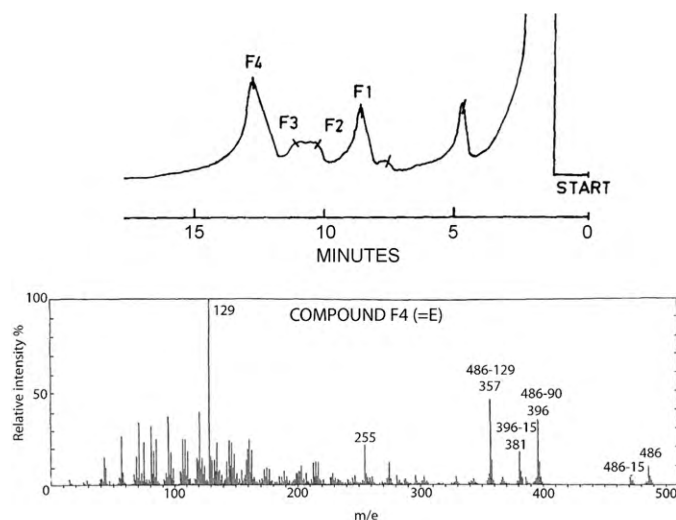

Fig. 1. The Ancestor. The first published study using an on-line chromatography/mass spectrometry system applied to a human steroid investigation. A GC/MS chromatogram of a fraction of fecal sterols as TMS derivatives, and the mass spectrum of peak 4 identified as β-sitosterol.
From Eneroth et al. [7].

in Stockholm and the late Professors Evan and Marjorie Horning in Houston. Publications and spectral libraries from this era remain critically useful to this day.

Until the mid 1960s steroid GC/MS was limited to steroids with simple structure, typically sterols, androstanes and 17-deoxypregnanes. These could be analyzed underivatized or as trimethylsilyl ethers. The silylation methods used at the time were unable to adequately protect corticosteroids from thermal degradation. This changed with the introduction of methyloxime derivatization of carbonyl groups which when conducted before silylation protected most steroids, although for corticosteroids the hindered 11-carbonyl and 17-hydroxyl were resistant to derivatization using reagents available at the time [9]. The improved derivatization technique meant that by 1968 a full analysis or “profile” of all human excreted steroids of interest could be obtained. This is nicely illustrated in a urine steroid profile published by Horning et al. [10], which shows the separation of all steroids ranging in complexity from androsterone to the cortols. It was recognized that the free 17-hydroxyl still left corticosteroids to a certain extent vulnerable to side-chain cleavage so a more powerful silylation was sought. The introduction of trimethylsilylimidazole (TMSi) as silylating reagent finally allowed all steroid hydroxyls to be derivatized but this reagent had the disadvantage of being viscous and involatile, unsuitable for injection into a GC/MS. Axelson and Sjövall developed a derivative purification method based on lipophilic Sephadex [11] and this was utilized for many years by steroid profilers but was finally replaced by a water extraction method devised by Dr Norman Taylor. A key development around 1970 was the computerization of GC/MS data recording and processing, and soon all instrument operations were managed by data systems [12].

The adoption of glass capillary columns for steroid analysis in the early 1970s was a major development. Not only was it possible to achieve better separation of structurally closely related compounds but the low gaseous flow rate allowed dispensing of molecular separators permitting the column to exit directly into the ion source. Glass capillary chromatography became invaluable in profiling urinary steroid metabolites. An early example of glass capillary column clinical steroid profiling is illustrated in Fig. 2. This illustrates a GC/MS urinary steroid profile of an infant shown to have hyperaldosteronism caused by renal tubule insensitivity to the hormone [13]. The excess production of tetrahydroaldosterone

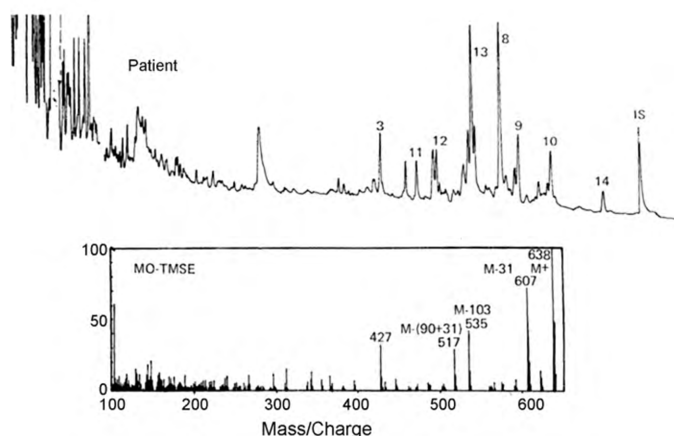

Fig. 2. Early glass capillary column urine steroid GC/MS analysis in diagnosis of a mineralocorticoid defect in a child. The figure shows GC chromatogram of the methyloxime-trimethylsilyl ether derivative and mass spectrum of tetrahydroaldosterone, Peak 13. The mass spectrum is of the fully derivatized "open" form of the steroid with molecular ion at *m/z* 638 and prominent ion form by loss of an oxime residue at *m/z* 607. This baby clearly had hyperaldosteronism. Other steroids of note are tetrahydro-11-dehydrocorticosterone (peak 11), 5αtetrahydrocorticosterone (peak 12), tetrahydrocortisone (peak 8) and 1β-hydroxycortolone (peak 14) Cholesteryl butyrate is the internal standard.
From Shackleton and Snodgrass [13].

was easily determined. By 1979 commercially made flexible fused silica columns had replaced the fragile glass ones used prior to that time.

GC/MS was widely used for clinical steroid analysis during the 1970s both employing the repetitive scanning techniques for urinary "profile" analysis metabolite analysis and selected-ion-monitoring (SIM) for targeted serum steroids, the latter technique in that era being termed "mass fragmentography", a misnomer since molecular ions were often those selected. Ingemar Björkhem and his colleagues at the Karolinska institute introduced accurate quantitative methods utilizing labeled internal standards of many of the important compounds we are measuring today by tandem MS [14]. It will be discussed in detail later but from the late 1970s GC/MS SIM was developed as definitive methodology for authentication of other analytical techniques. In addition to Ingemar Björkhem notable early researchers in this field have been Lothar Siekmann, Linda Thienpont and their co-workers [15–18] and studies were carried out on testosterone, estradiol, progesterone, cortisol and aldosterone.

In terms of sample work-up procedures one important development in the field was our introduction of C18 cartridges for sample extraction [19], the technique now known as Solid Phase Extraction (SPE). Most steroid methodologies in use today still rely on derivatives of this method of steroid recovery from biological matrices.

