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LC–MS/MS analysis of steroids in the clinical laboratory

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ABSTRACT

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) is a powerful tool that is changing the way we analyse steroids in the clinical laboratory. It is already opening up the field of steroid analysis in endocrinology and is providing new applications for individual steroids and panels of steroids in different clinical conditions. LC–MS/MS is now well-accepted technology and is increasingly being used to replace problematic immunoassay methods because of greater sensitivity and specificity. Improved sample preparation, modern chromatography methods, and sensitive, faster scanning mass spectrometers have all played a role in improving LC–MS/MS. LC–MS/MS is also playing a key role in improving the quality of assays through the development of reference measurement procedures, characterisation of reference materials and multi-site calibration programmes. There is increasing interest in multiplexing steroid assays into panels of diagnostic tests to aid and improve the diagnosis and monitoring of disease.

1. Introduction

Liquid chromatography tandem mass spectrometry (LC–MS/MS) is increasingly becoming the method of choice for steroid hormone analysis due to small sample volumes, fast analysis times, improved specificity compared to immunoassays, and the ability to multiplex panels of analytes. Applications for routine clinical methods for steroid analysis are now becoming possible because of improvements in sample preparation technology, liquid chromatography column technology, and mass spectrometer design [1]. Mass spectrometry (MS) enables the measurement of many steroids across a wide concentration range and provides us with a great opportunity to improve standards in steroid analysis. This is already starting to open up the field of steroid analysis in endocrinology and is providing new applications for individual steroids and panels of steroids important in different clinical conditions. Mass spectrometry is a well-known technique in clinical laboratories and the introduction of gas chromatography linked to a mass spectrometer (GC–MS) over 20 years ago has contributed greatly to our current knowledge on inborn errors of steroid metabolism. Urinary steroid profiling is an analytical technique that remains unrivalled for the unequivocal identification of nearly all steroid metabolic disorders [2]. GC–MS is an ideal steroid metabolomic tool that still leads to discoveries of novel steroids, even for such well-characterised conditions as 21-hydroxylase deficiency [3]. GC–MS has been largely superseded in the past 10 years by liquid chromatography tandem mass spectrometry (LC–MS/MS) because derivatisation is not needed to make steroids volatile in the gas phase, as is the case with GC–MS and it is much quicker and simpler to use in routine laboratories. Despite the increased complexity of sample preparation, it is recognised that GC–MS still has an important part to play in determining the steroid metabolome [4]. However, LC–MS/MS has also been applied to the area of steroidomics which describes the untargeted analysis of steroids in a sample. This approach is some way from routine use because of the lack of suitable reference compounds for many steroid metabolites to fully exploit the technology and the poor ionisation of some steroids. The current analytical methods for assessing steroid changes have been reviewed and compared to the steroidomics approach [5], but for the routine targeted determination of individual or panels of steroids, LC–MS/MS is now the method of choice for use in the clinical laboratory [6–9]. (See Table 1.)

LC–MS/MS has much to offer the clinical laboratory because the limitations of steroid measurement by direct immunoassay (IA) have been well documented [10,11] and recently prompted a recommendation from the editors of an endocrine journal to avoid using such assays and use instead LC–MS/MS for the measurement of sex steroids [12]. This resulted in a review of journal policies on hormone measurements, but while originally focussing on sex steroid assays, it became evident that other steroid assays also suffered from poor performance [13]. The Endocrine Society now recognises that high-quality, well-validated steroid assays are required to improve the quality of work. [14]. LC–MS/MS is well placed to meet these demanding challenges and this review will attempt to highlight the many strengths of LC–MS/MS for the analysis of steroids in the clinical laboratory.

1.1. Sample preparation

Sample preparation is essential before LC–MS/MS as a minimum requirement to remove protein which would otherwise block injectors

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Table 1
LC–MS/MS methods for measurement of steroids.

