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LC-MS/MS the First 20 years: A Personal View

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Clinical LC-MS/MS applications have been in routine service for just over 20 years. The introduction of electrospray ionisation first allowed development of new-born screening methods, but it was not until the start of the new millennium when routine applications for immunosuppressant drugs and then steroids began to emerge. Method development was driven by the increased sensitivity and specificity offered by LC-MS/MS over existing immunoassay (IA) and HPLC methods. Compared to HPLC, the new LC-MS/MS methods were faster, sample preparation was often simpler and drugs such as ciclosporin which have no chromophores could be measured for the first time using chromatographic methods. Immunosuppressant drugs continue to be measured by LC-MS/MS in the larger transplant centres and methods have now evolved to measure finger prick samples collected by the patient at home. This is proving to be a useful strategy in the COVID era with many clinics being performed remotely.[1]

Vitamin D became an early target for analysis because of the poor performance observed across EQA schemes, mainly due to cross-reactivity with metabolites, but the huge workload has become unmanageable for some departments and many have reverted to IA methods. This has highlighted the main deficiencies of LC-MS/MS compared to established IA methods: lack of automation, expensive equipment and the requirement for highly trained staff. Other potential analytical targets for LC-MS/MS were the small molecules either difficult to measure or poorly measured by IA. Steroid hormones were obvious candidates and these were initially developed as single test methods, but as LC-MS/MS instrumentation has advanced, there is now a trend to move towards multi-steroid panels. Methodological differences including instrumentation, internal standards, sample extraction and chromatography still mean that interlaboratory comparability is not as good as it should be.[2] Typically, it is the steroids that are infrequently measured (such as 17OHP) that suffer the worst comparative performance between laboratories.[3]

Improvements in interlaboratory performance are ongoing and have been driven by the increasing awareness that robust thoroughly validated methods using high quality isotopic internal standards are needed.[4] The production of commercially available calibration material in recent years has also helped because routine labs find it difficult to make and maintain calibrators, especially for a wide range of analytes. Quality is also being underpinned by initiatives such as the CDC hormone standardisation programme[5] and vitamin D standardisation certification programme.[6] Having LC-MS/MS target values assigned to EQA material in these schemes allows assessment of bias from the true result and LC-MS/MS assigned target values have since been adopted by other national external quality assessment schemes, for example, UKNEQAS, but as yet only for select steroids. Improvements in formulation and performance of some IAs have been made to address specificity issues, but there remain concerns around specificity in individual methods, for example, many oestradiol IAs perform poorly when measuring in the low concentration range found in children, men, post-menopausal women and patients taking aromatase inhibitors.[7] Importantly, LC-MS/MS methods do not suffer cross-reactivity problems with the oestrogen receptor antagonist fulvestrant[8] used in breast cancer treatment.

Achieving the necessary sensitivity to measure oestradiol using LC-MS/MS has been difficult without using derivatisation, but using the most sensitive instrumentation has

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enabled the development of assays capable of meeting the required sensitivity of 3.7 pmol/L advocated by the Endocrine Society.[9] Derivatisation of analytes to improve detection limits may reduce the specificity of the assay, thus negating the benefits of LC-MS/MS analysis and should be avoided if possible.

Cortisol IA methods also exhibit specificity problems when patients are taking prednisolone or metyrapone, and some still have issues with regard to releasing cortisol from binding proteins, a problem seen particularly with women on oestrogen therapy.[10] LC-MS/MS can also provide the added value of simultaneously measuring dexamethasone with cortisol to confirm adherence and drug absorption, when interpreting dexamethasone suppression tests.[11]

Modern instruments with improved sensitivity, faster scan speeds and improved chromatography have enabled the measurement of several analytes within one run. Early examples of this of this approach include the multiplexed analysis of immunosuppressant drugs[12] and azole antifungal drugs.[13] Multiplexed LC-MS/MS panels are now opening up the field of steroid analysis and are driving studies into the investigation of polycystic ovarian syndrome (PCOS)[14] and congenital adrenal hyperplasia (CAH).[15] PCOS is a multi-phenotypic syndrome and there is evidence that measuring a profile of steroids may help in its diagnosis and can discriminate between PCOS and NCCAH.[16] There is increasing interest in newer LC-MS/MS markers such as 11 keto testosterone (11 KT) which is now thought to be the dominant androgen in children during adrenarche[17] and may prove to be an effective androgenic marker in PCOS[18] and CAH.[19,20]