GC/MS as a truly routine technique was finally established by the introduction of computer controlled bench-top instruments by Hewlett-Packard culminating in the mass selective detector (MSD) marketed in 1982. Its descendents are still the workhorses of laboratories to this day. While in many respects LC tandem MS now dominates the steroid analysis field GC/MS is still important, and of course also can have MS/MS capability if an ion-trap instrument is used [5]. GC–MS/MS selected-ion-monitoring does not give such good data as a single-stage quadrupole and there is little reason to use it for urine steroid analysis when sample is plentiful. However, one recent problem we have had was in the prenatal diagnosis of Smith–Lemli–Opitz syndrome when sensitive analysis of 8-dehydroestriol in maternal serum was required. This diagnostic analyte can be present in very low concentrations so high background was a problem in a single-stage instrument and obtaining a definitive spectrum of the analyte could be challenging. Using a Thermofinnigan PolarisQ ion-trap we could eliminate background interferences, thus increasing the overall sensitivity. Moreover, the high specificity achieved allowed a reduction in the overall run time (from 30 min to 15 min), by modifying the temperature program on the GC. As an example of specificity improvement Fig. 3 shows the

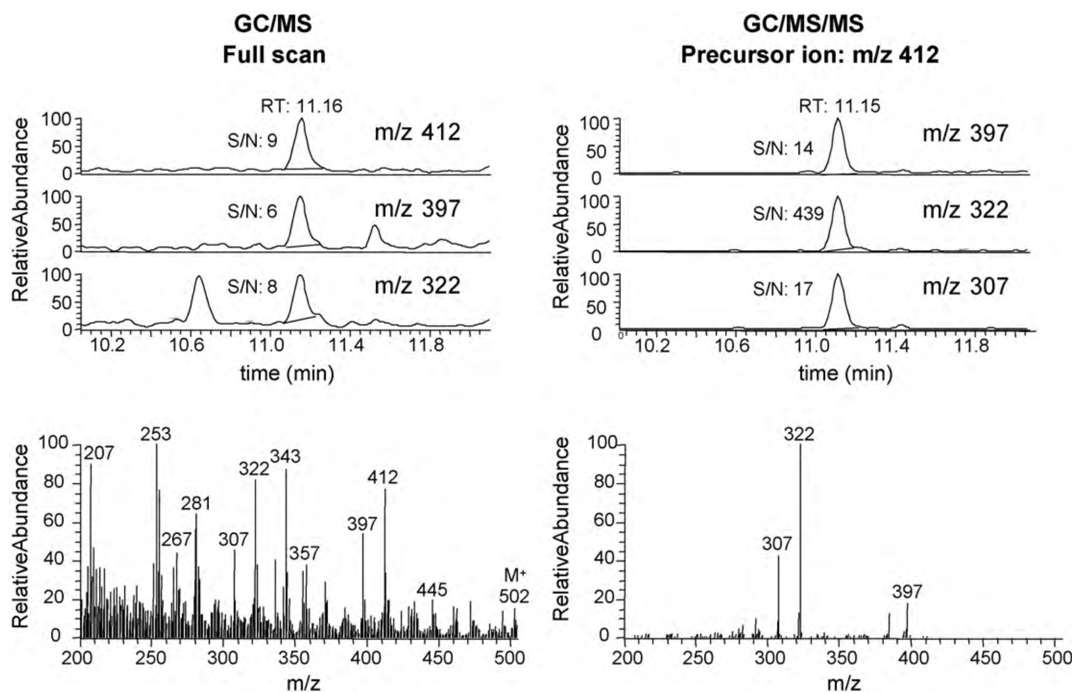

Fig. 3. Utilizing GC ion-trap MS/MS for improving specificity and sensitivity. This shows the full scan and product-ion MS/MS spectrum for the trimethylsilyl (TMS) derivative of serum 8-dehydroestriol, a diagnostic analyte for prenatal diagnosis of Smith–Lemli–Opitz syndrome (SLOS). Due to low concentration its presence may be not detected by classical GC/MS approaches even when using the most sensitive SIM mode.
From Shackleton [5], with permission.

full scan and product-ion MS/MS spectrum for 8-dehydroestriol. When using full scan mode, the detection of the most abundant ions (m/z 412, 397, 322, and visual appearance of the full spectrum) was compromised by the high background (Fig. 3, left). In contrast, acquisition in MS/MS mode (precursor ion m/z 412) provides a cleaner mass spectrum and a superior signal to noise ratio (S/N), especially for the transition product ion m/z 322 (Fig. 3, right).

The current role of GC/MS in clinical steroid analysis is discussed in papers by Shackleton [5] and Krone and co-workers [20, this issue].

3. Fast Atom Bombardment (FAB)

Steroid hormones are metabolized by oxidation and reduction (Phase 1 reactions) and conjugated (Phase 2) prior to excretion and it is these metabolites that when deconjugated are analyzed by GC/MS profile analysis. The analysis of intact glucuronides can be achieved, but in reality is not very practical [21].

From early in biological mass spectrometry a goal has been the analysis of polar and fragile compounds without chemical derivatization. The year 1981 saw the introduction by Barber et al. [22] of Fast Atom Bombardment, the first mass spectrometric technique to allow such analyses. In this ambient temperature technique the sample is mixed with an involatile matrix on a target which, following placement in the ion source is bombarded by a stream of atoms (Xe). The analyte is "sputtered" out of the matrix as an ion which is then detected and recorded by the mass spectrometer. This ionization can be also achieved with heavy ions such as Cs+, and this sister technique is called liquid-SIMS (secondary ion mass spectrometry), although FAB is often used as generic term for both techniques. Little molecular fragmentation occurs and ions produced are largely negative or positive molecular ions. The technique was principally adopted by peptide and protein analysts but we soon found it was ideal for analyzing steroid conjugates [23]. These gave simple mass spectra in negative mode essentially comprising just the molecular anion which meant that urinary steroids could be separately detected providing they differed in mass. We realized that unfractionated urine could be put on the probe and different forms of steroid biosynthetic disorders distinguished by the pattern of metabolites seen in the FAB spectra (Fig. 4). Each analyte