Reference	Analyte	Mode	LOQ ng/mL	Column	Time,min	Number of steroids	Sample type, volume	Sample prep
[52]	A-THF, E,F, THE,THF,	neg	0.3, 3, 0.6, 0.6, 0.3 ng/mL	Biphenyl	18	5	Urine, 2 mL	SPE
[53]	E, F, THE, THF, A-THF	pos	0.1, 0.1, 1.0, 1.0, 1.0 ng/mL	C18	18	5	Urine, 1 mL	SPE
[54]	E, F, The, THF, A-THF	pos	0.05, 0.05, 0.1, 0.1, 0.1 ng/mL	C18	25	5	Urine, 0.48 mL	PP
[17]	F	pos	5 ng/mL	C8	2	1	Serum, 0.02 mL	PP
[49]	F, E, DHEAS	neg	2 pg/mg	C18	20	3	Hair, 20 mg	SPE
[63]	A	neg	0.01 ng/mL	C18	4.4	1	Plasma, 0.25mL	LLE/2D
[64]	A	neg	0.01 ng/mL	PFP	5	1	Plasma, 250 uL	SLE
[77]	17βE2, 17αE2, E1	pos	62 pg/mL	Phenyl hexyl	2.7	3	Plasma	LLE
[82]	E1, E2	neg	0.5, 0.3 pg/mL	C8	9	2	Plasma, 0.5 mL	LLE/SPE
[81]	E2	pos	0.5 pg/mL	Phenyl hexyl	8	1	Plasma	Dialysis
[79]	E3	pos	0.05 ng/mL	C18	16	1	Serum	LLE
[108]	Salivary T	pos	2.2 pg/mL	C8	9	1	Plasma, 0.2 mL	SPE
[113]	ADTG, Etio-G, androstandiol 17G	neg	4.0, 4.0, 0.1 ng/mL	C18	6	3	Serum, 0.2 mL	SPE
[91]	T, A4, DHEA	pos	0.03, 0.03, 0.3 ng/mL	C8	9.1	3	Serum, 0.1 mL	LLE
[114]	DHEAS, Preg S, AlloPreg S, E1S, ADTS	neg	0.25 ng/mL	C18	9	5	Serum, 0.075 mL	WAX SPE
[107]	T	pos	0.07 ng/dL	C8	5	1	Saliva, 0.25 mL	LLE
[106]	T	pos	1.4 pg/mL	C18	5	1	Saliva, 0.25 mL	LLE
[96]	T, A4, DHEA	pos	10, 10, 50 ng/L	C18	3	3	Serum, 0.2 mL	LLE
[25]	E1S, E2S, A4S, 16OH DHEAS, PregS, DHEAS	neg	0.08 ng/mL	C18	6	6	Serum, urine, cells	SPE
[25]	E1, E2, E3, 16OH DHEA, A4, DHEA, T, 17OH Preg, P, Preg	pos	0.5 ng/mL	C18	10	11	Serum, urine, cells	SPE
[109]	E2, F, E, T, P, DHEA, B	pos	<5 pg/mL	C18	5.2	7	Saliva, 0.1 mL	Online SPE
[109]	A4, F, E, T, P, DHEA, B	pos	<0.9 pg/mg	C18	5.2	7	Hair, 30mg	Online SPE
[99]	E1, E2, T, DHT, DHEA, A4, 5 Diol.	pos	1 pg/mL	C18	6	7	Serum, 0.5mL	LLE
[117]	A, A4, androst, B,F, E, S, 11 deoxyB, DHEA, DHEAS, E2, E1, etiochol, P, T.	pos	0.01 ng/mL	C18	20	16	Serum	SPE
[118]	F, B, 11DOC, A4, 21OHP, T, 17OHP, P	pos	0.86, 0.24, 0.38, 0.15, 0.04, 0.08, 0.05, 0.25 nmol/L	C18	10	8	Serum, 0.2 mL	LLE
[120]	F, E, prednisolone, prednisone, 11DOC, dexamathasone, T, A4, P.	pos	2, 1.6, 0.5, 1, 0.1, 0.1, 0.02, 0.1, 0.2, 0.1 nmol/L	C18	6.1	9	Serum, 0.085 mL	LLE
[123]	17OHPreg, 17OHP, A4, DHEA, T, Preg, P	pos	0.01–0.1 ng/mL	c18	7	7	Serum, 0.15 mL	LLE
[124]	B, 11 DOC, 11deoxyB, 17OHP,P.	pos	0.18, 0.3, 0.06, 0.18,0.12 ng/mL	c18	6	5	Serum, 0.25 mL	LLE

and column frits. Sample preparation techniques including protein precipitation(PP), solid phase extraction (SPE), and liquid–liquid extraction (LLE) are deemed necessary to reduce matrix effects and in some instances to improve sensitivity by allowing sample concentration [6–8]. Protein precipitation works well for steroids in high concentration such as DHEAS and cortisol when a high sample dilution effectively removes matrix while maintaining acceptable signal-to-noise ratios. For less abundant steroids, SPE and LLE give a cleaner extract and can be used to concentrate the sample to improve sensitivity but may not be good for more polar steroids. These methods have been extensively reviewed [7,8], but there are newer techniques becoming available. SLE is a variant of LLE which uses similar strong solvents, e.g. dichloromethane and ethyl acetate. Sample is adsorbed onto a diatomaceous earth support in a 96-well plate and solvent is then passed through the packed bed. The large surface area of the packing material affords good separation and only hydrophobic compounds are eluted leaving salts and proteins in the packing material. Eluent is directly collected into a 96-well collection plate for blowing down. The method is fast, gives clean samples, and does not suffer from emulsion formation often found with LLE [8].

The AC extraction plate (Tecan, Switzerland) is a newer device also in 96 deep well microplates but contains a coating on the inner surface of the well to extract sample. The plate is washed to remove matrix effects, including phospholipids, and analytes are eluted using selective solvents and can then be directly injected from the plate. The system is more amenable to automation than SPE or SLE because there are fewer steps and samples transfers. Comparison with SLE has shown lower extraction efficiency but lower noise [15].