Salivary steroid methods have been developed to take advantage of the convenience of saliva collection. Salivary cortisone is a good surrogate marker for serum free cortisol[21] as it is present in greater than four-fold concentrations compared to salivary cortisol, closely reflects free serum cortisol after adrenal stimulation and is unaffected by CBG changes. Post-dexamethasone salivary cortisone may be a superior discriminatory marker between healthy subjects and patients with Cushing's syndrome or with autonomous cortisol secretion than salivary cortisol[22] and salivary cortisone improves the specificity of the late night test.[23] Salivary cortisol is also superseding urinary free cortisol in the investigation of Cushing's syndrome, often because of problems with patient compliance and incomplete collections leading to inaccurate results.

LC-MS/MS is now the preferred method for measuring plasma metanephrines for the diagnosis of phaeochromocytoma. Using plasma or serum instead of urine has also been shown to be useful for measuring 5HIAA in the diagnosis of carcinoid syndrome and negates the need for dietary restrictions.[24]

In the field of drug analysis, LC-MS/MS can measure a wide range of parent compounds as well as their metabolites, many of which are easy to ionise and give good sensitivity often with crude sample preparation. The need for rapid or out-of-hours analysis has mitigated against LC-MS/MS analysis for drugs such as anticonvulsants which are more conveniently measured on random access, routine platforms. LC-MS/MS measurement of antimycotic drugs such as voriconazole is increasing in demand as are steroid drugs such as prednisolone and dexamethasone to measure adherence. Adherence studies are gaining in popularity because of the emergence of expensive biological drugs. It is becoming increasingly important to confirm that commonly prescribed drugs such as steroids are being taken before therapy is changed to a more expensive treatment.[25]

The field of proteomics is also subject to growing interest as users are becoming increasingly cognisant of the specificity issues with assays that rely on antigen–antibody reactions. However, protein analysis using LC-MS/MS remains challenging because proteins often need to be digested with proteolytic enzymes to produce manageable fragments for the mass spectrometer to measure[26] and the absence of well-characterised certified reference material for many proteins means assay standardisation is inherently difficult. Nevertheless, smaller peptides such as angiotensin 1 (Ang 1) are easily measured using LC-MS/MS and can be used in a routine hospital laboratory setting. These methods provide sensitive interference-free measurement of Ang 1 and offer a good alternative to immunoassay-based methods for the estimation of plasma renin activity[27] to help diagnose primary hyperaldosteronism or to monitor fludrocortisone replacement in CAH patients.

The recent government funded moon-shot initiative for the measurement of COVID-19 using LC-MS/MS has shown that COVID-19-related peptides can be measured with high specificity and sensitivity using antibody enrichment of peptides after protein digestion. However, some of the steps are time critical and a degree of automation is needed to improve the workflow.[28] There have been many candidate methods described for peptides and proteins but very few have made it into routine clinical practice because of their complexity.[26] Thyroglobulin and insulin analysis following protein digestion are perhaps the best examples of clinically useful large proteins measured by LC-MS/MS in routine use; however, their availability remains limited. LC-MS/MS analysis of thyroglobulin is now the method of choice for many large contract laboratories particularly in the US because it is free from thyroid antibody interference.[26]

Artificial intelligence is perhaps the biggest advance that we will see in the future; it is currently being used to interpret the data generated by multiplexing steroid testing in the discrimination of benign from malignant adrenal tumours.[29,30] Other diagnostic areas likely to benefit from this approach include the differentiation of adrenal from pituitary Cushing's syndrome and in the diagnosis of CAH.[31] There is also some promising work on the use of multiplexed LC-MS/MS assays coupled with machine learning in the investigation of Cushing's syndrome[32] and

pheochromocytoma.[33] Machine learning represents a great advance that reduces the need for expert and highly subjective interpretative skills and will become increasingly important.

The automation of mass spectrometry is still in its early stages and is only available from one or two manufacturers, but whilst the equipment is excellent, the test repertoire is limited, and the systems are expensive. For established LC-MS/MS users, financing systems from existing budgets means sacrificing staff or LC-MS/MS instruments, and as a result, uptake for the technology has been slow. This is not surprising because the introduction of automation for IA methods 30 years ago followed a similar course. Other vendors are developing automation and the situation will change, as it did with IA, with increased competition and reduction of costs with volume sales.