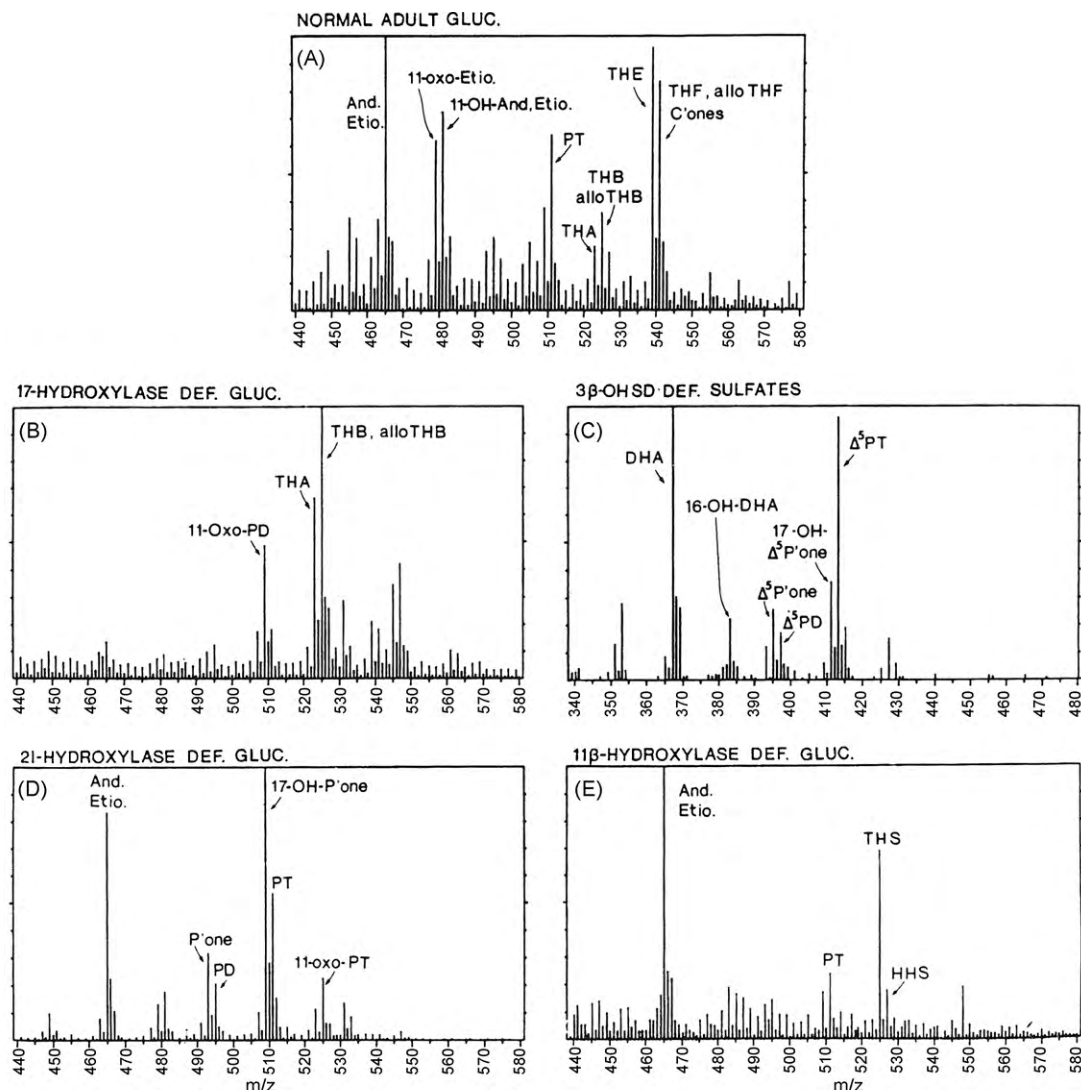

Fig. 4. FAB spectra of unresolved urine steroid conjugates. Urine extracts were put directly onto the probe. Since negative ion FAB spectra are composed almost exclusively of the molecular anion, conjugates can be separated in a spectrum provided they differ in mass. We were able to differentiate three forms of CAH using this technique: 3βHSD deficiency and 21- and 17-hydroxylase. Selected abbreviations: And/Etio, androsterone and etiocholanolone; THB/alloTHB, tetrahydrocorticosterones; THE, tetrahydrocortisone; THF/alloTHF, tetrahydrocortisols; c'ones, cortolones; DHA, dehydroepiandrosterone; Δ^5PT, pregnenetriol; Δ^5P'one, pregnenolone; Δ^5PD, pregnenediol; 17-OH-P'one, 17-hydroxypregnanolone; PT, pregnanetriol; 11-oxo-PT, pregnanetriolone; THS, tetrahydro-11-deoxycortisol; HHS, hexahydro-11-deoxycortisol.
From Shackleton and Straub [23], with permission.

differing in mass appeared separately within the mass spectrum. FAB was progressively improved by the introduction of continuous flow FAB allowing on-line HPLC FABMS [24] and its use with tandem MS instruments vastly increased its utility. FAB MS has been used in describing a new bile-acid biosynthetic defect [25] and as a diagnostic method for another sterol disorder cerebrotendinous xanthomatosis [26]. While a groundbreaking technique its use for steroid or bile-acid analysis was eclipsed by the introduction of HPLC/MS.

4. HPLC/MS

Blakley and Vestal [27] described the first practical HPLC/MS based on thermospray. We first utilized the technique for steroid analysis in 1987 and showed that it was particularly valuable for the analysis of steroid conjugates in negative ion mode where the molecular anion dominated [28]. Unconjugated steroids were also readily analyzed. We utilized the technique clinically for the diagnosis of Recessive X-linked Ichthyosis through the measurement of serum cholesterol sulfate [29] and we also published the first clinical assay of intact DHEAS [30]. Few clinical assays were reported using this technique although one of note is the profiling of serum corticosteroids by Shibasaki et al. [31].

LC/TSP MS was superseded by electrospray MS following its description in 1989 by Fenn et al. [32]. Professor Fenn was awarded the 2002 Nobel prize for this development. This latter technique could utilize narrow bore columns and solvent flow rates much lower than TSP MS. Capillary column/"nanospray" technology was also realized. In that era the focus was on using electrospray ionization (ESI) for macromolecules, particularly proteins but it was shown to be equally effective for small analytes including steroids and steroid conjugates. By this time Tandem MS was available which gave much needed specificity through the ability of monitoring the fragmentation of steroid specific daughters. This allowed reduction of background signal and separate analysis of poorly chromatographically separated steroid isomers which had different daughter ion fragments. In spite of the availability of potentially useful LC/MS methodology throughout the 1990s, there are few reports of the technique being used in clinical investigations. A good early paper on profiling corticosterone metabolites by ESI MS/MS is that of Miksik et al. [33]. This paper reproduces spectra of many A-ring saturated steroids which is useful as there is a paucity of tandem LC/MS studies of such compounds. Another recent article that includes chromatograms and spectra of reduced metabolites is that of Hauser et al. [34]. Yamashita et al. [35] have recently published a method for analyzing a panel of urinary steroid metabolites (cortisone, cortisol, tetrahydrocortisone, tetrahydrocortisol and 5α-tetrahydrocortisol) by LC/MS/MS using the piconyl derivatives which gives much greater sensitivity of measurement. However, with these few exceptions tandem MS has not been widely adopted for metabolite measurement, a role where GC/MS is still preeminent [20].

Bill Griffiths, now Professor at the University of Wales, Swansea, worked with Jan Sjövall and colleagues at the Karolinska institute developing high-sensitive, capillary column nano ESI/MS analysis of brain steroids, sterols and steroid sulfates [36–38]. A notable feature of their work was the novel use of Girard derivatives of carbonyl groups which resulted in excellent chromatographic properties and improved mass spectral fragmentation. While these techniques for tissue steroid profiling were invaluable on a research basis they are unsuitable for routine use in hormone measurement. Retention times are long and chromatographic systems not sufficiently robust for high-volume routine use. We carried out a pilot study showing this methodology could be used for diagnosis of Smith–Lemli–Opitz syndrome prenatally through quantitation of cholesterol and dehydrocholesterol [39]. However, to be effective this method would have to be transferred to a UPLC tandem MS platform.

5. Tandem MS as a mainstream technique in diagnostics

Only in the current decade have clinical laboratories made major attempts to utilize the technique as primary analytical procedures, but this field is now very active. In the US this work has been spearheaded by groups at the Mayo Clinic (mayomedicallaboratories.com), ARUP (aruplab.com), Esoterix (esoterix.com), Quest Diagnostics (questdiagnostics.com) and Georgetown University. In Europe the players are generally academic affiliated such the German groups in Hannover, Erlangen and Leipzig, whose participants can be located within the papers cited in the bibliography.

The recent push towards adoption of mass spectrometry for clinical assays came not from analysts but from neonatologists and endocrinologists with little knowledge of the technique, but dissatisfaction with the status quo. There are several, but two major origins for this development: (1) the necessity of follow-up ("second-tier") analysis on newborn screening results for congenital adrenal hyperplasia and (2) a realization that many hormone assays based on RIA were irreproducible, inaccurate and gave rise to compromised diagnoses.

