Review Article

# LC-MS/MS in the Clinical Laboratory – Where to From Here?

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**Abstract**
Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has seen enormous growth in clinical laboratories during the last 10–15 years. It offers analytical specificity superior to that of immunoassays or conventional high performance/pressure liquid chromatography (HPLC) for low molecular weight analytes and has higher throughput than gas chromatography-mass spectrometry (GC-MS). Drug/Toxicology and Biochemical Genetics/Newborn Screening laboratories were at the vanguard of clinical LC-MS/MS use, but have been eclipsed by Endocrine laboratories. In USA reference/referral laboratories, most steroids and biogenic amines are now assayed by LC-MS/MS, and the technology has started to penetrate into smaller laboratories. Assays for mineralo- and gluco-corticoids and their precursors, sex steroids, metanephrines and 25-hydroxy vitamin D highlight the advantages of LC-MS/MS.

However, several limitations of LC-MS/MS have become apparent, centring on the interacting triangle of ***sensitivity – specificity – throughput***. While sample throughput is higher than for conventional HPLC or GC-MS, it lags behind automated immunoassays. Techniques which improve throughput include direct sample injection, LC-multiplexing and sample-multiplexing. Measures to improve specificity and sensitivity include sample clean-up and optimising chromatography to avoid interferences and ion suppression due to sample-matrix components. Next generation instrumentation may offer additional benefits.

The next challenge for clinical LC-MS/MS is peptide/protein analysis. The quest for multi-biomarker profiles for various diseases has largely failed, but targeted peptide and protein testing by LC-MS/MS, directed at analytical and clinical questions that need to be answered, is proving highly successful. We anticipate that this will result in similar growth of clinical protein/peptide LC-MS/MS as has been seen for low molecular weight applications.

## Introduction

Mass spectrometry (MS) is now around one hundred years old. Its basic principles were first described by Nobel laureate Sir Joseph John ('J. J.') Thomson during a lecture he gave to the Cambridge Philosophical Society in 1897,[1] followed in 1913 by experimental validation, when he separated a stream of ionised neon gas into two isotopic components by applying a magnetic and an electric field to it.[2]

During the following decades, MS underwent rapid technical development and became a widely used analytical technique in the physical and chemical sciences. However, use for biological specimens remained limited, principally because the ionisation techniques available were only suitable for fairly low molecular weight compounds (~200 Da or less), and because no good methods existed for easy introduction of biospecimens into the high-vacuum of the mass spectrometer. This situation changed dramatically in the 1980s when John Fenn (Nobel Prize 2002, for electrospray ionisation (ESI)) perfected soft ionisation of large bio-molecules by ESI and developed some of the key technologies that allowed easy sample introduction into the mass spectrometer.[3] Thereafter, the use of MS for biospecimen analysis grew swiftly, initially within research and the pharmaceutical industry, followed by clinical laboratories. The growth of MS use for biospecimen analysis accelerated further during the mid and late 1990s, which saw a shift from gas-chromatography (GC) as a mass spectrometry front-end technology to liquid-chromatography

(LC), a technique that allowed much simpler work flows and significantly faster analytical turnaround times.

Now, as we are entering the second decade of the 21st century, LC mass spectrometry (LC-MS) has become a widespread technology within clinical reference and referral laboratories world-wide, and has started to penetrate into large and medium sized hospitals and regional clinical laboratories. Its applications within medical laboratories now span hundreds of different tests, ranging from rare and highly esoteric analytes to high volume tests in drug/toxicology, newborn screening and endocrinology.[4] With growing experience, the strengths and the weaknesses of the technology as well as future opportunities and challenges have become apparent, and it seems timely to review both of these aspects. Because of our background as endocrine laboratorians, this review focuses primarily on hormone testing and is therefore not comprehensive. It does, however, cover many aspects that can be generalised to other areas of clinical LC-MS testing.

We have structured this review into four parts:

1. We will first briefly review the current mass spectrometry technology and instrumentation that dominates within clinical laboratories, i.e. LC-MS/MS and describe the types of experiments that can be performed with these instruments.
2. Next, we will give some perspective as to what factors have driven the rapid adoption of LC-MS/MS and what its current uses are.
3. Following this, we will explore several key limitations of LC-MS/MS that have become apparent during the last decade, and will highlight various approaches to address these problems.
4. Finally, we will discuss a new breed of up-and-coming applications for LC-MS/MS, which are focused on clinical, targeted measurement of peptides and proteins.

**Current Clinical Mass Spectrometry – Instrumentation and Basic Principles**

A mass spectrometer is a device that measures the mass-charge ratio of charged particles (abbreviated as m/Q, m/q, m/Z, or m/z). In its incarnation for use in bio-specimen analysis it consists of four fundamental components (Figure 1A):

1. a sample inlet device that mediates the transition of a solid or liquid bio-specimen into the gaseous phase, a fundamental requirement for all subsequent steps of mass analysis,
2. an ionisation device that ionises vaporised bio-samples,
3. an ion path that transitions ions from the near-atmospheric pressure of the source into the high vacuum of the actual mass analyser and moves them towards a detector while separating them from each other based on their m/Q,
4. an ion detector to detect and quantify ions.

Overall, there are numerous variations on how these basic components are combined into a mass spectrometer, all with their various pros and cons, strengths and weaknesses. An excellent online overview of the technology can be perused at the Scripps Centre for Metabolomics and Mass Spectrometry website.[5]

In an actual instrument, some of these basic components may be combined into a single physical part. In particular, sample inlet/vaporisation and ionisation are usually highly interdependent and coupled with each other, and, hence, reside typically in a single physical device, called the source (Figure 1B and 1C). Similarly, in some designs (e.g. certain ion traps) ion separation and detection might be accomplished by physically inseparable processes.

In addition to these fundamental components, every mass spectrometer requires two necessary auxiliary components:

1. a device to deliver samples in a suitable form to the inlet/vaporiser – this is usually some form of chromatography device, or a solid target for matrix assisted laser desorption (MALDI) and related techniques for handling of solid samples,
2. a device for signal processing/data reduction of detector signals, basically some form of analogue-digital converter (usually part of the mass spectrometer) and a combination of firmware and computer software for data reduction, display, analysis and quantification of detection events.

Combining the various options of mass spectrometer designs, front-end components and back-end components, one comes up with a bewildering array of options. However, in clinical practice almost all mass spectrometers are single- or tandem mass filter (quadrupole) designs, fronted by either GC or LC. This review is focused on tandem MS with LC front-ends and specifically triple quadrupole (also referred to as 'tandem') MS (LC-MS/MS), the instrumentation that over the last 15 years has gradually superseded GC-MS and LC with a single quadrupole mass spectrometer detector (LC-MS). LC-MS/MS now makes up most of the instruments in clinical laboratories.

A LC-MS/MS instrument consists of an (i) atmospheric pressure ionisation source, typically an ESI source (Figure 1B) or an atmospheric pressure chemical ionisation (APCI) source (Figure 1C), coupled by an (ii) ion-inlet and focusing component (Q0), which provides both transition from atmospheric pressure to vacuum and ion-focusing, into a

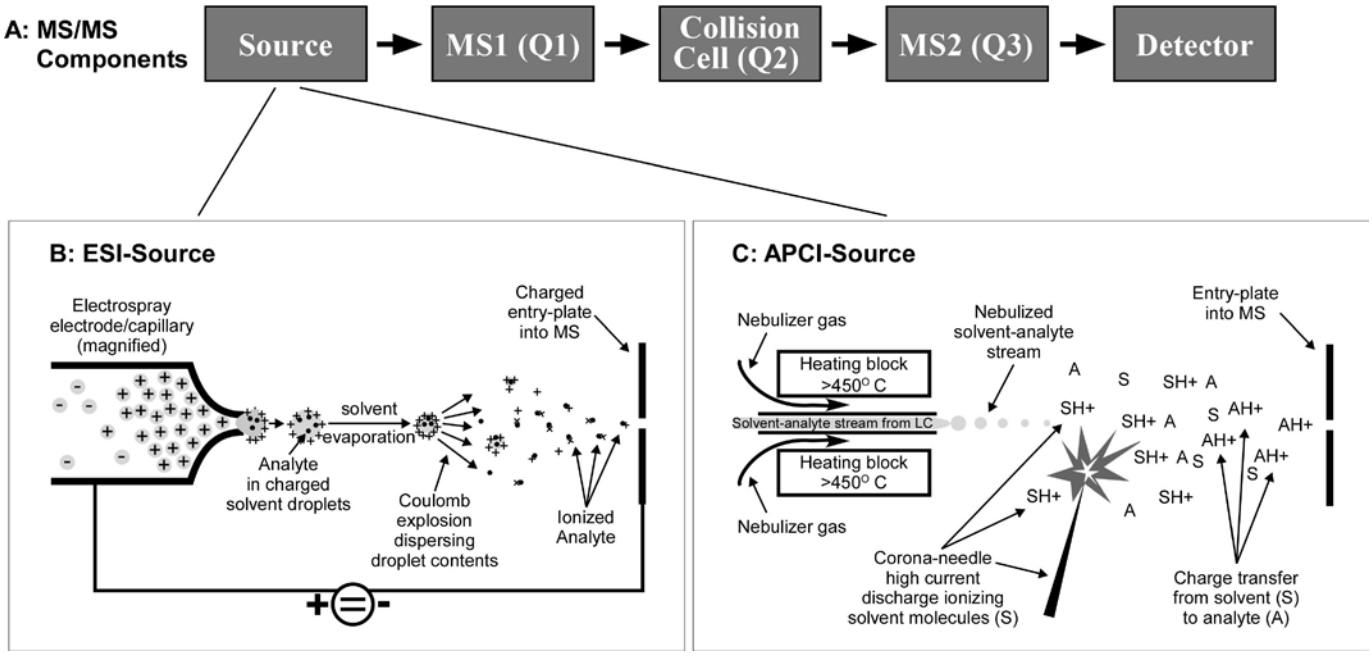


**Figure 1.** Principal components of a tandem mass spectrometer. (A) The sample is ionised in the source, passes into the 1st mass filter (Q1), then into the collision cell (Q2), followed by the 2nd mass filter (Q3), and finally the detector. (B) and (C) depict schematically the two principal types of ionisation-sources that are in use in current clinical LC-MS/MS instruments, electrospray ionisation (ESI, B) and atmospheric pressure chemical ionisation (APCI, C). In ESI, the solvent-analyte flow from the LC passes into the source through a positively charged, very narrow capillary, and gets nebulised as microscopic, positively charged solvent-analyte droplets. These droplets fly towards the negatively-charged faceplate, with solvent evaporating on the way, until they disintegrate in a Coulomb explosion, when the repulsive charge of their ionised components exceeds their surface tension. The individual ionised analyte molecules then pass through the faceplate entry hole into the mass spectrometer. In APCI, the solvent-analyte stream from the LC is vaporised by heated nebuliser gas and the polar components of the solvent(s) vapour are ionised by a high-current discharge of a Corona needle. The solvent molecules subsequently transfer their charge to ionisable analyte molecules, which pass through the faceplate entry hole into the mass spectrometer.