Steroids and their metabolites are a heterogeneous mix of compounds with wide differences in chemical properties ranging in polarity. Thus, there is no single sample preparation technique that can be successfully applied for sample clean-up [6–8]. Choice of extraction method may be dictated by the availability of exhaust devices such as fume hoods and prevailing health and safety regulations. PP is certainly simple and inexpensive, does not require expensive exhausts, and is extremely powerful when coupled with on-line SPE. Newer off-line SPE plates are designed to minimise tedious washing steps and to improve throughput and ease of use. In general, the use of SPE and SLE in a 96-well plate format reduces sample transfers and makes sample handling much simpler, reducing the time to prepare a plate to less than 1 h even with manual pipetting. Use of robotic pipetting stations is becoming popular to improve assay precision and provide positive sample identification but it can be difficult to configure pipetting stations for multiple tube types; as a consequence, these instruments work best with only one or two different types of tube.

1.2. LC columns

Traditionally, LC–MS methods for steroid analysis have used standard, relatively large HPLC columns packed with 3 or 5 μm particles [7,16]. In recent years, the introduction of higher efficiency small particle size columns has been made possible by the introduction of ultra high-performance liquid chromatography (UPLC) technology to cope with the much higher back pressures generated by these columns. These smaller particulate columns often packed with sub 2 μm particles offer increased separation efficiency, increased sensitivity, lower solvent usage, and faster analytical methods [8]. Good chromatography is essential for the development of robust steroid methods and it is important to ensure the adequate separation of isobaric compounds, i.e. with the same mass-to-charge ratio. Examples of isobaric compounds include 21 deoxycortisol, 11 deoxycortisol, and corticosterone, which all share the same nominal molecular weight and are indistinguishable in the mass spectrometer [7,8]. For those laboratories without access to UPLC equipment, the use of fused core particle technology columns may provide nearly as much chromatographic separation but with much lower system pressures (Kinetex™ Phenomenex, UK; Accucore™

Thermo Scientific, UK; Cortex™ Waters, UK). Core shell columns also have the advantage of faster re-equilibration after gradient formation which can reduce injection-to-injection times and give faster throughput [17]. Throughput is important for productivity and can influence turnaround times, e.g. an assay with injection-to-injection times of 5 minutes will permit the overnight run of two full 96-well plates without encroaching into the next day's work. In this way, routine work can be carried out overnight leaving the instrument free for development work during the day.

The choice of column chemistry now exceeds the tried and tested C18 packing materials, with phenyl biphenyl and pentafluorylphenyl phases particularly useful for the separation of isobaric compounds [18–20]. The differing selectivity shown by these newer phases is due to π–π interaction between the aromatic ligand attached to the stationary phase and the steroid molecule.

1.3. Mass spectrometry

The most common detectors in use for steroid analysis are triple quadrupole instruments also known as tandem mass spectrometers. Most recent advances in mass spectrometry systems used in clinical laboratories have been evolutionary rather than revolutionary. Incremental changes have been made to improve specificity, sensitivity, and stability by improvements in ionisation efficiency, ion transfer and detector design [1,21].

The differences in instrument sensitivity and performance are wide both within and between vendors. There are a number of vendors that supply excellent instruments but it should be noted that each vendor will have several different models ranging from entry level to high-grade research instrument. It is difficult to make general comparisons of instrument performance between vendors because instrument sensitivity, one of the key performance criteria, is usually analyte specific and will vary between compounds, generally because of differences in ionisation source design.

The scan speeds of modern instruments are faster and permit optimal peak integration for multiple transitions, even for very narrow chromatography peaks produced by UPLC instruments. Faster scanning instruments thus make possible the analysis of multiple steroids in one run and crucially the measurement of multiple transitions to achieve greater analytical security [7].

Measurement of both quantifier and qualifier ions is now an absolute requirement to compensate for any possible interference in the assay to improve specificity [7,22]. This is especially the case for steroids because of the similarity in structure between different compounds, exacerbated by ionisation changes such as water loss, M + 2 isotope effects, and adducts formation which can all increase the risk of isobaric interference [23]. Steroids can form adducts with different mobile phase additives and fragmentation of the precursor ion invariably differs in positive and negative ionisation mode, the ionisation of steroids and metabolites has been recently reviewed [24]. Multiplexing steroids in the same analytical run can therefore be difficult because of the need to remove isobaric interference chromatographically but also the huge concentration differences between some steroids, differences in ionisation mode, and differences in ionisation characteristics [24]. Formation of adducts, or conversely cleavage of side chains, can occasionally convert even quite disparate steroid moieties into structurally very similar isobars/isomers of each other, and in these cases, better chromatography is necessary to resolve interference [25].