Many would view LC-MS/MS as a powerful analytical tool that has permitted the development of novel methods and provided an interface with clinical researchers to improve diagnostic testing. However, some have had a poor experience with this technology because in reality to make it work effectively requires a lot of support from enthusiastic staff, and this may not always be the case in a routine laboratory struggling for resources. Automation may eventually provide the solution to this problem, but it is difficult to see how it would cope with the more difficult assays requiring some form of enrichment/concentration step to improve sensitivity. Improvements in sensitivity have been impressive over the past 20 years mainly due to improvement in ion transmission and detection, but the main problem with mass spectrometry is in the poor efficiency of electrospray ionisation. Consequently, some form of sample clean-up will always be required to improve signal-to-noise in methods requiring high sensitivity.

Improvements in instrument design with improved scan speeds, greater sensitivity and robustness have improved the scope of laboratories to measure some challenging analytes over the past 20 years. It could be argued that much of the low hanging fruit has already been picked but technology drives this field and continuing improvements in instrumentation ensure that existing methods can always be improved. LC-MS/MS is also well placed to discover and exploit new diagnostic tests and will have a major part to play in clinical laboratories in the future.

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References

1. Marshall DJ, Kim JJ, Brand S, et al. Assessment of tacrolimus and creatinine concentration collected using Mitra microsampling devices. *Ann Clin Biochem*. 2020; 57:389-396
2. Buttler RM, Martens F, Ackermans MT, et al. Comparison of eight routine unpublished LC-MS/MS methods for the simultaneous measurement of testosterone and androstenedione in serum. *Clin Chim Acta* 2016; 454:112-118.
3. Greaves RF, Ho CS, Loh TP, et al. Working group 3 "harmonisation of laboratory assessment" European cooperation in S, Technology Action BMD. Current state and recommendations for harmonization of serum/plasma 17-hydroxyprogesterone mass spectrometry methods. *Clin Chem Lab Med* 2018; 56:1685-1697.
4. French D. Advances in bioanalytical techniques to measure steroid hormones in serum. *Bioanalysis* 2016; 8:1203-1219.
5. Cao ZT, Botelho JC, Rej R, et al. Impact of testosterone assay standardization efforts assessed via accuracy-based proficiency testing. *Clin Biochem* 2019; 68:37-43.
6. Erdman P, Palmer-Toy DE, Horowitz G, Hoofnagle A. Accuracy-based vitamin d survey: six years of quality improvement guided by proficiency testing. *Arch Pathol Lab Med* 2019; 143:1531-1538.
7. Middle JG, Kane JW. Oestradiol assays: fitness for purpose? *Ann Clin Biochem* 2009; 46:441-456.
8. Owen LJ, Monaghan PJ, Armstrong A, et al. Oestradiol measurement during fulvestrant treatment for breast cancer. *Br J Canc* 2019; 120:404-406.
9. Rosner W, Hankinson SE, Sluss PM, et al. Challenges to the measurement of estradiol: an endocrine society position statement. *J Clin Endocrinol Metab* 2013; 98: 1376–1387.
10. Owen LJ, Adaway JE, Davies S, et al. Development of a rapid assay for the analysis of serum cortisol and its implementation into a routine service laboratory. *Ann Clin Biochem* 2013; 50:345-352.
11. Vogg N, Kurlbaum M, Deutschbein T, et al. Method-specific cortisol and dexamethasone thresholds increase clinical specificity of the dexamethasone suppression test for Cushing syndrome. *Clin Chem* 2021; 67:998–1007.
12. Koster RA, Dijkers EC, Uges DR. Robust, high-throughput LC-MS/MS method for therapeutic drug monitoring of cyclosporine, tacrolimus, everolimus, and sirolimus in whole blood. *Ther Drug Monit*. 2009; 31:116-125.
13. Decosterd LA, Rochat B, Pesse B, et al. Multiplex ultra-performance liquid chromatography-tandem mass spectrometry method for simultaneous quantification in human plasma of fluconazole, itraconazole, hydroxyitraconazole, posaconazole, voriconazole, voriconazole-N-oxide, anidulafungin, and caspofungin. *Antimicrob Agents Chemother*. 2010; 54:5303-5315.
14. Keevil B. Steroid mass spectrometry for the diagnosis of PCOS. *Med Sci* 2019; 7:78.
15. Fiet J, LeBouc Y, Guéchot, J, et al. A liquid chromatography/tandem mass spectometry profile of 16 serum steroids, including 21-deoxycortisol and 21-deoxycorticosterone, for management of congenital adrenal hyperplasia. *J Endocr Soc*. 2017; 1: 186–201.
16. Oriolo C, Fanelli F, Castelli S, et al. Steroid biomarkers for identifying non-classic adrenal hyperplasia due to 21-hydroxylase deficiency in a population of PCOS with suspicious levels of 17OH-progesterone. *J Endocrinol Invest* 2020; 43:1499-1509.
17. Rege J, Turcu AF, Kasa-Vubu JZ, et al. 11-Ketotestosterone is the dominant circulating bioactive androgen during normal and premature adrenarche. *J Clin Endocrinol Metab* 2018; 103: 4589–4598.
18. O'Reilly MW, Kempegowda P, Jenkinson C, et al. 11-Oxygenated C19 steroids are the predominant androgens in polycystic ovary syndrome. *J Clin Endocrinol Metab* 2017; 102: 840–848.
19. Jha S, Turcu AF, Sinaii N, et al. 11-Oxygenated androgens useful in the setting of discrepant conventional biomarkers in 21-hydroxylase deficiency. *J Endocr Soc*. 2021; 5(2): bvaa192.
20. Turcu AF, El-Maouche D, Zhao L, et al. Androgen excess and diagnostic steroid biomarkers for nonclassic 21-hydroxylase deficiency without cosyntropin stimulation. *Eur J Endocrinol* 2020; 183:63-71.