(iii) first mass-filtering device (Q1), which leads into a (iv) collision chamber (Q2) that can be filled with low-pressure gas for collision-induced dissociation (CID), followed by a (v) second mass-filtering device (Q3), and finally an (vi) ion-impact detector (electron multiplier) (Figure 1A).

This instrumentation allows the user to perform, within the limits of the instrument's sensitivity and mass resolution, five different kinds of experiments:

1. ***Full Scan***: Scan across the entire (or part of the) mass range of both mass filters (Q1 and Q3) while Q2 does not contain any collision gas – this experiment allows the user to see all ions contained in a sample (Figure 2A).
2. ***Product Ion Scan***: Select one specific m/Q in Q1, fill Q2 with collision gas to fragment the selected m/Q, and then scan across the entire (or part of the) mass range of Q3 – this experiment allows the user to see all fragment/product ions of the selected precursor ion (**Figure 2B**).
3. ***Precursor Ion Scan***: Scan across the entire (or part of the) mass range of Q1, fill Q2 with collision gas to fragment all ions in the scan range, and then select one specific m/Q in Q3 – this experiment allows the user, by temporal correlation of detection of the product ion and the m/Q that just preceded its detection, to determine which m/Q precursor ion(s) might have given rise to the selected product ion (Figure 2C).
4. ***Neutral Loss Scan***: Scan across the entire (or part of the) mass range of Q1, fill Q2 with collision gas to fragment all ions in the scan range, and then scan Q3 across a predetermined range that corresponds to a fragmentation-induced loss of one specific mass having

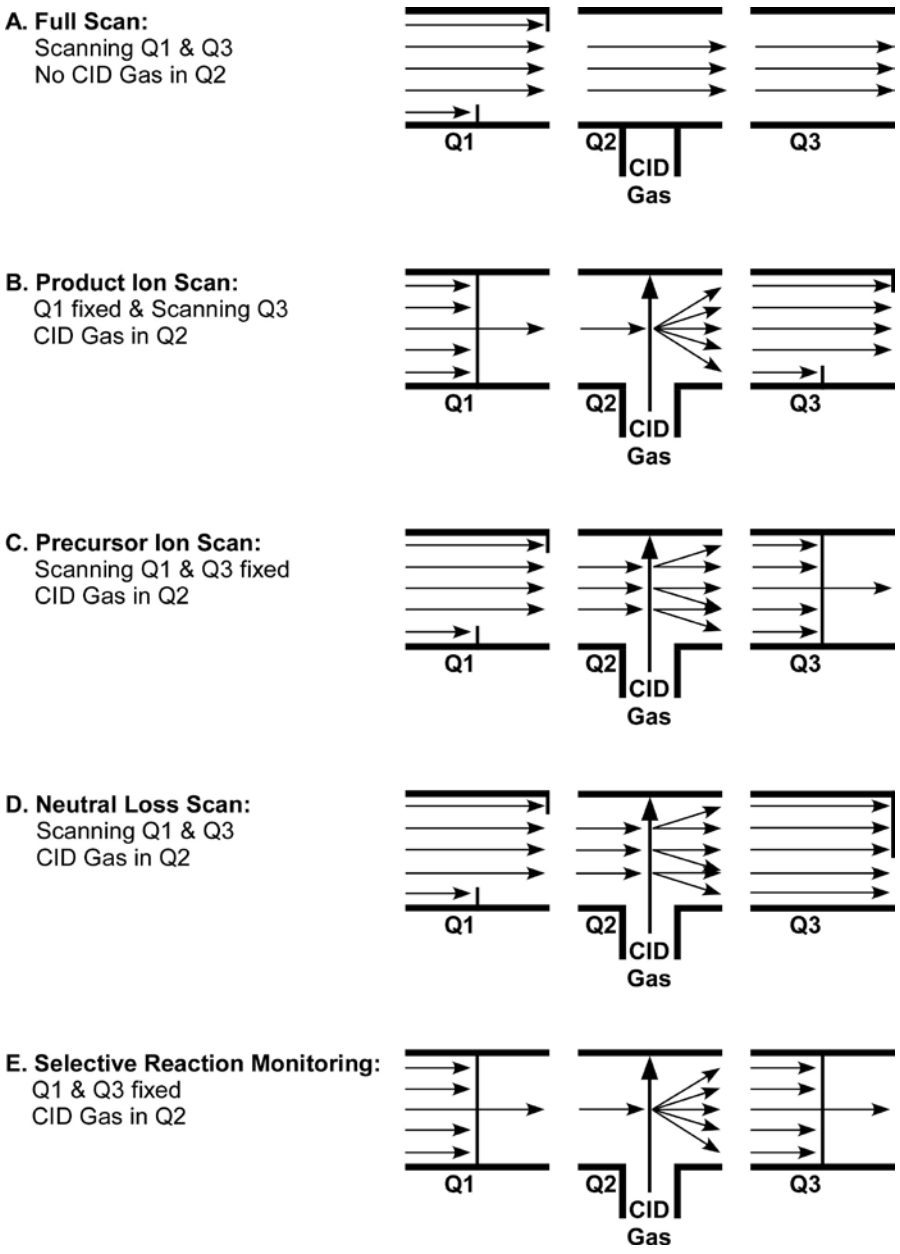


**Figure 2.** The five principal experiments that can be performed with mass filtering tandem mass spectrometers: Full Scan (A), Product Ion Scan (B), Precursor Ion Scan (C), Neutral Loss Scan (D) and Selective (Multiple) Reaction Monitoring (E). CID, collision-induced dissociation.

occurred for every potential ion in the precursor scan range. This experiment allows identification of all precursors that have lost a selected common chemical group, e.g. all precursors that have lost the mass corresponding to a methyl-group (Figure 2D).

5. ***Selective (or Multiple) Reaction Monitoring (SRM or MRM)***: Select one specific m/Q in Q1, fill Q2 with collision gas to fragment the selected m/Q, and then select one specific m/Q of these fragments in Q3 – this experiment allows highly specific detection of an analyte with the m/Q selected in Q1 that is known to fragment specifically into the product ion selected into Q3. For this specific ion-pair the detection sensitivity is also vastly increased, since the detector now processes primarily (or exclusively) this single analyte-specific ion-pair, and can do so repeatedly over several cycles (Figure 2E).

The first three of these experiments are frequently used during

method development, (i) to identify the precursor m/Q of a molecule of interest (Full Scan), (ii) to determine the m/Qs it fragments into (Product Ion Scan), and (iii) to confirm that in a biological matrix only the molecule of interest gives rise to the m/Q product ion (Precursor Ion Scan) that is selected for the final method. This final method will be SRM in most situations. For select applications, when looking for common metabolites or a family of related compounds, Neutral Loss Scans might be used instead, but well over 90% of final LC-MS/MS methods in clinical use are SRM methods.

**Current Clinical Use of Mass Spectrometry**

***Growth of Clinical LC-MS/MS Use***

As indicated in the introduction, clinical use of LC-MS/MS has seen unprecedented growth during the last 10 to 15 years. When used in SRM mode, LC-MS/MS combines high analytical specificity with high analytical sensitivity, often allowing relatively short chromatography run-times. In our own clinical specialist laboratories, which use LC-MS/MS (Drug & Toxicology, Biochemical Genetics/Newborn screening, Endocrine, Cardiovascular/Lipids, Renal), the number of LC-MS/MS instruments has grown from none in 1998 to over 60 in 2010. We performed about 2,000,000 tests by LC-MS/MS in 2010. Other large referral/reference laboratories in the USA have experienced similar increases in LC-MS/MS use. Almost all of this growth has occurred in LC-MS/MS applied to low molecular weight analytes, with the main drivers having been:

1. the limitations of immunoassays for low molecular weight compounds,
2. the easier workflows and higher throughput than conventional HPLC or GC-MS, which hitherto were the chief alternatives to immunoassays for these analytes, and
3. the often significantly lower cost of using LC-MS/MS equipment for testing, compared with the alternative techniques.

Traditional competitive radio-immunoassays (RIAs) for low molecular weight compounds require handling and disposal of radioactive materials, often require prolonged incubation times, and may need organic extraction and chromatography before the actual assay procedure to minimise non-specific and specific interferences (cross-reactivity). While converting these assays to non-radioactive detection formats addresses the issue of use of radioactivity, the other problems listed above affect alternate formats just as much as RIAs. In addition, all competitive immunoassays, even when used on automated instruments, suffer, by their very design, from a limited dynamic range. Different immunoassays for the same analyte are also often in poor agreement with each other, making patient follow-up over time, or between laboratories, as well as longitudinal studies, extremely difficult. If no commercial immunoassay is available for an analyte of interest, designing and validating a new in-house immunoassay is a major undertaking, compared with the much easier development of a new LC-MS/MS assay. Finally, reagent costs are negligible for LC-MS/MS assays, but can be substantial for esoteric immunoassays. Consequently, there can be significant cost savings when an assay is switched from an immunoassay format to LC-MS/MS technology.