The most common ionisation techniques are electrospray ionisation (ESI) and atmospheric chemical ionisation (APCI). ESI is an efficient method for converting analytes in the column eluate into gas phase ions suitable for analysis by the processes of desolvation and ion desorption. APCI uses a much hotter ionisation source with a corona discharge region where gas phase chemical reactions take place. APCI gives a more selective ionisation and importantly for some compounds, it has been shown to have much lower matrix effects [7], but ESI is still popular for steroid analysis and any differences in performance compared to APCI for steroid analysis but may be steroid dependent

[26]. Atmospheric pressure photoionisation (APPI) is a complementary technique to APCI and ESI, whereby ion formation takes place through photoionisation of a dopant, which ionises non-polar molecules through proton transfer and charge exchange [7]. APPI may be beneficial for molecules with low polarity, including many steroids and its main advantages, along with APCI are reduced matrix effects and more selective ionisation.

Gains in instrument sensitivity can be achieved by increasing the aperture of the sampling cone, thus allowing more ions into the instrument. Unfortunately, this approach also lets in more interfering substances which invariably compromise the signal-to-noise ratio, a problem that some manufacturers have addressed using offset ion guides after the sampling cone to remove interference. The triple quadrupole instrument consists of two mass analysis quadrupoles separated by a collision cell and further gains in sensitivity have arisen from improved transmission of ions through the mass detectors. The strengths of this instrument lie in the ability to filter ions of a pre-determined mass in the first quadrupole and then fragment these ions in a compound-specific way in the collision cell. Monitoring these fragment ions, filtered in the second quadrupole, which can only have come from the parent ion, gives rise to high analytical specificity. It should be noted that when operating at near maximum resolution, the tandem mass spectrometer will only separate ions with one Dalton resolution so interference from structurally related compounds or metabolites is still possible and this can only be reduced by performing good sample clean-up and chromatography. Instrument parameters chosen for analysis are determined by careful tuning and both positive and negative ion mode ionisation should be explored.

Assay sensitivity can be greatly affected by mobile phase constituents and while negative and positive ionisation can be used in the same run, formic acid which may be necessary to generate positive ions will suppress negative ionisation and will compromise sensitivity. Steroids without the 3 oxo-4-ene structure are difficult to fragment [1] and derivatives have been used to improve sensitivity in some cases [7], although newer instruments have sufficient sensitivity to measure these without derivatisation. [8].

Methods can be tailor made to suit a given set of circumstances including sample type, throughput, and sensitivity. Development of a method will require judicious use of sample clean up, mobile phase composition, and chromatography to minimise matrix effects and get the best possible results. LC–MS/MS is reference laboratory technology but clinical laboratories do not have the luxury of time and need to get results out quickly while still retaining quality. Validation of a LC–MS/MS assay requires investigation into a number of parameters such as selectivity, sensitivity, stability, reproducibility, and matrix effects; this should be completed during method development for each steroid [22].

1.4. Calibration

Standardisation or harmonisation issues (Annesley 2009 21) still remain a problem because there is a lack of available standard reference materials. Improving the quality of assays by making them traceable to a certified standard is not yet available for all steroid hormones, but is an important goal and work to rectify this has already started with initiatives such as the Hormone Standardization Program (HoSt) [27].

Good calibration practice is essential to the development of reference ranges applicable to all assays that can be shown to align to the reference method and will enable harmonisation of test results. There is still a long way to go because currently only testosterone (T), estradiol (E2), and 25 hydroxy vitamin D are covered by this scheme and there is a real and present need to underpin all of the commonly requested steroid methods with high-quality reference materials and reference measurement procedures. Cortisol is a highly characterised analyte

and for many years the National Institute for Standards and Technology (NIST) has provided cortisol standard reference material (SRM) to underpin assay calibration. Reference measurement procedures have been described that utilise SRM 921 for calibration, these methods have subsequently been used to produce higher-order certified reference materials that are metro logically traceable to SI units [28].

A welcome development is the production of commercially available certified reference standards ready to use in a liquid form. These are not matrix based but nevertheless enable the easier production of matrix-based calibrators in the laboratory and these are now becoming available for a wide range of steroids.

There is a dearth of commercially available serum-based calibrators for routine steroid analysis. This has been well described [7,8,21] and is still a major block to the wider roll out of LC–MS/MS, because many laboratories lack the technical skills or the time to make calibrators. This is an important issue because interlaboratory agreement can be improved up to a point if common calibration material is used [29]. Assay calibration is hugely important but may not be the whole reason for the variability between vitamin D and testosterone methods [30], poor validation of assays including variable ion suppression and choice of internal standard may also be important factors [23,31]. Subsequent experience with vitamin D assays has shown that the inter laboratory variation improved after the introduction of common calibration material but has since remained relatively constant with a between laboratory coefficient of variation of approximately 10% [32].