Regarding the methodologies in use by the major players. Apart from electrospray ionization, two other LC interfaces were developed in the late 1990s and are in common use in steroid analysis; APCI (atmospheric chemical ionization) and APPI (atmospheric photoionization). Each analytical group, using different methodologies and equipment have determined the most sensitive system for each steroid. Electrospray is certainly the favorite for conjugates, and APCI seems to be in first place for most neutral steroids, although ESI remains popular. Nearly all groups utilize positive-ion MS and with the latest generation of equipment forgo any chemical derivatization. There are exceptions reported, particularly for the analysis of aldosterone and the estrogens. Aldosterone is analyzed in negative mode by some groups [40,41] and other researchers utilize the piconyl derivative for a 10-fold improvement in sensitivity [42]. Dansylation of estrogens is favoured to improve sensitivity of measurement, particularly in children [43] but other groups have achieved needed sensitivity without derivatization [44], and the major US commercial laboratories universally measure aldosterone and estrogens without modification. Reading the papers from the various groups you will find that while the goals are the same there are many differences in technique. Some have on-line extraction by HPLC or turbulent flow, some have off-line SPE or solvent extraction. Some do protein precipitation, some do not. Some derivatize to achieve needed sensitivity. Some use the new micro particle UPLC (Ultraperformance LC) chromatography systems and others conventional columns.

While the focus for the remaining part of the paper is on blood analysis (serum or blood spot), tandem MS is now widely used for analyzing steroids in other matrices. Taylor et al. validated a method for free urine cortisol and cortisone in 2002 [45] and a similar method that includes the ring-A saturated metabolites of cortisol has been recently published [42]. Saliva has become an important medium for diagnosis and recent papers describe the quantitation of salivary cortisol as a surrogate marker for free hormone concentration, and in screening for Cushing's syndrome [46,47].

5.1. CAH diagnosis

Diagnosing CAH in the newborn period has long been a challenge [48]. In fact this is relatively easily and accurately carried out

on random urine samples by GC/MS [49,50]. With the more ubiquitous serum analysis of 17-hydroxy-progesterone (17OHP) the false positive rate is very high generally due to cross contamination by fetal steroids. In an early ESI/MS and GC/MS study we showed that the major source of contamination was 17-OH-pregnenolone sulfate [51], and the topic has been recently discussed by Fingerhut and co-workers [52] who confirm the likelihood of steroid conjugates being partially responsible for elevated 17OHP levels, as titres dropped markedly if non-polar solvent extraction preceded RIA. They propose that a second reason for high values is stress induced increase in 17OHP levels, particularly in premature newborns. Many researchers would agree with this interpretation. While compromised 17OHP values in newborns has been long known, the severity of the problem has been accentuated by the introduction of blood-spot screening for CAH which is now required in all of the States which comprise the USA. The false positive rate is the greatest of all the mandated screens carried out. Clearly some form of confirmatory or "second-tier" testing was necessary for infants found positive for CAH at newborn screening. It was mentioned that GC/MS is an excellent technique for neo-natal CAH diagnosis but the technique would not be useful for confirmation of newborn screening results where hundreds of samples may need to be analyzed monthly.

Significant progress has now been made in developing a second-tier test based on LC–MS/MS for use in confirming the disorder in cases found positive by the initial radioimmunoassay screen [53]. The accuracy of diagnosis is greatly improved when a result panel including 17OHP, androstenedione and cortisol is produced, particularly if quantitative levels are obtained by the use of labeled internal standards [54]. An excellent systematic study has been reported by Schwarz et al. [54] which is based on experience in newborn CAH screening by the State of Utah. From an initial RIA 17OHP screening of 64,000 newborns a large number were considered to have elevated results, possibly indicative of CAH. Analyzing the steroid panel of the samples initially suspected of having high 17OHP greatly reduced the number of false positives. Fig. 5 shows comparative chromatograms of the diagnostic steroids in a normal blood spot and one from an infant subsequently shown to have CAH, visually these are very distinctive and the high levels of androstenedione and 17OHP in the CAH infant can be clearly seen. The criteria for a positive second-tier test was a 17OHP level > 12.5 ng/ml and an androstenedione + 17OHP/cortisol ratio > 1.0. Of 64 samples giving positive second-tier results from a very large cohort only 6 were confirmed clinically as having

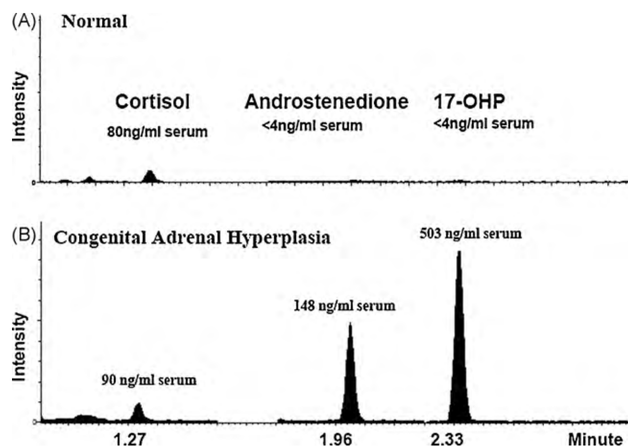

Fig. 5. CAH newborn screening: second-tier testing. UPLC–MS/MS steroid profiles of a normal (A) and CAH infants (B) were obtained using positive-ion ESI in selective reaction monitoring (SRM) mode. Deuterated internal standards were used for quantification; the illustration represents equivalent volumes of serum for the two infants.
From Schwarz et al. [54], with permission.

CAH showing that even measuring these three parameters there is still a high false positive rate. I have long believed that this is a rationale for quantifying 21-deoxycortisol as an important analyte in addition to 17OHP. This steroid is "one hydroxylation step" closer to cortisol than 17OHP so inherently must be the better analyte. It has been know for 40 years in the urine metabolite field that the 21-deoxycortisol metabolite pregnanetriolone is the single best analyte for confirming CAH by GC/MS. While 17OHP has a *central* role in steroid biosynthesis, being at a branch-point, with its options being either routes to cortisol or the androgens; 21-deoxycortisol is on a "spur" or "tangent", a compound *only* formed when there is deficient 21-hydroxylase. There are many reasons why 17OHP may be increased which is why it varies so greatly in the neonate, its production changing with prematurity and birth-weight, for example, probably attributable to stress. Cristoni et al. [55] first described the measurement of 21-deoxycortisol for CAH diagnosis by HPLC–MS/MS. Janzen and colleagues expanded this observation and showed that quantifying this analyte in newborns allows accurate diagnosis of the condition, independent of prematurity, birth weight or other factors [56,57]. Fig. 6 is from their 2006 publication and illustrates their results for 17OHP and

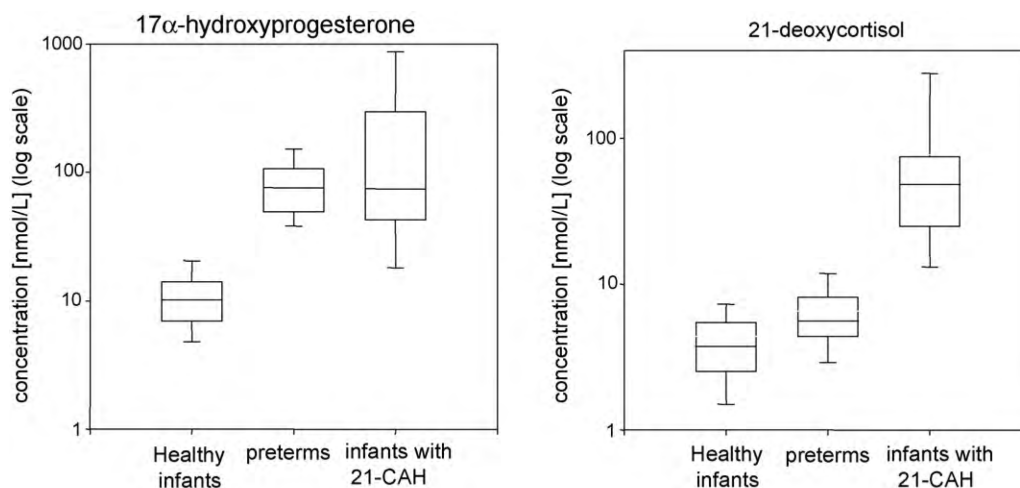

Fig. 6. Improved accuracy of CAH diagnosis by including 21-deoxycortisol in HPLC–MS/MS methods. There can be overlap between 17OHP levels in normal and CAH infants, particularly if babies are premature. By contrast, 21-deoxycortisol is only abnormal in CAH infants.
From Janzen et al. [57], with permission.