Laboratories with a strong tradition of HPLC or GC-MS, which ran costly, esoteric immunoassays and had a need for rapid assay development for low molecular compounds, were therefore early adopters of LC-MS/MS, with the pharmaceutical industry being the earliest adopters, followed by Drug & Toxicology[6] laboratories and Biochemical Genetics/Newborn Screening[7] laboratories.

By contrast, Endocrine laboratories started using LC-MS/MS relatively late, in part because the concentration ranges of many endocrine analytes are fairly low, which presented a challenge to early tandem mass spectrometers. Moreover, while most specialist endocrine laboratories had a strong tradition of running esoteric immunoassay, as well as of designing new in-house immunoassays, they frequently lacked chromatographic experience. However, for steroid hormones, cross-reactivity between the numerous closely-related endogenous steroids has been a long standing challenge for endocrine laboratories, and has led to the gradual conversion of most, if not all, clinical steroid assays from RIAs/Immunoassays to LC-MS/MS assays.[8] Other analytes that have been switched to LC-MS/MS in endocrine laboratories have been the biogenic amines and vitamin D metabolites.

We will discuss some of these applications, which are in high demand and are currently being adopted by many clinical laboratories. We will not cover pre-analytical variables (sample collection) that may affect analyte stability and may have an impact on the performance of the clinical diagnostic tests.[9-11]

***Examples of LC-MS/MS in Endocrine Testing***

*Primary Aldosteronism*

Aldosterone measurement is performed to confirm autonomous aldosterone secretion by an adrenal tumor (primary aldosteronism), which is one of the more common causes of potentially curable secondary arterial hypertension. The accurate measurement of circulating aldosterone concentrations is essential for the correct diagnosis of hyper-aldosteronism. GC-MS measurement of aldosterone in serum, plasma or urine was considered the reference method.[12-14]

The GC-MS method was accurate and specific, but required derivatisation after laborious aldosterone extraction from serum or plasma. Due to this complexity of sample preparation, GC-MS methods for aldosterone were not adopted in routine clinical laboratories. Instead, the vast majority of clinical measurements were until recently performed using in-house developed RIAs. These methods were either direct measurements, or used additional sample extraction before RIA to remove interferences from plasma or serum. All these RIAs lacked standardisation or harmonisation. In our lab we have developed and validated LC-MS/MS methods for aldosterone in urine and plasma.[15] We were able to enhance the sensitivity by 10-fold using offline solid phase extraction (SPE) and concentrating the extract from one mL of serum or urine. In comparison to GC-MS, LC-MS/MS methods are more robust and can be performed in a semi-automated environment.[16,17] The lower limit of quantification is 55 pmol/L (2.0 ng/dL), with inter- and intra-day analytical precision of <10%.

*Cortisol Measurements and Cushing's Syndrome*

Cushing's syndrome is a hormonal disorder caused by prolonged exposure of the body's tissues to high levels of cortisol. Cushing's syndrome is relatively rare and most commonly affects adults aged 20 to 50. No single laboratory test is perfect for diagnosis and usually several different tests need to be performed, most of which are centred on cortisol measurements. The three tests used most commonly to diagnose Cushing's syndrome are 24-hour urinary free cortisol measurement (UFC), measurements of midnight plasma cortisol or late-night salivary cortisol, and the low-dose dexamethasone suppression test.

Historically, analysis of urinary free cortisol was the test of choice for the diagnosis of Cushing's syndrome, along with plasma cortisol and adrenocorticotropic hormone (ACTH). Although all immunoassays for UFC use liquid-liquid extraction to eliminate interfering compounds, these methods are still susceptible to interferences from cortisone and several other endogenous steroid metabolites, as well as from synthetic glucocorticoids, such as prednisolone.[18,19] Another limitation of immunoassays is the lack of an internal standard to monitor variable recovery of cortisol during the extraction process. Several recent papers comparing immunoassay and chromatographic methods for UFC measurements clearly indicate that only chromatographic methods can accurately measure UFC.[19-21] These limitations of immunoassays for UFC have led to the development of more specific methods based on liquid chromatography with ultraviolet detection (LC-UV), LC-MS, and GC-MS.[22,23] The chromatographic methods suffer from less interference, and allow simultaneous quantification of cortisone, an endogenous metabolite of cortisol. Before the tandem MS era, the GC-MS method for the quantification of cortisol had a limit of detection (LOD) of ~30 nmol/L (1 µg/dL) in biological fluids, required derivatisation and had run times of 45 min. LC-MS/MS assays have improved analytical specificity further, provide much shorter run times, and offer a superior LOD (6 nmol/L, 0.2 µg/dL) to GC-MS, while maintaining a respectable inter-assay imprecision of <10% for both cortisol and cortisone.

Published LC-MS/MS methods for UFC measurement use either sample extraction or direct sample injection ('dilute and shoot'). Direct methods reduce the labour requirements and decrease the potential for human error. Both extracted and direct methods compare well with traditional HPLC methods. In our own laboratory we found a regression fit between LC-MS/MS ($y$) and HPLC ($x$) for cortisol of: $y = 1.11x + 0.03$ µg cortisol/24 h ($r^2 = 0.992$; n = 99), while, cortisol concentrations were systematically overestimated by an immunoassay (immunoassay ($x$), LC-MS/MS ($y$): $y = 0.66x - 12.1$ µg cortisol/24 h ($r^2 = 0.67$; n = 99)),[24] most likely due to cross-reacting cortisol metabolites (Figure 3). In a later study, a 'dilute and shoot' LC-MS/MS method with on-line clean up was developed. It showed comparable performance to the earlier standard LC-MS/MS method (direct injection-MS/MS ($y$) versus standard LC-MS/MS ($x$): $y = 0.99x + 1.24$, r = 0.999), including a similar proportional downward bias of 56% compared with a cortisol immunoassay.[25] No interferences were found with prednisolone, prednisone, dexamethasone, desoxycorticosterone, fludrocortisone, and 11-desoxycortisol. However, MS/MS, despite its greater analytical specificity is not entirely free from interferences. Under routine test conditions occasional interferences have been reported, including the prednisolone metabolite tetrahydroprednisolone, and fenofibrate.[26]

*Sex Steroids – Oestrogens and Testosterone*

Measurement of serum oestradiol (E2) is an integral part of the assessment of female reproductive function, including studies of fertility, oligo-amenorrhoea, and menopausal status. In addition, it is widely used for monitoring of ovulation induction as well as during preparation for *in vitro* fertilisation. Most E2 assays have traditionally been optimised for these clinical scenarios, which place only modest demands on assay sensitivity but require fast assay turn around times. However, a more sensitive E2 assay, simultaneous measurement of oestrone (E1), or both are needed in many other clinical situations. These include inborn errors of sex-steroid metabolism, disorders of puberty, oestrogen deficiency in men, and increasingly, therapeutic drug monitoring, either in the context of low-dose female hormone replacement therapy or anti-oestrogen treatment. There is also an increasing research-driven demand for high-sensitivity E1 and E2 assays, e.g. to

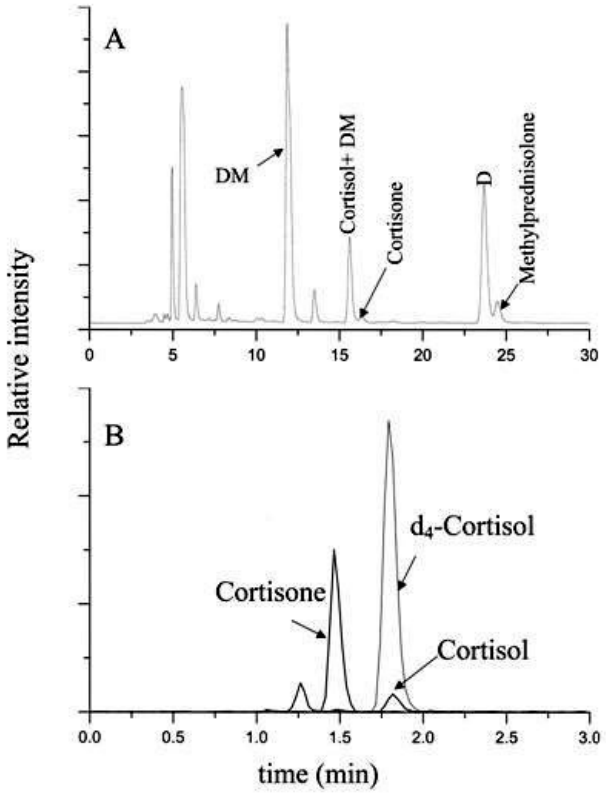


**Figure 3.** Comparison of HPLC-UV (A) and LC-MS/MS (B) chromatograms of urinary free cortisol measurements of the same patient sample that contains potentially interfering substances (carbamazepine). (A) Several potentially interfering peaks are visible on HPLC-UV, with one of the carbamazepine metabolites co-eluting with cortisol and the cortisone peak being barely separated from the cortisol peak, despite a run time of 30 minutes. (B) No interferences are seen by LC-MS/MS with only 3 minutes run time and the cortisone peak shows baseline separation from the cortisol peak. Note the cortisol isotopic internal standard overlaying the cortisol peak. Reprinted from Taylor *et al*. (Clin Chem 2002;48:1511-9) with permission from Clinical Chemistry.

study breast cancer, male osteoporosis, Alzheimer's disease, and cardiovascular disorders.[27,28]