2. Cortisol

Cortisol is important in the diagnosis and management of the hypophyseal pituitary adrenal axis [33]. It is the major glucocorticoid in humans and it is approximately 90% bound to its complementary binding globulin (cortisol binding globulin, CBG) and 6% bound to albumin in the circulation [34]. It is the remaining 4% unbound or free cortisol concentration that exerts physiological activity. As the protein-bound fractions are too large to be freely filtered by the glomerulus, urine, saliva [33], and sweat cortisol measurements provide clinicians with a more accurate indicator of free cortisol concentrations [35].

Cortisol is not widely measured in routine laboratories using LC–MS/MS but a candidate reference method has been developed to assess the performance of routine assays [36].

This method is being used to underpin the UKNEQAS scheme for cortisol measurement (UKNEQAS, Clinical Chemistry Birmingham, and PO Box 3909, Birmingham, B15 2UE, UK) and preliminary work performed using commercially available immunoassay methods shows large variations in performance and substantial variable interference from drugs such as prednisolone and metyrapone [17,37]. Serum cortisol immunoassays are particularly susceptible to exogenous steroid interference, since these are structurally similar to cortisol and are widely used therapies for many disorders [37], although LC–MS/MS measurement of cortisol in serum is very simple and it can be easily assimilated into a routine laboratory [17].

Quantification of cortisol in saliva provides an index of the concentration of free serum cortisol [38], but cortisol at the low concentrations found in saliva is not easy to measure using some immunoassays and non-linear relationships have been found between immunoassays and LC–MS/MS precluding direct comparison between assays [39]. Methods for free cortisol in human serum have been reported and utilise ultrafiltration [38,51], although salivary cortisol/cortisone may provide as much information and are technically less challenging or expensive. Ultrafiltration is technically simpler than equilibrium dialysis (ED) and requires centrifugation of the device for 30 min at 37°C, whereas ED frequently requires an overnight step. Both techniques are challenging when low concentrations of the free steroid fraction are present but ultrafiltration is suitable for the separation of glucocorticoids [51].

Salivary assays are particularly useful in those patients with limited venous access and those with CBG polymorphisms or altered CBG concentrations [33]. Salivary cortisol is now a mainstay in the diagnosis of endogenous Cushing's syndrome [40,41] but is subject to interference from cortisol medication. In these situations, Cortisol contamination can be deduced by demonstrating normal salivary cortisone and a high cortisol-to-cortisone ratio [42] measured using LC–MS/MS.

This is because salivary cortisone is produced by the action of 11βHSD2 from endogenous cortisol and is unaffected by exogenous cortisol. This has led others to claim that salivary cortisone may be a better marker of serum-free cortisol than cortisol itself [38] and may be especially useful for monitoring patients on hydrocortisone replacement therapy [43].

Salivary cortisol and cortisone are simple to measure either by LLE [44] or SPE [45] and the response of salivary cortisol and cortisone to a synacthen challenge has been assessed for the investigation of adrenal insufficiency [46–48].

Salivary measurement may also be useful when monitoring mitotane therapy. Mitotane is an adrenocytolytic agent used in the treatment of adrenal cancer which causes adrenal insufficiency necessitating cortisol replacement therapy and the monitoring of cortisol levels. Salivary analysis shows significant interference in immunoassays for cortisol compared to LC–MS/MS when measuring samples from patients taking mitotane [49]. Cortisol is a major stress hormone and because of its ease of collection, salivary cortisol has found application in stress studies [50].

Alternative sampling strategies have shown that hair cortisol may prove to be a useful of prolonged exposure to hydrocortisone [52].

Although LC–MS/MS methods have been in use for many years for the quantitation of urinary free cortisol in the investigation of hypercortisolism [53], some believe that LC–MS/MS may be too specific because cortisol metabolites are not simultaneously measured. It is postulated that cortisol metabolites cross-react with immunoassays giving them a positive bias compared to LC–MS/MS which may improve the diagnostic sensitivity compared to LC–MS/MS albeit with worse specificity [54].

In a different clinical context, cortisol metabolites in 24 h urine samples have been used to assess the activity of 11 β-HSD1 and 11β-HSD2 for the investigation of essential hypertension and the metabolic syndrome. 11β-HSD2 is assessed using the cortisol/cortisone ration and 11β-HSD2 is assessed using the tetrahydrocortisol + allotetrahydrocortisol/tetrahydrocortisone ratio. Measurement of total metabolites using deconjugation with glucuronidase, although requiring extra steps, is technically easier because of the much greater concentrations involved compared to free metabolites. However, measuring both free and conjugated steroids may give complementary information [55].

Measuring the much lower free concentrations without deconjugation saves time and reduces the chance of inaccurate results due to incomplete hydrolysis and variability in enzyme preparations. Care must be taken to separate the two stereoisomers A-THF and THF from isobaric endogenous interference after SPE [56]. This work has since been replicated using protein crash rather than SPE with similar results [57].