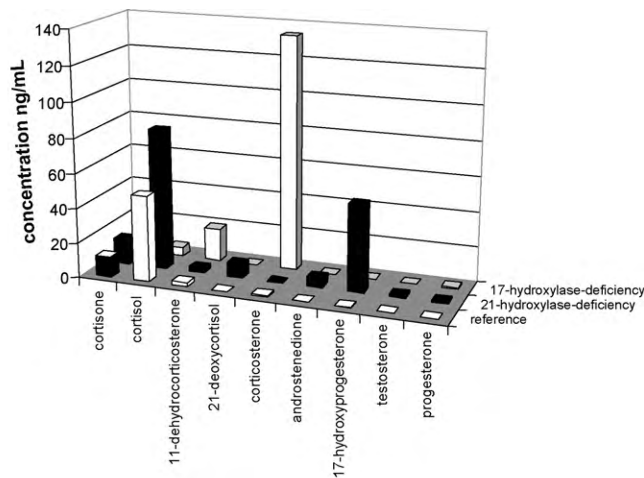

Fig. 7. Tandem MS plasma steroid profiles of three individuals: patient with normal adrenal function (reference) and CAH patients with 17α- and 21-hydroxylase deficiencies.
From Rauh [59], with permission.

21-deoxycortisol in normal and premature newborns and in CAH babies. In fact, in treated CAH infants *only* 21-deoxycortisol give elevated values. While I focused on the Janzen and co-workers' measurement of 21-deoxycortisol [57], their serum and blood-spot steroid panel also includes 17OHP, testosterone, androstenedione, dihydrotestosterone, corticosterone, 11-deoxycortisol and cortisol, making it a very comprehensive test. These workers have also demonstrated the use of their method for diagnosis of 11β-hydroxylase deficiency [56–58]. Rauh and co-workers have also contributed greatly to this field and have adopted an LC/tandem MS panel also incorporating 21-deoxycortisol and corticosterone and a nice example from their work is in Fig. 7 showing profiles delineating the 21- and 17-hydroxylase deficiency forms of CAH [59]. Since initial review of this current paper one more important study has been published: Rossi et al. [60] describe the second-tier testing for CAH using a panel of 17OHP, androstenedione, 11- and 21-deoxycortisol and cortisol. They used only 50 μl of serum, solvent extraction, and UPLC/MS/MS with electrospray ionization. All steroids could be quantified within 3 min.

Summarizing this section; I believe a panel containing 17OHP, cortisol, 21-deoxycortisol and androstenedione should be employed by all doing second-tier testing for newborn screening of 21-hydroxylase deficiency CAH. I would also suggest that diagnosis could be clinched by "third-tier" testing using urinary steroid GC/MS, a non-invasive technique with close to zero false positives.

5.2. Improving accuracy of hormonal measurement

Currently LC/tandem MS is transforming clinical steroid measurement. Historically, steroid hormone assays carried out by different laboratories using different methodologies, typically radioimmunoassay, have shown unacceptable variability. This is an old problem, it having been addressed since the beginning of hormonal measurement [61]. Isotope dilution GC/MS was the original technique used for validation of routine methods; so-called "definitive" or "reference" methodologies [14,15]. Early-on a commission of the European community addressed these issues and there are multiple publications on production and validation of authenticated calibrators [16–18]. Methodologies employing these calibrators were named Reference Measurement Procedures (RMPs). In contrast to the EU, the United States has paid less attention to standardization of hormonal analysis.

While accuracy and reproducibility concerns have been around a long time and hold true for all serum steroid measurements, it has recently surfaced in the US as a particular problem in testosterone and estradiol measurement. Accurately diagnosing hypogonadism in men is compromised, and determining hypo- and hyperandrogenism in women and children with naturally lower levels is an even greater problem. For low testosterone conditions the problem is not just interlaboratory variation, but the existing kits and methods themselves are inadequate for measurement at low levels. A groundswell of dissatisfaction with testosterone assays has been documented in recent papers such as those of Rosner et al. [62], and a panel of experts met at the Center for Disease Control (CDC) to address this problem [63].

Over the last few years several prominent academic and commercial laboratories have introduced LC/MS/MS methods of analysis in an attempt to improve the measurement of testosterone and other hormonal steroids [64–66]. These facilities can now do testosterone measurement at rates of thousands of samples per month with the high sensitivity required for the female and pediatric population. Typically these laboratories have good within facility reproducibility and comparisons of measured amounts between laboratories has greatly improved over results obtained by radioimmunoassay. However, there is still unacceptable variation and further standardization of materials and methods is needed. This is actively been addressed with the help of the National Bureau of Standards (NBC) and the CDC. A key development was publication and use of a candidate RPM method for testosterone reported by Tai et al. from the National Institute of Standards and Technology (NIST) [67]. The repeatability of within-set and between set values were <1.0%. This method was used as the gold-standard in a study published by Vesper and colleagues for comparing eight commercially used methods [68], and while the results showed that accuracy had improved over previous techniques the results emphasized the work that still had to be done to standardize methodology, materials and calibrators. Thienpont et al. [69] have made a similar study comparing four published LC tandem MS methods to a GC/MS RMP. It should be noted that this RMP is one of two listed by the Joint Committee for Traceability in Laboratory Medicine (JCTLM). It has been in constant use in a reference laboratory for 15 years emphasizing how old this problem is. The study demonstrated fairly good accuracy and standardization of the LC–MS/MS procedures but calibration issues remained, again emphasizing the need for thorough validation, including traceability of all methods in routine clinical use.

In the era of ultrasensitive LC/MS assays not only does much more attention need to paid to authentication of steroid standards and calibrators but also to quality of often-used solvents and plastics. Contaminants that were previously below sensitivity threshold can now be a major problem.

While the focus on assay improvement has been with testosterone, all hormonal steroid measurements would be improved by the adoption of tandem MS and now several labs are offering analysis of multiple steroids, including hard-to-measure steroids such as aldosterone and the estrogens [34,40,44,53,64,65,70]. LC/MS/MS has now become the preferred technique for quantitation of the seco-sterol 25-hydroxy-vitamin D3 [64,71]. A candidate reference method (RMP) for progesterone has recently being published by Tai and colleagues at the NIST [72].