High-sensitivity E2 immunoassays are challenging because physiologic serum concentrations of E2 are typically <140 pmol/L (40 ng/L) in adult men and postmenopausal women and in both sexes during infancy and childhood. None of the commercially-available automated direct E2 assays appears to have sufficient sensitivity for the evaluation of E2 in the sera of children and men. Assays with higher sensitivity are available, but they have traditionally been manual RIAs. Although some of these E2 and E1 RIAs provide better sensitivity, they have several important drawbacks, in particular suboptimal agreement between different assays. Similar issues are also relevant with regard to automated, chemiluminescence-based, direct E2 immunoassays. The College of American Pathologists' survey results for the past few years confirm that the performance of direct E2 immunoassays needs to improve with respect to analytical accuracy, between-assay harmonisation and detection limit.[29]

Earlier LC-MS/MS SRM experiments in our laboratory with negative ion-pairs corresponding to underivatised E1 (*m/z* 269/145) and E2 (*m/z* 271/145) gave a method with good precision, linearity, and functional sensitivities of 37 pmol/L (10 ng/L) and 370 pmol/L (100 ng/L) for E1 and E2, respectively. This functional sensitivity was insufficient for most clinical applications, particularly with regard to E2, and we therefore pursued derivatisation to improve ionisation efficiency of E2, with the aim of maximising detection sensitivity. Dansyl chloride (Figure 4) has been described in the literature[30] as an effective E2 derivatisation reagent for LC-MS/MS methods, and it proved indeed highly effective, resulting in a ~10 fold improvement in analytical sensitivity. For E2 the critical concentration (95% certainty that the analyte is undetectable with the method) was 6.2 pmol/L

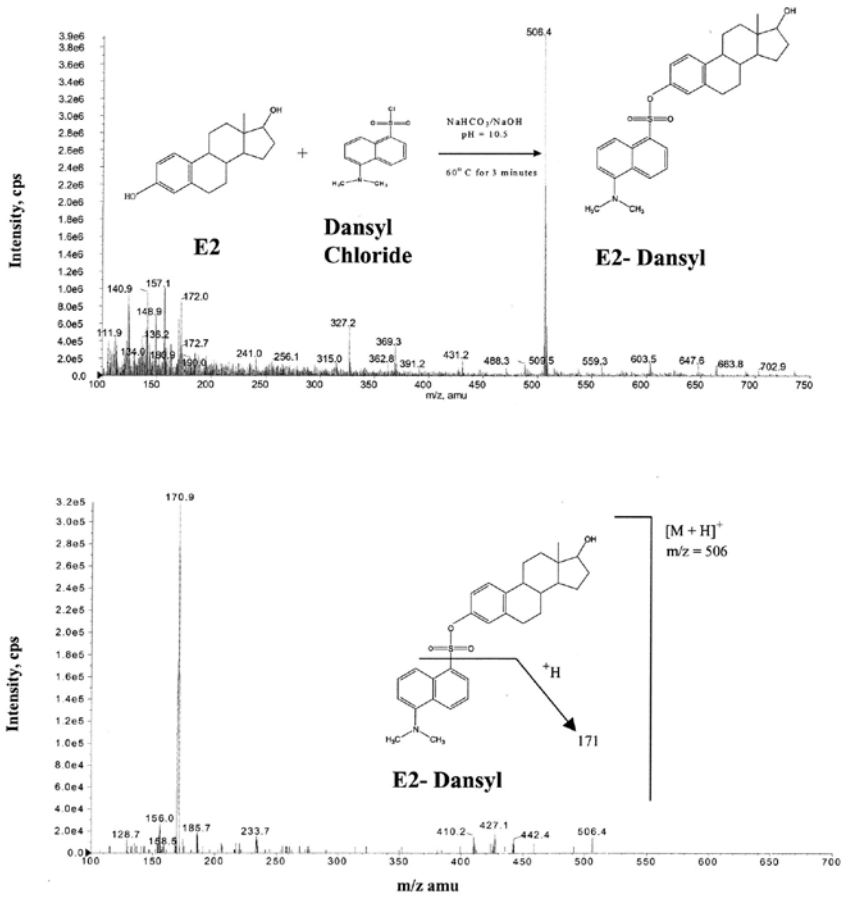


**Figure 4.** Atmospheric pressure chemical ionisation precursor ion (top) and product ion (bottom) scan of oestradiol derivatised with dansyl chloride. Reprinted from Nelson *et al*. (Clin Chem 2004;50:373-84) with permission from Clinical Chemistry.

(1.7 ng/L), the detection limit was 10.3 pmol/L (2.8 ng/L; CV for replicates = 13%), the detection range was 10.3–2205 pmol/L (2.8–600 ng/L), and the functional sensitivity was 23.2 pmol/L (6.3 ng/L).[31] GC-MS/MS (see also next section) can achieve an even lower limit of detection of 0.1 pg/ml (0.36 pmol/L). The method does, however, require a larger sample volume, as well as more extensive sample clean-up and longer chromatography.[27,28] In general, for all analytes that are present at extremely low concentrations, thorough optimisation of chromatography is critical to separate as much as possible any interfering matrix components or cross-reactants from the analyte of interest (Figure 5, see also discussion next section).[32,33]

Testosterone, the major androgenic hormone in humans, is commonly measured to aid in the diagnosis of clinical conditions related to its excess or deficiency. In addition, testosterone measurements are used to monitor testosterone replacement or anti-androgen therapy. Measurement of serum testosterone has in many ways suffered from similar problems as oestrogen measurement. Automated direct immunoassays, requiring minimal human intervention, are commonly used because of their high sample throughput. These assays compare quite well with each other at high serum testosterone concentrations. However, similar to E2 assays, many automated testosterone immunoassays suffer from poor accuracy at concentration levels below 1.73 nmol/L (<50 ng/dL), making them less than ideal for use in women and children, as well as in men undergoing anti-androgen therapy. In addition, there is increased interest amongst endocrinologists to measure testosterone concentrations in serum with high absolute accuracy. This is reflected in a recent Endocrine Society recommendation, which states that laboratory proficiency testing should be based on the ability to measure accurately and precisely testosterone concentrations in patient samples, rather than on the usual approach of agreement with other labs using the same method.[34-36]

Almost a decade ago we developed a sensitive and rapid testosterone assay based on LC-MS/MS, which was based on on-line sample extraction and multiplexing. Our validation criteria for precision, accuracy, and linearity included accuracy and linearity within of ±10% the target concentrations, along with a total imprecision of <15% throughout the reportable

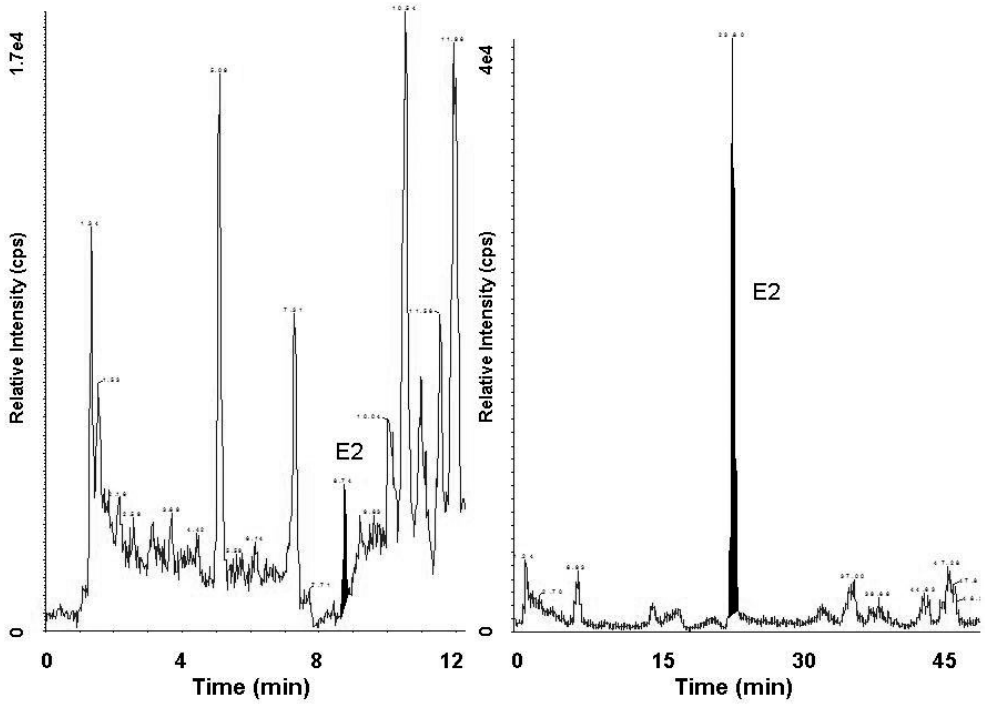


**Figure 5.** Oestradiol (E2) measurement in cell culture medium shows relatively poor signal-to-noise for a sample containing approximately 2 pg/mL of E2 (left panel). After optimisation of chromatography, with a longer run time, the signal intensity is improved by a factor of ~10 and the signal-to-noise ratio shows even greater improvement (right panel).

range. During method comparison with the automated ACS-180 (at the time Bayer Diagnostics) testosterone assay we found a slope of 1 and an $R^2$ of 0.999 for samples obtained from healthy adult males. By contrast, for female samples there was substantial disagreement between the ACS-180 assay and the LC-MS/MS assay, with the automated immunoassay failing to provide reproducible and accurate results in a large proportions of females. Extracting and concentrating the samples to remove interferences before running them on the ACS-180 improved the performance of this assay, but it continued to show significant systematic and random bias compared with the LC-MS/MS method (LC-MS/MS = ACS-180 x 0.7, $R^2$: 0.86). Recently we have compared our LC-MS/MS method with the USA Center of Disease Control reference LC-MS/MS method and an excellent agreement was obtained (slope: 1 and $R^2$: 0.99).