Analysis of a much larger panel of corticosteroids in urine has been described after metabolite deconjugation with glucuronidase and then LLE. LODs below 1 ng/mL were typically obtained for steroids with a 3 oxo-4-ene structure whereas LODs below 15 ng/mL were common for the other steroids. LOQs ranged from 1 to 50 ng/mL depending on the analyte [58]. This method has been used to study the urinary steroid metabolome in patients with acute intermittent porphyria showing a decreased output of cortisol and its metabolites [59].

2.1.1. Congenital adrenal hyperplasia

Individuals with classical CAH are known to have elevated levels of 17-α-hydroxyprogesterone (17-OHP) due to deficiency of 21-α-hydroxylase, but there may be other enzyme blocks leading to a build-up of other metabolites including 11-deoxycortisol, 21-deoxycortisol,

androstenedione, corticosterone, and 11-deoxycorticosterone [61]. Second-tier testing of these steroids, after initial dried blood spot testing for 17 OHP, shows that the positive predictive value of the screening can be improved and all false-positive tests can be eliminated [61].

Rather than just looking at metabolite concentration, it may be more useful to investigate precursor/product ratios. Thus, in classical CAH, the elevated 17-OHP/S ratio is a biomarker of diminished 21-α-hydroxylase activity, and the elevated 17-OHP/A4 ratio is a biomarker of adrenal androgen excess via increased 17, 20-lyase activity. The similar S/F ratio indicates that the rate of production via 11-β-hydroxylase and disappearance of F is maintained in CAH [62,63].

2.2. Aldosterone

Diagnosis of primary aldosteronism (PA) is made by screening, confirmation testing, and subtype diagnosis (computed tomography and adrenal vein sampling). Screening is performed using the aldosterone-to-renin ratio. Aldosterone has proven difficult to measure by LC–MS/MS because of poor ionisation in the mass spectrometer source. To overcome this inherent lack of sensitivity, it has been necessary to extract relatively large volumes of sample in order to introduce a concentration step. The main techniques adopted for use have been LLE but methods have been developed with long chromatographic run times [64,65], making them less suitable for many clinical laboratories. Simpler SPE methods have been developed [66,67], and SPE agrees well when compared to SLE, but the SLE sample preparation is simpler, less time consuming, and showed improved method sensitivity. This enabled the samples to be analysed using a less expensive mass spectrometer, while still achieving adequate sensitivity for the screening of primary aldosterone's [68].

Therapeutic intervention in cases of PA depends on the correct identification of unilateral and bilateral tumours. This is currently performed using expensive and technically challenging adrenal venous sampling techniques (AVS), but measurement of peripheral steroids has been advocated to stratify patients with PA [69–71]. Likely candidates for this include the 'hybrid steroids' 18 oxocortisol and 18 hydroxycortisol [69,71].

2.3. Oestrogens

The span of physiologically relevant E2 concentrations is wide and covers <5 pg/mL in women on aromatase inhibitor therapy to 2000 pg/mL for women undergoing ovulation induction. While suitable for measuring the higher concentrations, direct immunoassay struggles in the lower concentration range [72].

Direct E2 immunoassays lack accuracy and validity for measurement of serum E2 in men [72], children [74], postmenopausal [75,76], or aromatase inhibitor-treated women [77].

The recent Endocrine Society position statement has addressed the deficiencies of direct immunoassays for the measurement of E2 [78]. Estradiol, like aldosterone, is also difficult to measure using LC–MS/MS because it is difficult to ionise and will preferentially ionise in negative ion mode [8].

2.3.1. Derivatives

Derivatisation of the oestrogen phenolic moiety to a dansyl ester has been used to improve assay sensitivity [79–81]. The effects of exemestane, a potent and selective third-generation steroidal aromatase in activator, have been studied on 12 endogenous oestrogens and their metabolites, using a dansylation method for free oestrogens and negative ionisation for conjugates [82]. Another highly sensitive method used a new derivatisation procedure which forms analytes as pre ionised N-methyl pyridinium-3-sulphonyl derivatives. They suggested that the metabolite 4-MeO-E2 could be a potential biomarker for breast cancer cases [83].

A method for 18 oestrogens using accurate mass measurement of 1-methylimidazole-2-sulfonyl adducts was found to significantly improve the sensitivity 2–100-fold by full-scan MS and targeted selected ion monitoring MS over other derivatisation methods including dansyl, picolinoyl, and pyridine-3-sulfonyl products [84].

Derivatisation also makes it possible to measure free estradiol but dialysis is needed to prepare samples prior to dansyl derivatisation and then LC–MS/MS for accurately measuring free estradiol. The method was independent of total estradiol or sex hormone binding globulin concentrations [85].

Methods not relying on derivatisation have been unsuccessful but the discovery of increased sensitivity due to trace amounts of ammonium fluoride in the mobile phase has now made this possible [86,87], but separation of estrone from estradiol is necessary to prevent $M + H^+$ interference. The exact mechanism for this enhancement is unclear but is thought to involve the formation of a fluoride adduct which is easily cleaved to generate a greater yield of ions [88].