As these assays come on-line stringent controls will be needed to ensure within-lab and between-lab accuracy and reproducibility. When major labs adopt LC/MS/MS on a massive scale even more attention must be focused on assay quality and training. Systemic error, failure to follow precise protocols, quality control lapses in a commercial laboratory can now cause thousands of inaccurate results to be reported. This was the case a year ago for the 25-hydroxy-vitamin D assay offered by Quest Diagnostics. This

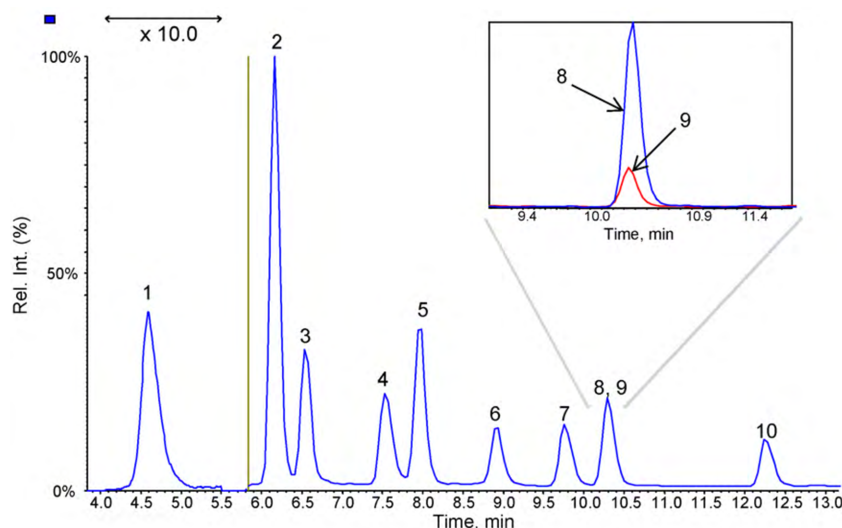

Fig. 8. Steroids quantified using the kit being developed by Perkin-Elmer. Total ion chromatogram of the 10 compounds in the calibrator: 1. aldosterone; 2. cortisol; 3. DHEAS; 4. corticosterone; 5. 21-deoxycortisol; 6. androstendione; 7. testosterone; 8. 17 OHP; 9. DHEA; and 10. progesterone. Data for aldosterone was acquired in negative ion mode, the remaining peaks being acquired in positive-ion mode: the insert shows the extracted ion chromatograms for the MRM transitions of peaks 8 and 9. Chromatogram supplied courtesy of Dr Blas Cerda, Perkin-Elmer Corporation.

well-respected company admitted to using faulty calibrants and incorrect procedures in four of its facilities. Training, vigilance and accurate calibrants are all vital if steroid assays are to be improved [73,74]. This episode emphasizes the importance of using common standards and calibrators in improving the reproducibility of this assay between laboratories.

A recent sea-change in clinical mass spectrometry results from the pro-activity of instrument and technology companies in listening to, and working with, analysts to improve methodologies. Until recently, once such companies had cashed the check for hardware their interest waned. Their sustained concern is not altruism; they see a potential revenue stream, so are spearheading the development of comprehensive kits to improve accuracy and reproducibility and work diligently to keep a close relationship with users. This is an important development because individual research scientists, and endocrinologists do not have the deep-pockets required to undertake the methodology development and proficiency evaluation required. Acquiring or producing needed labeled internal standards alone is often prohibitively expensive. This work is not readily funded by research agencies and many commercial laboratories would also find this investment daunting. The Waters [60] and Perkin-Elmer corporations are actively contributing to this development since both have active groups working on clinical steroid analysis. Perkin-Elmer are in the final stage of introducing a kit for the analysis of 10 steroid hormones and precursors. This kit will include all needed labeled internal standards, calibrators and controls. This application will work on any current generation LC–MS/MS platform, although some instruments may not have the sensitivity required for analytes in lowest concentration. Fig. 8 shows the selected ion/MRM transition total ion chromatogram of the steroids that will be quantified. We hope such kits will bring uniformity of steroid results produced worldwide closer to realization.

The days of immunoassay in serum steroid hormone measurement seem numbered. However, the advent of the LC/MS/MS era will result in consolidation of diagnostic services as few small diagnostic labs will be able to afford the hardware. Only the most expensive of current instruments have the sensitivity to measure the full range of steroid hormones and precursors. Large commercial entities in the US such as the Mayo clinic, Esoterix, ARUP and Quest Diagnostics quickly recoup their investments because of the large number of samples analyzed. This would not apply to smaller labs who may have to use more modest equipment and forgo some of the most taxing analytes. Such labs without state-of-the-art machines may have to restrict there offerings to easier analytes and make use of derivatization to improve sensitivity.

While tandem MS will replace immunoassay techniques for serum steroid analysis, GC/MS still has a role to play in the evaluation of individual patients, as argued in a separate paper in this issue [20]. GC/MS is still preeminent for studying complex metabolomes, and even for confirming pediatric CAH on a less invasive basis since only a urine sample is required.

Acknowledgements

This work was supported by the following grants: MRC Experimental Grant – DLAC.RRAK11881. Welcome Trust Programme Grant – DLAC.RCHX13057. The author thanks Drs Montserrat Serra and Xavier Matabosch for assisting with manuscript preparation.

References

[1] F.W. Aston, A positive ray spectrograph, Philos. Mag. Ser. 6 (38) (1919) 707–714.
[2] H.C. Urey, F.G. Brickwedde, G.M. Murphy, A hydrogen isotope of mass 2, Phys. Rev. 39 (1932) 164–165.
[3] R. Schoenheimer, D. Rittenberg, Deuterium as an indicator in the study of intermediary metabolism, Science 82 (1935) 156–157.
[4] W.J. Griffiths, C.H.L. Shackleton, J. Sjövall, Steroid analysis, in: R. Caprioli (Ed.), The Encyclopedia of Mass Spectrometry, Elsevier, Amsterdam, 2006, pp. 447–473.
[5] C.H.L. Shackleton, Genetic disorders of steroid metabolism diagnosed by mass spectrometry, in: N. Blau (Ed.), Laboratory Guide to the Methods in Biochemical Genetics, Springer-Verlag, Berlin, 2008.
[6] C.C. Sweeley, E.C. Horning, Microanalytical separation of steroids by gas chromatography, Nature 187 (1960) 144–145.
[7] P. Eneroth, K. Hellström, R. Ryhage, Identification and quantification of neutral fecal steroids by gas–liquid chromatography and mass spectrometry: studies of human excretion during two dietary regimens, J. Lipid Res. 5 (1964) 245–262.
[8] R. Ryhage, Use of a mass spectrometer as a detector and analyzer for effluent emerging from high temperature gas liquid chromatography columns, Anal. Chem. 36 (1964) 759–764.
[9] H.M. Fales, T. Luukkainen, O-methyloximes as carbonyl derivatives in gas chromatography, mass spectrometry, and nuclear magnetic resonance, Anal. Chem. 37 (1965) 955–957.
[10] E.C. Horning, C.J. Brooks, W.J. Vanden Heuvel, Gas phase analytical methods for the study of steroids, Adv. Lipid Res. 6 (1968) 273–392.
[11] M. Axelson, J. Sjövall, Analysis of unconjugated steroids in plasma by liquid–gel chromatography and glass capillary gas chromatography mass spectrometry, J. Steroid Biochem. 8 (1977) 683–692.

[12] R. Reimendal, J. Sjövall, Analysis of steroids by off-line computerized gas chromatography–mass spectrometry, Anal. Chem. 44 (1972) 21–29.

[13] C.H. Shackleton, G.H. Snodgrass, Steroid excretion by an infant with an unusual salt-losing syndrome: a gas chromatographic–mass spectrometric study, Ann. Clin. Biochem. 11 (1974) 91–99.