Many other laboratories have also developed LC-MS/MS testosterone assays during the last 10 years. One can now find many different versions of extraction and LC-MS/MS methods in many different laboratories.[35,37] Because of high test-volumes and paediatric testing requirements, LC-MS/MS-based testosterone assay should not simply offer accurate, precise, and unambiguous testosterone measurement with a functional sensitivity of 173–346 pmol/L (5–10 ng/dL), but should also offer rapid pre-analytical sample processing with minimal manual manipulations, sample throughput of at least 30 samples per hour and low sample volume requirements.[38]

*Other Steroids - Congenital Adrenal Hyperplasia*

Most (>90%) cases of congenital adrenal hyperplasia (CAH) are due to mutations in the steroid 21-hydroxylase gene (CYP21A2). CAH, due to 21-hydroxylase deficiency, is diagnosed by confirming elevations of 17-hydroxy-progesterone and androstenedione, along with a decreased cortisol. By contrast, in two less common forms of CAH due to 3-β-hydroxysteroid dehydrogenase and 11-hydroxylase deficiency, respectively, 17-hydroxyprogesterone and androstenedione levels are not significantly elevated and measurement of pregnenolone/17-hydroxypregnenolone and deoxycortisol/deoxycorticosterone, respectively, are necessary for diagnosis. Various commercial and in-house RIA methods were traditionally used in clinical laboratories for the analysis of 17-hydroxyprogesterone, androstenedione, pregnenolone, 17-hydroxypregnenolone, deoxycortisol, and deoxycorticosterone in serum or plasma, but were found to lack standardisation and often yielded inconsistent results. Mass

spectrometric methods were considered gold standard, but they were not offered by most laboratories. In early 2000, a clinical LC-MS method for 17-hydroxyprogesterone was developed and validated for confirmation of CAH due to 21-hydroxylase deficiency.[39] Since then, LC-MS/MS methods for many other steroids have been reported and efforts are being made to harmonise these assays between performing labs.[8,33]

*Catecholamines and Metanephrines*

Phaeochromocytoma is a potentially lethal tumour of chromaffin cells of the adrenal medulla that produces episodes of hypertension with symptoms of palpitations, severe headaches, and sweating. The diagnosis of phaeochromocytoma is challenging; autopsy series suggest that many phaeochromocytomas are not diagnosed until after the patient's demise. The testing of catecholamines and metanephrines, in urine or plasma, or, of vanillylmandelic and homovanillic acid in urine, are commonly used to screen patients for phaeochromocytoma, who have difficult-to-treat hypertension or show symptoms/signs suspicious of a chromaffin tumour. Measurements of fractionated free plasma or urinary metanephrines (normetadrenaline and metadrenaline), the O-methylated metabolites of norepinephrine and epinephrine, are particularly sensitive and specific tests for the diagnosis of phaeochromocytoma.

Metanephrines are present in urine mainly as sulfate- and glucuronide-conjugated metabolites produced from free metanephrines by the actions of conjugating enzymes.[40] An acid hydrolysis step is usually performed to liberate the free metanephrines from the conjugated metabolites. This step minimises requirements for high analytical sensitivity, simplifying subsequent measurement. Urinary fractionated metanephrines are usually measured by HPLC with electrochemical detection (HPLC-EC). GC-MS and LC-MS/MS are more recent alternatives offering higher sample throughput and improved analytical specificity.[41,42]

Early on, chromatographic separation of normetanephrine (NMN) and metanephrine (MN) was accomplished by use of normal phase chromatography after solid-phase extraction. NMN, MN, $d_3$-NMN, and $d_3$-MN positive ions were detected in the multiple-reaction monitoring mode using the specific transitions of *m/z* 166→134, 180→148, 169→137, and 183→151, respectively, with an atmospheric pressure chemical ionisation source. Recently the same LC-MS/MS method has been optimised using automated online extraction.[43,44] Sample precipitation using isopropanol is another, less expensive, alternative to on-line or off-line solid-phase extraction.[45]

*Vitamin D – The Test of the Decade*

Vitamin D has gained great interest, largely due to its role in bone health and numerous reports of deficiencies within the general population. The majority of vitamin D circulates bound to a specific transport protein, vitamin D-binding protein. Vitamin D is hydroxylated in the liver at the carbon-25 position giving rise to 25-hydroxy vitamin $D_2$/$D_3$ (25OHD), the most abundant circulating, but biologically inactive form of the vitamin. Biological activity is conferred by the final hydroxylation step catalysed in the kidney by 1-α-hydroxylase, resulting in the production of 1,25-dihydroxyvitamin D.

Various methodologies including HPLC-UV, HPLC-EC, RIA (with low throughput), automated chemiluminescence immunoassays (high throughput), and LC-MS-MS have been described for the measurement of 25OHD in biological fluids[46] and most of these are being used by clinical laboratories for 25OHD measurement in patient care. One challenge that has both analytical and clinical implications is the variability encountered with some of these methods, a problem reflected in proficiency surveys. When the results of a recent College of American Pathologists survey are compared, the impact of methodology and lack of standardisation is dramatic. For one sample a mean of 187 nmol/L (75 ng/mL) was obtained using LC-MS/MS with <15% variability between the reporting laboratories. For the same sample, laboratories using chemiluminescence immunoassays reported a range of results from 102 to 240 nmol/L (41 to 96 ng/mL).[47,48] There could be many reasons for these variations, including drifts in the reagents being manufactured, but there is a clear and urgent need for assay standardisation and harmonisation. To address the issue of standardisation, the National Institute of Standards and Technology has developed quality control materials (human serum, SRM 972) which contain $25OHD_2$, $25OHD_3$, and their respective 3-epi isomers at four different concentrations as characterised by LC-MS-MS.[49,50] LC-MS-MS is perhaps positioned best to facilitate this standardisation process, as, with suitable chromatography, it allows unequivocal differentiation and accurate quantification of all these compounds, offering great sensitivity and specificity.[51] The method has also proved its mettle for 25OHD testing in terms of high throughput and day-to-day robustness: several laboratories in the US each perform many thousands 25OHD measurements every day by LC-MS/MS, roughly equal, or more, than are performed by immunoassays.

**Limitations and Problems of Clinical LC-MS/MS and their Potential Solutions**

Several key limitations of LC-MS/MS have become apparent with the exponential increase of its use in clinical laboratories. They centre on the following, interacting aspects of clinical LC-MS/MS: highly manual workflows, complexity

of operation and maintenance of instrumentation, sample throughput limits, insufficient detection sensitivity for some analytes and problems with detection specificity.

***Improving LC-MS/MS Workflows and Ease of Use***

The manual nature of LC-MS/MS workflows and the high complexity of the instrumentation's operation and maintenance have long been key factors hindering more rapid adoption of LC-MS/MS outside of specialised referral and reference laboratories. For smaller laboratories, entry into LC-MS/MS is not easy. The first hurdle is often the high initial cost of the equipment, which might deter institutions on a tight budget. Next, in the absence of previous MS experience, it has to be anticipated that a laboratory scientist will have to spend 3-6 months, often including extramural training, to acquire a basic skill and comfort level that allows him or her to successfully start implementing new LC-MS/MS assays. Usually, at least two individuals should be largely dedicated to LC-MS/MS in such a setting. New test implementation and troubleshooting will often still prove difficult, until more experience has been acquired. Subtle differences to published methods with regards to equipment, reagents and conditions, which incidentally may not have been listed in sufficient detail in a publication, can prove frustrating. Only a solid understanding of the underlying technologies and their peculiarities will ensure success. It is therefore recommended that either individuals with previous MS experience are specifically hired for this task, or that an extensive training/learning period (see above) is planned for existing staff. If existing staff are used, then the learning curve will be flattened if these individuals have significant prior experience with HPLC or GC-MS. Finally, initial new test development should probably focus on analytes that circulate in micromolar or higher concentrations.

Despite these hurdles, which can be formidable for smaller laboratories, the compelling analytical advantages of LC-MS/MS for many low molecular weight analytes and the sometimes substantial reagent cost savings compared to commercial immunoassays have prompted many smaller, or more general, laboratories to consider LC-MS/MS. This increased interest in LC-MS/MS by smaller laboratories has not gone unnoticed by the instrument manufacturers, and they have worked hard at improving the user-friendliness and robustness of their machines, while at the same time providing users with streamlined and tested recipes for relatively painless implementation of common assays.

At the same time, manufacturers of robotic liquid handlers are increasingly focused on making their instruments work better for pre-analytical and analytical LC-MS/MS workflows. LC systems have also improved in user-friendliness and integration with the MS, and sample clean-up or extraction has begun to be integrated into several LC front-ends. It is now conceivable to imagine that in the near future we will have integration of liquid handling, sample extraction/clean up and LC with each other in a single MS-front-end, which in turn will be highly integrated with the MS/MS instrumentation, with all being interfaced bi-directionally to a Laboratory Information System.

***Improving LC-MS/MS Throughput***

The improvements in LC-MS/MS workflows and ease of use have increased LC-MS/MS market penetration and will continue to encourage increased use. Unfortunately, these usability enhancements do not address directly the pressing need to improve sample throughput of LC-MS/MS systems. Depending on the test(s), or mix of tests, a LC-MS/MS system, with a single channel LC, can perform between 70 to 250 tests per working day, assuming a two-shift (14-16 hours), 5-6 days per week schedule (or half that number for a single shift laboratory). On the face of it, these numbers seem quite respectable. However, daily clinical test volumes of many hundreds of samples are not unusual for high volume tests such as $25OHD_2$ and $25OHD_3$, testosterone, oestradiol, and cortisol, even in mid-sized clinical laboratories. Large referral laboratories might perform many thousands of several, or of each, of these tests per day (Figure 6). Consequently, many laboratories continue to use automated immunoassays for these analytes, despite the often compelling analytical and financial advantages of LC-MS/MS. Those laboratories, who do use LC-MS/MS for high volume tests, face the dilemma of recurring, costly instrument purchases, increasingly cramped laboratory space, and escalating use, storage and disposal of the toxic and flammable solvents used in LC-MS/MS workflows.