The role of mass spectrometry in the analysis of oestrogen metabolites in breast cancer has been reviewed, with only nine urinary steroidal oestrogens representing 90% of measured urinary oestrogen metabolites [89].

2.4. Androgens

The use of sex steroids measured using LC–MS/MS for the investigation of hyper- and hypo-androgensim has been recently reviewed [90] and when compared to LC–MS/MS, immunoassay perhaps not surprisingly, can over recover [91]. The 3-oxo-4-ene structure found in steroids, such as androstenedione, progesterone, and testosterone is amenable to protonation and confers efficient ionisation characteristics during ESI. On the other hand, the ionisation efficiencies of 3ß-hydroxy-5-ene steroids, such as DHEA and pregnenolone, and of 5 α/5ß androstane and pregnane are low because of their low proton affinities, resulting in poor responses using ESI or APCI [6]. The use of a range of derivatising reagents designed to improve the sensitivity of these poorly ionised compounds in different biological samples, including tissue, hair, and nails has been reviewed [92].

There have been a number of published methods for the multiplexing of androgens, sample preparation included SPE [93–98], derivatisation [99], and LLE [100].

Comparison of LC–MS/MS methods for testosterone, androstenedione, and DHEA between laboratories has shown good agreement. However, some of the assays had differences in standardisation, and others showed high variation [101]. Again, this highlights the need for better standardisation to improve interlaboratory agreement. In a comparison of an LC–MS/MS method against seven currently available immunoassays, while there was generally good correlation, some of the commercially available DHEAS methods showed standardisation problems and/or high imprecision. These problems may potentially have clinically adverse consequences [102].

Derivatisation of oestrogens has been successfully coupled with non-derivatised androgens; this allows the ionisation of oestrogens and androgens in positive mode without the need to change mobile phase constituents. Dansyl chloride was used to improve the sensitivity of estrone and estradiol after LLE but did not derivative the androgens present [103]. Measurement of androstenedione may be an important marker for hyperandrogenaemia in polycystic ovary syndrome [99] and has been shown to be elevated when serum testosterone is normal.

There have been a number of population-based reference intervals published using LC–MS/MS [104–106]. However, it has been postulated that different cutoff values should be considered depending on the clinical/investigative setting, e.g. the large overlap invariably found in testosterone levels between controls and PCOS subjects may be caused by applying inappropriately high cutoffs. This study recommended a lower serum total testosterone cut of 1.24 nmol/L in the clinical setting,

when there is a high pre-test probability of disease, to improve the negative predictive value [107].

Methods for salivary testosterone have been developed as a surrogate for serum-free testosterone in men [108] and in the more technically challenging samples from women [109,110]. Passive drool is the ideal method of collection for salivary testosterone because swab devices have been shown to cause problems [111].

Salivary testosterone has been studied in female-to-male transgender adolescents during treatment with intra-muscular injectable testosterone esters [112], but a recent study shows that although salivary testosterone correlates with equilibrium dialysis testosterone in men, this is not the case in women [111]. Testosterone is thought to bind to salivary proteins which substantially affect the low salivary testosterone found in women but not the higher salivary testosterone found in healthy adult men. This explains why free serum testosterone measured after using equilibrium dialysis is directly comparable to salivary testosterone in males but not females [111].

Testosterone and cortisol have been measured separately in saliva but the simultaneous measurement of estradiol, cortisol, cortisone, testosterone, progesterone corticosterone, and DHEA has also been demonstrated after online SPE [113].

2.5. Multiplexed analysis

Adrenocortical carcinoma is rare but differentiation from adrenal adenomas presents a diagnostic challenge in patients presenting with adrenal incidentalomas. The investigation of incidentally discovered adrenal masses by routine imaging techniques is thus becoming a clinical problem. Urine steroid metabolomics is a sensitive and specific biomarker tool for the investigation of disease which has been used to discriminate between benign and malignant adrenal tumours. Steroid profiling of 32 steroids using GC–MS identified nine candidate discriminatory steroids that gave comparable sensitivity and specificity to the full profile [114], all of which could be measured using LC–MS/MS.

The classical approach to steroid measurement in urine is to deconjugate the steroids before analysis, but hydrolysis with β-glucuronidase presents some limitations that may result in the underestimation of some conjugates. Differences in the hydrolysis efficiency with different β-glucuronidase preparations have been documented for steroids and the reaction conditions are compound specific. It has been shown that it may be better to measure some conjugates directly without deconjugation. [115].

The role of direct measurement of androgen glucuronides and its possible role in the study of breast and prostate cancer have been reviewed. The glucuronides are acidic, are easily extracted using SPE, and are easily ionisable in negative ion mode [116]. However, baseline separation of serum androsterone glucuronide, etiocholanolone glucuronide, and androstan-3a, 17b diol 17-glucuronide is mandatory to avoid isotopic interference [117].