[14] I. Björkhem, O. Lantto, L. Svensson, Serum testosterone determination by mass fragmentography, Clin. Chim. Acta 60 (1975) 59–66.

[15] L. Siekmann, Determination of steroid hormones by the use of isotope dilution–mass spectrometry: a definitive method in clinical chemistry, J. Steroid Biochem. 11 (1979) 117–123.

[16] L. Thienpont, L. Siekmann, A. Lawson, E. Colinet, A. De Leenheer, Development, validation, and certification by isotope dilution gas chromatography–mass spectrometry of lyophilized human serum reference materials for cortisol (CRM 192 and 193) and progesterone (CRM 347 and 348), Clin. Chem. 37 (1991) 540–546.

[17] L.M Thienpont, V.I. De Brabandere, D. Stockl, A.P. De Leenheer, Use of cyclodextrins for prepurification of progesterone and testosterone from human serum prior to determination with isotope dilution gas chromatography/mass spectrometry, Anal. Chem. 66 (1994) 4116–4119.

[18] L.M. Thienpont, A.P. De Leenheer, Efforts by industry toward standardization of serum estradiol-17β measurements, Clin. Chem. 44 (1998) 671–674.

[19] C.H. Shackleton, J.O. Whitney, Use of Sep-pak cartridges for urinary steroid extraction: evaluation of the method for use prior to gas chromatographic analysis, Clin. Chim. Acta 107 (1980) 231–243.

[20] N.P. Krone, B.A. Hughes, G.G. Lavery, P.M. Stewart, W. Arlt, C.H.L. Shackleton, Gas chromatography/mass spectrometry (GC/MS) remains a pre-eminent discovery tool in clinical steroid investigations even in the era of fast tandem mass spectrometry (LC/MS/MS), J. Steroid Biochem. Mol. Biol., this issue.

[21] C.H. Shackleton, V.R. Mattox, J.W. Honour, Analysis of intact steroid conjugates by secondary ion mass spectrometry (including FABMS) and by gas chromatography, J. Steroid Biochem. 19 (1983) 209–217.

[22] M. Barber, R. Bordoli, R. Sedgwick, A. Tyler, Fast atom bombardment of solids (F.A.B.): a new ion source for mass spectrometry, J. Chem. Soc. Chem. Commun. 7 (1981) 325–327.

[23] C.H. Shackleton, K.M. Straub, Direct analysis of steroid conjugates: the use of secondary ion mass spectrometry, Steroids 40 (1982) 35–51.

[24] R.M. Caprioli, T. Fan, J.S. Cottrell, Continuous-flow sample probe for fast atom bombardment mass spectrometry, Anal. Chem. 58 (1986) 2949–2954.

[25] K.D. Setchell, F.J. Suchy, M.B. Welsh, L. Zimmer-Nechemias, J. Heubi, W.F. Balistreri, Δ^4-3-oxosteroid-5β-reductase deficiency described in identical twins with neonatal hepatitis. A new inborn error in bile acid synthesis, J. Clin. Invest. 82 (1988) 2148–2157.

[26] B. Egestad, P. Pettersson, S. Skrede, J. Sjövall, Fast atom bombardment mass spectrometry in the diagnosis of cerebrotendinous xanthomatosis, Scand. J. Clin. Lab. Invest. 45 (1985) 443–446.

[27] C.R. Blakley, M.L. Vestal, Thermospray interface for liquid chromatography/mass spectrometry, Anal. Chem. 55 (1983) 750–754.

[28] D.J. Liberato, A.L. Yergey, N. Esteban, C.E. Gomez-Sanchez, C.H. Shackleton, Thermospray HPLC/MS: a new mass spectrometric technique for the profiling of steroids, J. Steroid Biochem. 27 (1987) 61–70.

[29] C.H. Shackleton, S. Reid, Diagnosis of recessive X-linked ichthyosis: quantitative HPLC/mass spectrometric analysis of plasma for cholesterol sulfate, Clin. Chem. 35 (1989) 1906–1910.

[30] C.H. Shackleton, C. Kletke, S. Wudy, J.H. Pratt, Dehydroepiandrosterone sulfate quantification in serum using high-performance liquid chromatography/mass spectrometry and a deuterated internal standard: a technique suitable for routine use or as a reference method, Steroids 55 (1990) 472–478.

[31] H. Shibasaki, T. Furuta, Y. Kasuya, Quantification of corticosteroids in human plasma by liquid chromatography–thermospray mass spectrometry using stable isotope dilution, J. Chromatogr. B Biomed. Sci. Appl. 692 (1997) 7–14.

[32] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Electrospray ionization for mass spectrometry of large biomolecules, Science 246 (1989) 64–71.

[33] I. Miksik, M. Vylitova, J. Pacha, Z. Deyl, Separation and identification of corticosterone metabolites by liquid chromatography–electrospray ionization mass spectrometry, J. Chromatogr. B Biomed. Sci. Appl. 726 (1999) 59–69.

[34] B. Hauser, T. Deschner, C. Boesch, Development of a liquid chromatography–tandem mass spectrometry method for the determination of 23 endogenous steroids in small quantities of primate urine, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 862 (2008) 100–112.

[35] K. Yamashita, R. Nakagawa, M. Okuyama, S. Honma, M. Takahashi, M. Numazawa, Simultaneous determination of tetrahydrocortisol, allotetrahydrocortisol and tetrahydrocortisone in human urine by liquid chromatography–electrospray ionization tandem mass spectrometry, Steroids 73 (2008) 727–737.

[36] S Liu, W.J. Griffiths, J. Sjövall, Capillary liquid chromatography/electrospray mass spectrometry for analysis of steroid sulfates in biological samples, Anal. Chem. 75 (2003) 791–797.

[37] S. Liu, J. Sjövall, W.J. Griffiths, Neurosteroids in rat brain: extraction, isolation, and analysis by nanoscale liquid chromatography–electrospray mass spectrometry, Anal. Chem. 75 (2003) 5835–5846.

[38] Y Wang, W.J. Griffiths, Capillary liquid chromatography combined with tandem mass spectrometry for the study of neurosteroids and oxysterols in brain, Neurochem. Int. 52 (2008) 506–521.

[39] W.J. Griffiths, Y. Wang, K. Karu, et al., Potential of sterol analysis by liquid chromatography–tandem mass spectrometry for the prenatal diagnosis of Smith–Lemli–Opitz syndrome, Clin. Chem. 54 (2008) 1317–1324.

[40] T. Guo, R.L. Taylor, R.J. Singh, S.J. Soldin, Simultaneous determination of 12 steroids by isotope dilution liquid chromatography–photospray ionization tandem mass spectrometry, Clin. Chim. Acta 372 (2006) 76–82.

[41] U. Turpeinen, E. Hamalainen, U.H. Stenman, Determination of aldosterone in serum by liquid chromatography–tandem mass spectrometry, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 862 (2008) 113–118.

[42] K. Yamashita, M. Okuyama, R. Nakagawa, et al., Development of sensitive derivatization method for aldosterone in liquid chromatography–electrospray ionization tandem mass spectrometry of corticosteroids, J. Chromatogr. A 1200 (2008) 114–121.

[43] M.M. Kushnir, A.L. Rockwood, B. Yue, A.W. Meikle, High sensitivity measurement of estrone and estradiol in serum and plasma using LC–MS/MS, Methods Mol. Biol. 603 (2010) 219–228.