*'Dilute and Shoot'*

The most obvious factor responsible for the limited throughput of LC-MS/MS is the time required for sample introduction into the LC and the subsequent time necessary for chromatography. Once a chromatographic system has been fully optimised to minimise the time needed to remove interferences and to separate analytes from solvent fronts, there is no room for further improvement in throughput from a chromatographic standpoint. The only way to save additional time in a simplex LC setup is to dispense with analytical chromatography altogether. The sample is simply diluted and injected directly into the MS, with or without in-line sample clean-up or guard column. For this so called 'dilute and shoot' approach, the new speed limit is determined by the combination of the mechanical speed of the autosampler, and the time it takes for the sample to traverse tubing, pumps and any guard columns or in-line clean-up columns/loops, if present. Depending on

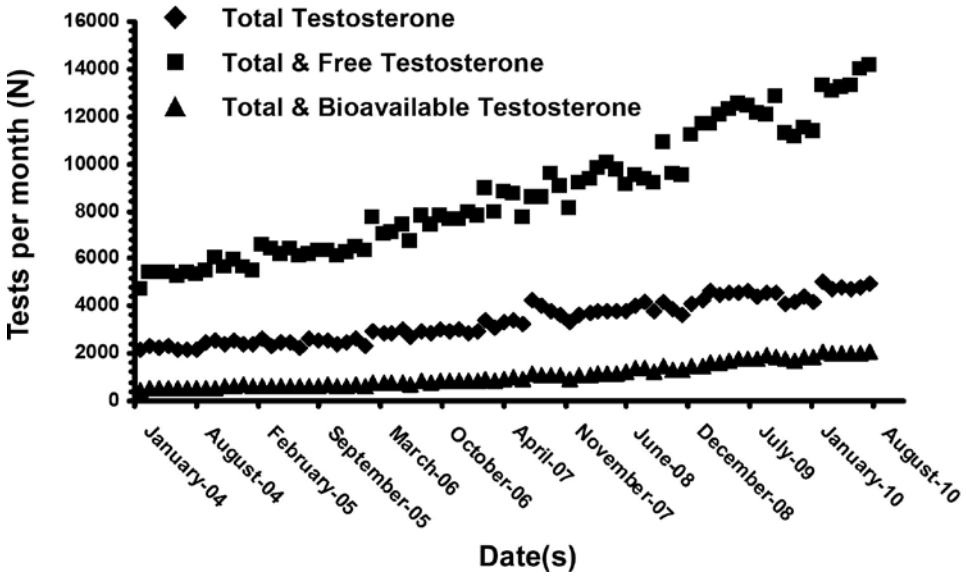


**Figure 6.** Monthly testosterone test volumes at the Mayo Clinic Rochester Endocrine laboratory from January 2004 to August 2010. All three depicted testosterone assays require measurement of total testosterone by LC-MS/MS.

the setup and instrumentation, this translates into a time of 45-120 seconds per injection for a theoretical throughput of about 400 to 1300 samples per 14-16 hours working day (or half that number for a single shift). Increased instrument cleaning and maintenance due to the large amounts of sample matrix injected, tend to cut the theoretical throughput figure by around 50%, still a sizable improvement on the baseline throughput of single channel LC LC-MS/MS systems.

For select analytes the 'dilute and shoot' approach can solve throughput problems. Unfortunately, only a minority of analytes is suited for this approach. For many analytes, chromatographic separation from solvent fronts and matrix interferences is crucial to minimise background interferences and ion suppression, both of which can profoundly impact detection specificity and sensitivity. Moreover, interfering non-specific matrix components with ion-pairs that match those of the analyte might prevent accurate quantification even at high analyte concentrations. This problem is amplified, if specific isobaric interferences are present; in particular if these substances are true isobars (same elemental composition) and belong to the same chemical family. In these cases, precursor and major product ions are often, if not always, identical and analytical specificity is severely compromised in the absence of chromatographic separation. The increasing awareness of these pitfalls has dampened the initial enthusiasm for 'dilute and shoot' that prevailed in the late 1990s and the technique is now used quite selectively, principally in some drug/toxicology and newborn screening applications.

*LC-Multiplexing*

An alternative approach for increasing throughput, without forgoing the advantages of chromatography, is to use a multiplexed LC-system like the Thermo Fisher TLX4® online Sample Preparation System or the Applied Biosystems MPX™-2 system. These instruments include up to four separate chromatographic systems, which operate simultaneously, but in a staggered fashion. This allows the specimens to be introduced into the mass spectrometer only at the time when the peak(s) of interest elute, thereby increasing throughput up to four-fold when compared to a single LC front-end (Figure 7).[52-55]

The maximum throughput of such a system is achieved when the speed of chromatography divided by the multiplexing factor equals the minimum time in which the autosamplers can introduce specimens to the system. This works out to a new sample being introduced every 30-60 seconds in a 4-plexed system, which corresponds to a minimal individual chromatography time of 2-4 minutes per sample. More commonly, analytes require somewhat longer chromatography times for optimal separation. However, even with, by LC-MS/MS standards, long chromatography run times of 10-12

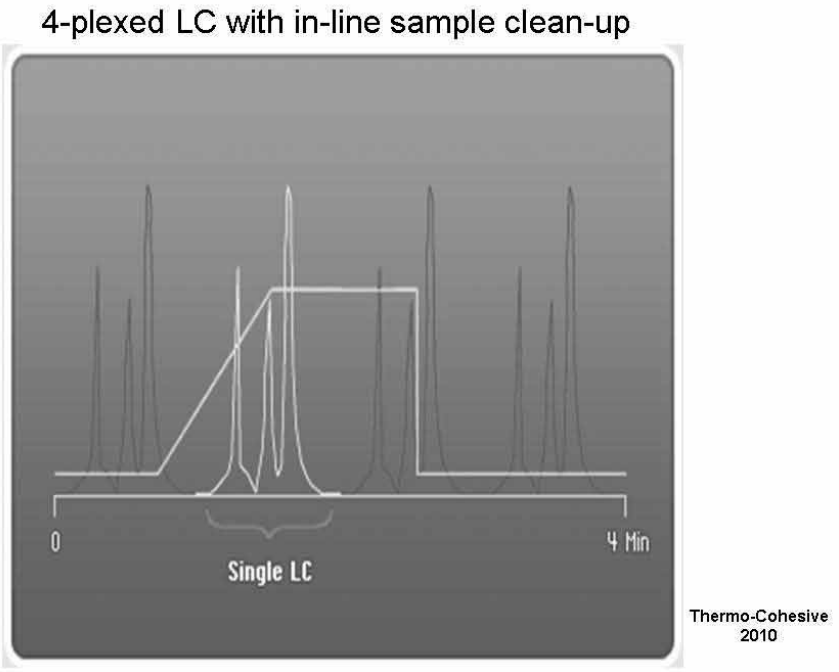


**Figure 7.** Diagram depicting the principle of LC-multiplexing (courtesy Thermo). The total chromatographic run time is 4 minutes for each sample. However, the data-window of interest is only 1 minute wide. By staggering injections from four LC systems – a different injection every minute – and letting the LC flow before and after the window of interest go to waste, four injections can be 'squeezed' into a 4 minute usage of the tandem mass spectrometer.

minutes, this leads to a different sample being introduced into the system every 2.5 to 3 minutes. Averaged over a range of different analytes, a 4-plexed LC system therefore achieves at least the same throughput as the 'dilute and shoot' approach, without forgoing the advantages of chromatography. We have achieved throughputs of >220,000 samples per instrument per year for some analytes with 4-plexed systems. In addition, such systems facilitate in-line sample clean-up, and thereby also improve overall workflows. The principal drawback of multiplexing LC is that the complexity of the equipment, plumbing and instrument settings increases dramatically and staff usually require significant additional training. This solution is therefore best suited for laboratories with significant LC-MS/MS experience.

*Sample Multiplexing*

'Dilute and shoot' or LC multiplexing address the throughput limitations of LC-MS/MS in part, but these techniques can still only barely achieve the lower range of throughput offered by the fastest automated immunoassay analysers. These analysers can achieve peak throughputs of 150-400 samples per hour under ideal conditions (single test with short incubation) and ¼ to ½ of these figures under real-world conditions (multiple tests, with variable incubation times). While additional small improvements in speed can be expected for multiplexed LC-MS/MS and for direct sample injection, both techniques are approaching the theoretical mechanical and physical limitations of current equipment.

One solution to this conundrum is to introduce more than one specimen per injection. As few as two specimens per injection immediately double the overall capacity of an instrument, assuming that the chromatographic conditions remain static. Each further increment in the number of simultaneously introduced specimens will increase throughput in a linear fashion, up to the point when the electronic switching time of the MS/MS detector becomes limiting. Since the latter is at least an order of magnitude faster than the best mechanical front-end systems that are currently available to clinical laboratories, throughput increases of 10-fold or more can easily be imagined.

In order to achieve such multiplexing of samples, different samples must be modified in a way, that allows the MS/MS to identify unambiguously that a given analyte and internal standard peak have originated from one specific of several multiplexed samples. One solution to this challenge is differential mass-tagging. A different, but closely related,

derivatisation reagent is used to derivatise each separate sample that is to be multiplexed. Each of these different reagents needs to be able to react equally well with characteristic group(s) that are specific to the analyte of interest.