Combination of conjugated and unconjugated steroids in serum and other body fluids has been demonstrated using SPE, but [26] the choice of SPE used can influence protein binding of some compounds resulting in inaccurate measurement. Development of a combined assay for 5 steroid sulphates showed that bias in the estrone sulphate assay could be effectively eliminated by using weak anion exchange SPE [118].

Incidentalomas are generally hormonally inactive benign adenomas but some patients show some degree of hypercortisolism. The patients with subclinical hypercortisolism (SH) cannot be phenotypically defined as Cushing's but nevertheless they may be at risk of severe outcomes. A steroid profile in serum showed that the androgens DHEA and androstenedione showed good accuracy in predicting hypercortisolism and the SH patients also showed increased production of 21deoxycortisol and 11 deoxycorticosterone after ACTH stimulation [119].

Multiplexed steroids have also been used in the investigation of PCOS [120], Addison's disease [121], and developing paediatric reference ranges [122] and in the study of endometriosis [123].

There is a commercial kit available for multiplexing steroids (Biocrates kit method) which uses SPE with 2 solvents to extract DHEAS for a separate run [121].

A novel extraction method using hollow fibre liquid phase microextraction has also been developed to extract 16 steroids form a range of fluids. There are currently no commercially available devices but the technique is said to perform better than PP but it has not been tested against other extraction methods [124].

In a bid to improve adrenal vein sampling (AVS) for the detection of primary aldosteronsim, methods for multiple steroids offer the possibility of better steroid markers than cortisol for assessing positioning of AV sampling catheters [125–127].

Differential ion mobility has been used to remove isobaric interference from progesterone and 11 deoxycorticosterone to study levels of these hormones in postmenopausal women. The specificity gained facilitated the use of a simpler sample clean up procedure, decreasing run times and increasing speed of analysis [128].

2.5.1. Pre-analytical concerns

Steroid concentrations may vary throughout the day and also throughout the menstrual cycle. Diurnal variation occurs with almost all steroids except for progesterone [129] and correct timing of sample collection for steroids is necessary to allow correct interpretation of results. The development of age- and sex-specific reference ranges for samples taken both in the morning and the evening is recommended, otherwise any results are of dubious value. Apart from the well-described surges in progesterone and oestradiol which occur during the menstrual cycle, the concentration of other steroids, notably T, A4, and 17HPRG also increase during the follicular phase [120], thus making it prudent to take samples for sex steroids in the follicular phase of the menstrual cycle.

3. Summary

Improvements in chromatographic and mass spectrometry hardware have been rapid over the past decade and the roll out of steroid assays into routine practice and away from the research and specialist laboratory has increased. Many of the barriers to introduction of LC–MS/MS have been overcome, methods have become simpler, faster, and results can be turned around in some cases just as fast as with immunoassays. The main thrust of early development work has seen the replacement of poorly performing immunoassays with high-quality LC–MS/MS methods but the next phase will undoubtedly focus on multiplexed assays and the development of clinically relevant panels of steroids to aid in the diagnosis and monitoring of disease. This approach is already being guided by metabolomics screening, whereby candidate steroids for inclusion in diagnostic panels can be identified.

Increasingly stringent criteria for laboratory accreditation mean that all methods must be traceable to certified calibrators. Further development of LC–MS/MS in clinical laboratories must therefore be underpinned by access to certified reference materials. It cannot be overstated that calibration is vitally important to enable harmonisation between laboratories and the use of common well-characterised reference ranges. Calibration, including production of easy-to-use field calibrators and quality control materials, is a central issue holding back LC–MS/MS analysis of steroids in routine laboratories. Thorough assay validation is also vitally important to ensure that methods destined for routine use perform to a reproducibly high standard. Calibration is only part of the story because even with good calibration, assay bias can be introduced with inadequate sample clean up and poor chromatography. To overcome this, cross-validation using real clinical samples compared with other laboratories can identify bias that may not be shown up with pure or spiked serum standards. Even after thorough

validation, constant vigilance through the use of internal and external quality assessment is needed to ensure that performance is safeguarded. The introduction of LC–MS/MS–based target values in external quality assessment schemes can only improve this situation.

There are some diagnostic kits available for steroid measurement but whether these will prove cost effective for routine use remains to be seen. LC–MS/MS instruments are becoming easier to use and are more robust but still require an analytical skill set which has been lost in some laboratories. I would contest that this is a training and laboratory culture issue and an LC–MS/MS service can be maintained in a routine laboratory. Finally, accurate mass measurement instruments will become increasingly popular for steroid measurement because of the even greater specificity conferred by increased resolution. This technology is currently held back by higher costs and also because of its current inferiority compared to triple quadrupole instruments for quantitative work. This will undoubtedly change with technological improvement as we have witnessed with LC–MS/MS over the past decade.

LC–MS/MS is here to stay and because of its many advantages over immunoassay, it will remain the method of choice for ensuring the quality of steroid assays used in the diagnosis and monitoring of endocrine disease.

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