[44] T. Guo, J. Gu, O.P. Soldin, R.J. Singh, S.J. Soldin, Rapid measurement of estrogens and their metabolites in human serum by liquid chromatography–tandem mass spectrometry without derivatization, Clin. Biochem. 41 (2008) 736–741.

[45] R.L. Taylor, D. Machacek, R.J. Singh, Validation of a high-throughput liquid chromatography–tandem mass spectrometry method for urinary cortisol and cortisone, Clin. Chem. 48 (2002) 1511–1519.

[46] U. Turpeinen, M.J. Valimaki, E. Hamalainen, Determination of salivary cortisol by liquid chromatography–tandem mass spectrometry, Scand. J. Clin. Lab. Invest. 69 (2009) 592–597.

[47] I. Perogamvros, L.J. Owen, J. Newell-Price, D.W. Ray, P.J. Trainer, B.G. Keevil, Simultaneous measurement of cortisol and cortisone in human saliva using liquid chromatography–tandem mass spectrometry: application in basal and stimulated conditions, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877 (2009) 3771–3775.

[48] C.H. Shackleton, F.L. Mitchell, J.W. Farquhar, Difficulties in the diagnosis of the adrenogenital syndrome in infancy, Pediatrics 49 (1972) 198–205.

[49] M.P. Caulfield, T. Lynn, M.E. Gottschalk, et al., The diagnosis of congenital adrenal hyperplasia in the newborn by gas chromatography/mass spectrometry analysis of random urine specimens, J. Clin. Endocrinol. Metab. 87 (2002) 3682–3690.

[50] S.A. Wudy, M. Hartmann, J. Homoki, Hormonal diagnosis of 21-hydroxylase deficiency in plasma and urine of neonates using benchtop gas chromatography–mass spectrometry, J. Endocrinol. 165 (2000) 679–683.

[51] T. Wong, C.H. Shackleton, T.R. Covey, G. Ellis, Identification of the steroids in neonatal plasma that interfere with 17α-hydroxyprogesterone radioimmunoassays, Clin. Chem. 38 (1992) 1830–1837.

[52] R. Fingerhut, False positive rate in newborn screening for congenital adrenal hyperplasia (CAH)-ether extraction reveals two distinct reasons for elevated 17alpha-hydroxyprogesterone (17-OHP) values, Steroids 74 (2009) 662–665.

[53] C.Z. Minutti, J.M. Lacey, M.J. Magera, et al., Steroid profiling by tandem mass spectrometry improves the positive predictive value of newborn screening for congenital adrenal hyperplasia, J. Clin. Endocrinol. Metab. 89 (2004) 3687–3693.

[54] E. Schwarz, A. Liu, H. Randall, et al., Use of steroid profiling by UPLC–MS/MS as a second tier test in newborn screening for congenital adrenal hyperplasia: the Utah experience, Pediatr. Res. 66 (2009) 230–235.

[55] S. Cristoni, D. Cuccato, M. Sciannamblo, et al., Analysis of 21-deoxycortisol, a marker of congenital adrenal hyperplasia, in blood by atmospheric pressure chemical ionization and electrospray ionization using multiple reaction monitoring, Rapid Commun. Mass Spectrom. 18 (2004) 77–82.

[56] N Janzen, M. Peter, S. Sander, et al., Newborn screening for congenital adrenal hyperplasia: additional steroid profile using liquid chromatography–tandem mass spectrometry, J. Clin. Endocrinol. Metab. 92 (2007) 2581–2589.

[57] N. Janzen, S. Sander, M. Terhardt, M. Peter, J. Sander, Fast and direct quantification of adrenal steroids by tandem mass spectrometry in serum and dried blood spots, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 861 (2008) 117–122.

[58] M. Peter, N. Janzen, S. Sander, E. Korsch, F.G. Riepe, J. Sander, A case of 11beta-hydroxylase deficiency detected in a newborn screening program by second-tier LC–MS/MS, Horm. Res. 69 (2008) 253–256.

[59] M. Rauh, Steroid measurement with LC–MS/MS in pediatric endocrinology, Mol. Cell Endocrinol. 301 (2009) 272–281.

[60] C. Rossi, L. Calton, G. Hammond, et al., Serum steroid profiling for congenital adrenal hyperplasia using liquid chromatography–tandem mass spectrometry, Clin. Chim. Acta 411 (2010) 222–228.

[61] G. Rohle, H. Breuer, External quality control for hormone determinations in the Federal Republic of Germany, Horm. Res. 9 (1978) 450–454.

[62] W. Rosner, R.J. Auchus, R. Azziz, P.M. Sluss, H. Raff, Position statement: utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement, J. Clin. Endocrinol. Metab. 92 (2007) 405–413.

[63] W Rosner, H. Vesper, Preface. CDC workshop report improving steroid hormone measurements in patient care and research translation, Steroids 73 (2008) 1285.

[64] O.P. Soldin, H. Sharma, L. Husted, S.J. Soldin, Pediatric reference intervals for aldosterone, 17α-hydroxyprogesterone, dehydroepiandrosterone, testosterone and 25-hydroxy vitamin D3 using tandem mass spectrometry, Clin. Biochem. 42 (2009) 823–827.

[65] S.J. Soldin, O.P. Soldin, Steroid hormone analysis by tandem mass spectrometry, Clin. Chem. 55 (2009) 1061–1066.

[66] R.J. Singh, Validation of a high throughput method for serum/plasma testosterone using liquid chromatography tandem mass spectrometry (LC–MS/MS), Steroids 73 (2008) 1339–1344.

[67] S.S. Tai, B. Xu, M.J. Welch, K.W. Phinney, Development and evaluation of a candidate reference measurement procedure for the determination of testosterone

in human serum using isotope dilution liquid chromatography/tandem mass spectrometry, Anal. Bioanal. Chem. 388 (2007) 1087–1094.
[68] H.W. Vesper, S. Bhasin, C. Wang, et al., Interlaboratory comparison study of serum total testosterone [corrected] measurements performed by mass spectrometry methods, Steroids 74 (2009) 498–503.
[69] L.M. Thienpont, K. Van Uytfanghe, S. Blincko, et al., State-of-the-art of serum testosterone measurement by isotope dilution–liquid chromatography–tandem mass spectrometry, Clin. Chem. 54 (2008) 1290–1297.
[70] U. Ceglarek, L. Kortz, A. Leichtle, G.M. Fiedler, J. Kratzsch, J. Thiery, Rapid quantification of steroid patterns in human serum by on-line solid phase extraction combined with liquid chromatography–triple quadrupole linear ion trap mass spectrometry, Clin. Chim. Acta 401 (2009) 114–118.
[71] D.R. Bunch, A.Y. Miller, S. Wang, Development and validation of a liquid chromatography–tandem mass spectrometry assay for serum 25-hydroxyvitamin D2/D3 using a turbulent flow online extraction technology, Clin. Chem. Lab. Med. 47 (2009) 1565–1572.
[72] S.S. Tai, B. Xu, M.J. Welch, Development and evaluation of a candidate reference measurement procedure for the determination of progesterone in human serum using isotope-dilution liquid chromatography/tandem mass spectrometry, Anal. Chem. 78 (2006) 6628–6633.
[73] G.D. Carter, 25-Hydroxyvitamin D assays: the quest for accuracy, Clin. Chem. 55 (2009) 1300–1302.
[74] G.D. Carter, J.C. Jones, Use of a common standard improves the performance of liquid chromatography–tandem mass spectrometry methods for serum 25-hydroxyvitamin-D, Ann. Clin. Biochem. 46 (2009) 79–81.