We have used this approach successfully to increase sample throughput of our $25OHD_2$/$25OHD_3$ assay by injecting five differentially tagged samples per injection, increasing throughput on a single LC-MS/MS system with a 4-plexed chromatography front-end from 60 samples per hour to 300 samples per hour.[56] Vitamin $D_2$ and vitamin $D_3$ and its metabolites, including $25OHD_2$ and $25OHD_3$, are good candidates for such an approach, as they are efficiently and irreversibly derivatised[57] by dieneophiles, and specifically by Cookson-type reagents, or triazolinediones (TADs).[58-60] TADs have the ability to carry any number of different functional groups. The reactive portion of the molecule is synthesised from a substituted isocyanate molecule,[61] and the 'R' group can be altered at the choosing of the synthetic chemist. Individual specimens containing $25OHD_2$ and $25OHD_3$ can therefore be derivatised with different TADs, combined and run concurrently (Figure 8). Based on the different Q1 masses of the differential mass tags, the mass spectrometer can distinguish from which individual sample a given $25OHD_2$ and $25OHD_3$ peak (and their respective isotopic internal standard peaks) have originating (Figure 9). Since the derivatisation also increases ionisation efficiency, any increase in ion suppression due to the increased matrix components is negated sufficiently to allow the assay to have comparable analytical performance to underivatised methods that do not use sample pooling. With more recent LC-MS/MS instrumentation than was used in our study, improved switching times may allow the number of multiplexed samples to be increased further.

The main logistic challenge in sample multiplexing is to eliminate as completely as possible any chance of sample mix-up. This can be achieved best by automating sample

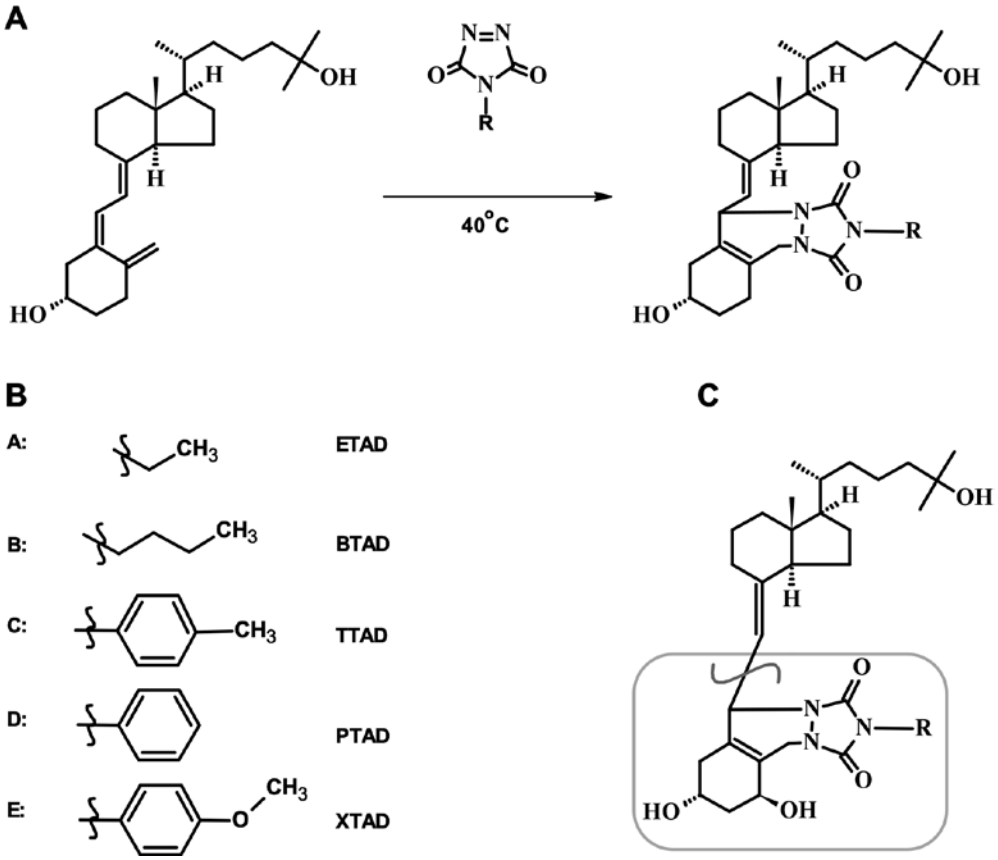


**Figure 8.** Derivatisation of 25-hydroxyvitamin D (25OHD) by triazoline-diones (TADs). 25OHD (*A*) reacts irreversibly with the reactive group of TADs, which can contain various different 'R' groups (examples in *B*), to form a derivatised 25OHD. The derivatised 25OHD fragments in Q2 through its molecular back bone (*C*), yielding analyte-specific product ions. Reprinted from Netzel *et al.* (Clin Chem 2011;57:431-40) with permission from Clinical Chemistry.

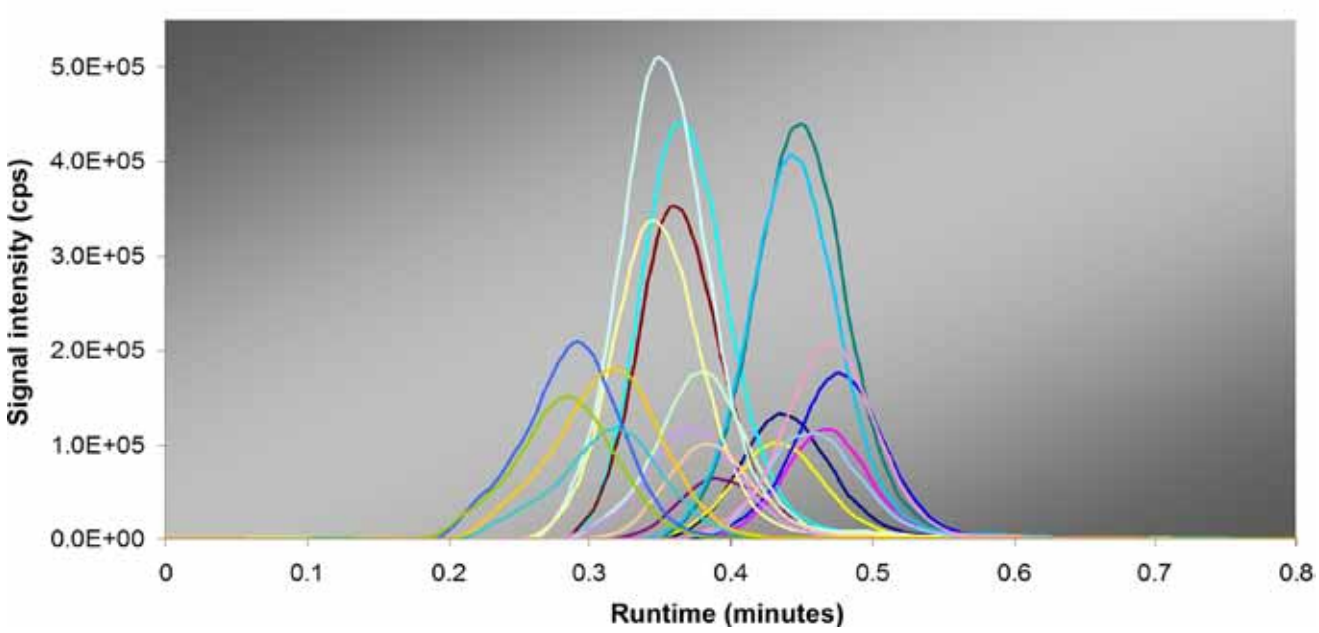


**Figure 9.** Chromatograms of five differentially TAD-labelled patient samples containing $25OHD_2$, $25OHD_3$ and their respective internal standards (20 peaks overall). Reprinted from Netzel *et al*. (Clin Chem 2011;57:431-40) with permission from Clinical Chemistry.

processing, derivatisation and subsequent sample-pooling, and by driving this entire process by bar codes, propagated from the original primary patient tubes.

The concept of differential mass tag derivatisation can be expanded to multiplexing different analytes, provided one can identify derivatisation reagents that are specific for a certain analyte or group of analytes. Finally, patient and analyte multiplexing could even be combined.

***Improving LC-MS/MS Detection Sensitivity***

LC-MS/MS detection sensitivity is not a major issue for most testing in drug/toxicology and newborn screening/ metabolism. In these applications, the analyte concentrations are at worst in the high picomolar concentration range, more frequently in the nanomolar, micromolar, or occasionally even millimolar range. By contrast, many steroid hormones circulate at low picomolar concentrations (or less) in at least some patient groups. For example, as indicated earlier, all sex steroids are in the low picomolar, or sub-picomolar range in pre-pubertal children, while oestrogen concentrations in men and postmenopausal women, and androgen concentrations in women are also exceedingly low. Normal free thyroid hormone levels are in the low picomolar range in all humans, as are 1,25-dihydroxyvitamin D levels. All of these analytes are now frequently measured by LC-MS/MS. Biogenic amines, cytokines and peptide hormones are less frequently measured, but their concentrations can be even lower.

For all of the above analytes, detection limits of clinical LC-MS/MS in serum, plasma or urine samples are about 1-2 orders of magnitude higher than what would be required to assure accurate quantification in all patient groups, despite the fact that the detection sensitivity of the most recent top-of-the-line MS/MS machines is between 10-fold and 20-fold better for pure samples in clean matrices than what was seen in instruments that were state of the art 5-6 years ago. Yet, performance in biological matrices has typically only improved 2-6 fold. This suggests that neither ion-optics nor detector performance are the major limiting factors in the quest for increased sensitivity, but that the biological sample matrix impacts negatively on detection sensitivity, either through elevating the non-specific background signal, or through interferences that obscure analyte peaks, or through suppression of analyte ionisation.

*Ion Suppression*

Ion suppression has been recognised as a significant problem since the early to mid 1990s.[62-65] Ion suppression is chiefly observed with ESI, but can also occur with APCI, though less commonly.[66-69] APCI can also be occasionally associated with apparent signal enhancement.[70,71]

The mechanisms of ion suppression are incompletely understood. In ESI (Figure 1B), analyte competes with itself and various matrix components for the relatively limited amount of excess charge that is available for full ionisation,

as well as for space within droplets. Saturation of droplets with surface-charged analyte or matrix components can also interfere with ejection of analyte trapped within the droplets. Neither of these mechanisms is likely to affect APCI (Figure 1C) as severely as ESI, since the heated gas stream used in APCI obviates the need for charge-initiated volatilisation. At the same time, the much higher charge density of the Corona needle discharge and the intrinsic charge transfer from solvent ions to analyte in APCI are likely sufficient to ensure analyte ionisation in most situations. However, increases in surface tension or viscosity of samples, due to matrix components, may impede volatilisation in both ESI and APCI. Similarly, co-precipitation of analyte with non-volatile matrix components might occur with either ionisation technique.