21. Perogamvros I, Keevil BG, Ray DW, Trainer PJ. Salivary cortisone is a potential biomarker for serum free cortisol. *J Clin Endocrinol Metab* 2010; 95: 4951-4958.
22. Ueland GA, Methlie P, Kellmann R, et al. Simultaneous assay of cortisol and dexamethasone improved diagnostic accuracy of the dexamethasone suppression test. *Eur J Endocrinol* 2017; 176:705-713.
23. Kannankeril J, Carroll T, Findling JW, et al. Prospective evaluation of late-night salivary cortisol and cortisone by EIA and LC-MS/MS in suspected cushing syndrome. *J Endocr Soc* 2020; 4:bvaa107.
24. Adaway JE, Dobson R, Walsh J, et al. Serum and plasma 5-hydroxyindoleacetic acid as an alternative to 24-h urine 5-hydroxyindoleacetic acid measurement. *Ann Clin Biochem* 2016; 53:554-560.
25. Alahmadi FH, Keevil B, Elsey L, et al. Serum inhaled corticosteroid detection for monitoring adherence in severe asthma. *Allergy Clin Immunol Pract*. 2021; S2213-2198(21)00669.
26. Kushnir MM, Rockwood AL, Roberts WL, et al. Measurement of thyroglobulin by liquid chromatography-tandem mass spectrometry in serum and plasma in the presence of antithyroglobulin autoantibodies. *Clin Chem* 2013; 59:982-990.
27. Owen LJ, Adaway J, Morris K, et al. A widely applicable plasma renin activity assay by LC-MS/MS with offline solid phase extraction. *Ann Clin Biochem*. 2014; 51:409-411.
28. Rauh M. LC-MS/MS for protein and peptide quantification in clinical chemistry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2012; 883-884:59-67.
29. Arlt W, Biehl M, Taylor AE, et al. Urine steroid metabolomics as a biomarker tool for detecting malignancy in adrenal tumors. *J Clin Endocrinol Metab* 2011; 96:3775-3784.
30. Wilkes EH, Rumsby G, Woodward GM. Using machine learning to aid the interpretation of urine steroid profiles. *Clin Chem* 2018; 64:1586-1595.
31. Hines JM, Bancos I, Bancos C, et al. High-resolution, accurate-mass (HRAM) mass spectrometry urine steroid profiling in the diagnosis of adrenal disorders. *Clin Chem* 2017; 63:1824-1835.
32. Masjkur J, Gruber M, Peitzsch M, et al. Plasma steroid profiles in subclinical compared with overt adrenal cushing syndrome. *J Clin Endocrinol Metab* 2019; 104:4331-4340.
33. Erlic Z, Kurlbaum M, Deutschbein T, et al. Metabolic impact of pheochromocytoma/paraganglioma: targeted metabolomics in patients before and after tumor removal. *Eur J Endocrinol* 2019; 181:647-657.