Regardless of its exact mechanism, ion suppression is most likely to occur if a sample contains high concentrations of basic components (in positive ion mode; acidic in negative ion mode) that elute in the same time window as the analyte, and have a similar mass. In these cases, profound signal loss can occur. Since this loss occurs before the MS/MS stage, it can never be compensated for by any tweaking of MS parameters or detector settings. Every LC-MS/MS method should therefore include a step during its development that evaluates ion suppression. The most popular way to do this is to infuse pure analyte at a constant rate while injecting matrix that has been subjected to the intended HPLC separation through a T-junction.[72] Suppression of signal can then be observed by monitoring the total ion count over time.

Methods to diminish ion suppression and other matrix interferences, centre on two approaches: sample clean up and optimising chromatography. With regards to the former, there is a fairly reproducible hierarchy of effectiveness of sample-dilution < protein-precipitation < SPE < liquid-liquid-extraction (LLE), while optimisation of chromatography shifts analyte peaks out of the maximum ion suppression time window. Optimised chromatography also allows one to avoid interfering peaks, thus further increasing sensitivity. In combination, an improved sample clean-up and better chromatography can have dramatic effects on detection sensitivity, as illustrated in Figure 5.

*Beyond Ion Suppression – Optimising Analyte-Specific Ionisation and Ion Transfer*

Optimised sample clean-up and chromatography can lead to substantial sensitivity gains, but are often still insufficient to allow detection of extremely low analyte concentrations, as may be required, when assessing completeness of androgen or oestrogen suppression in prostate cancer and breast cancer patients, respectively, who are treated with various drugs to suppress sex steroid production. For example, the aim of aromatase inhibitor treatment is complete suppression of oestrogen biosynthesis. For such purposes, measurement of E1 and E2 in sub-picomolar concentrations is necessary.

In addition, for high volume tests it is often not possible to perform the extensive sample clean-up and long chromatography required, due to the increased workload and limited instrument capacity. This can sometimes result in an inability to achieve detection sensitivities that would normally be expected to be within the capability of routine LC-MS/MS.

In both of the above scenarios, sample derivatisation can lead to profound improvements in analyte-specific ionisation, and consequently detection sensitivity. The key to success is to identify a derivatisation reagent that is selective for the analyte of choice, but not for competing specific- or non-specific interferences. Using such an approach, we see a 6-10-fold increase in detection sensitivity for E1 and E2 after derivatisation with dansyl chloride, a compound that is specific for the organic ring found in these steroids.[31] Other authors report similar, or even greater improvements for oestrogens and other analytes.[8,58-60,73-75] As reported earlier in the section on sample multiplexing, TADs reliably and specifically increase ionisation of vitamin D-related compounds, and are typically employed when 1,25-dihydroxyvitamin D needs to measured. The disadvantages of derivatisation are that it might further complicate LC-MS/MS workflows, and that in some instances the major fragment ion is the derivatisation reagent itself (or the substrate that was derivatised), with the accompanying potential loss of specificity.

Another approach to increase detection sensitivity is to employ immune-affinity purification before LC-MS/MS testing. Immune-affinity purification can result in samples that are remarkably free of ion suppression, non-specific interferences and specific interferences, in particular if the technique is combined with SPE or LLE. We use this approach in our laboratory for a high volume, high detection sensitivity test (1,25-dihydroxyvitamin D) and for a demanding peptide test that requires very high sensitivity (parathyroid hormone).[76] Potential problems of immune affinity purification centre on more complex workflows, and, rarely, sub-optimal specificity of the antibody used.

When all these measures prove insufficient to achieve the needed detection sensitivity for an analyte, optimisation of the flow/injection rate into the source and source temperature, manipulation of injection distance, use of multiple injection capillaries, changes to the geometry of the faceplate and the entry/transmission ion optics orifice can yield significant improvements in ionisation and transmission of desirable ions, while reducing ion suppression and transmission of

undesirable non-specific interferents.[77-80] Even without resorting to nano-flow techniques, which are cumbersome and impractical for clinical use in their current incarnations, slowing the flow rate and increasing the source temperature will typically increase analyte-specific ionisation in biological matrices, as well as improving ion transfer into the instrument's ion path.[77,80] Other parameters of source, ion path entry and ion path, are not generally under the user's control, but manufacturers have started to take notice of these factors and are beginning to incorporate them in their latest designs, promising improved sensitivity.[81,82]

GC, traditionally used as a front-end for single quadrupole MS, is making a comeback as a front-end for MS/MS instrument, promising increased detection sensitivity. The high heat within the GC source ensures complete volatilisation, while the very long chromatography capillaries (~30 m) lead to much better analyte-matrix and analyte-interference separation than what can be achieved by LC, as well as resulting in much narrower, taller peaks, further improving signal to noise ratio. Consequently, detection sensitivity can in some instances be improved dramatically, as can be seen in Figure 10, which shows clear detection of 0.1 pg/mL of oestradiol in a serum sample, at least 10-fold better than what we observe routinely with LC-MS/MS.

Finally, orbital ion-traps offer not only much better mass-resolution (and thereby specificity), but also improved sensitivity, compared to typical triple quadrupole designs.[83,84] Their principal draw back is their limited dynamic range, imposed by the intrinsic space charge problems of ion-traps.[85]

***Improving LC-MS/MS Specificity***

One reason LC-MS/MS was so enthusiastically embraced in clinical laboratories was that it seemed to combine high detection sensitivity with high analyte specificity when used in MRM mode. It seemed to offer all the advantages of GC-MS without the disadvantages of compromised detection sensitivity and long chromatography run-times. However, as we have discussed in the previous section, without proper attention to sample clean-up and chromatography its sensitivity promise can never be realised fully. The same is true for specificity. Isobaric compounds, especially true isobars, contained in the sample matrix are a particularly exacting problem, as precursor and product ions might be identical, as mentioned briefly above in the subsection on ion suppression. $MS^n$, or use of a second or third product ion can help identify such problems, but may still fail in allowing correct quantification.[86] Chromatography can usually resolve the problem, but may fail to do so for some isomeric compounds, unless special conditions are used.[51]

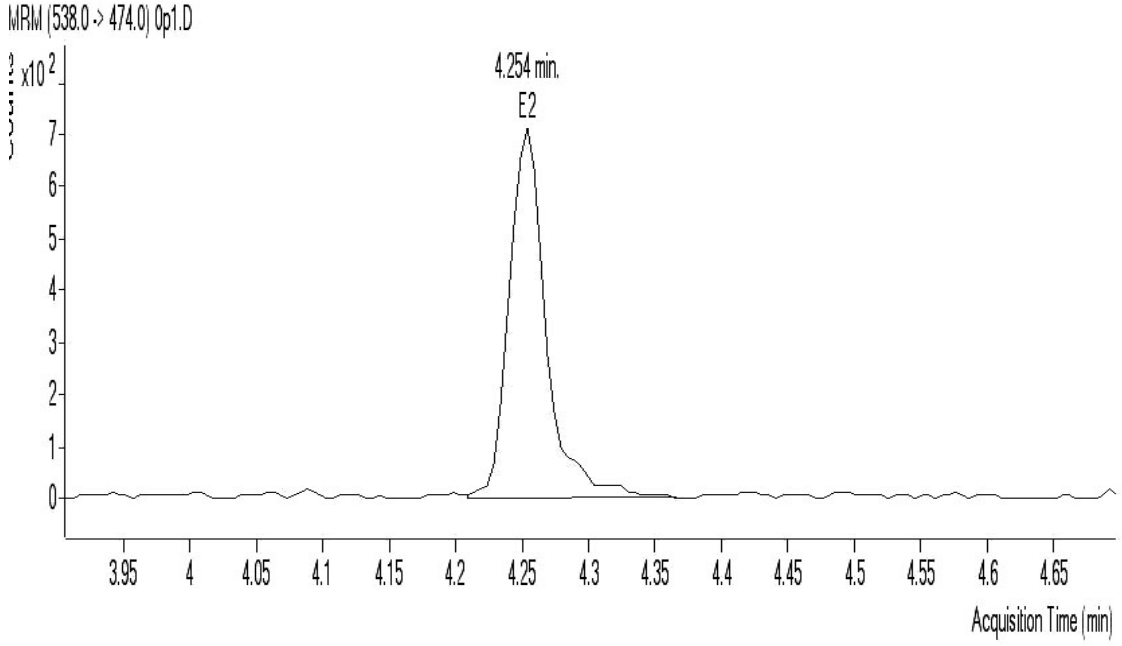


**Figure 10.** Gas-chromatography, selective reaction monitoring (SRM) tandem mass spectrometry (GC-MS/MS) of a sample containing 0.1 pg/mL of oestradiol. The long column (~30 metres) and high temperatures used in GC minimise background, ion suppression, and interferences, and allow the MS/MS to show its full potential in SRM mode. The analytical sensitivity in this example is nearly 10-fold better than what can be achieved by LC-MS/MS. Courtesy of Agilent.

These problems are all magnified for steroid hormones. Depending on their position within the steroid metabolic pathway, they can be challenging to distinguish from each other with standard LC-MS/MS. In addition, the concentration range of the lowest to the highest abundance steroids in serum and plasma spans several orders of magnitude and there can be vast differences in observed absolute and relative concentrations of groups of steroids between different groups of healthy individuals, or at different times of the day or month. These issues might be further compounded, or changed in a radical fashion, by the presence of inherited disorders of steroid metabolism or by drug treatments that interfere with steroid metabolism. Examples of the same steroid being hundred or thousand fold different in concentration between two different patients are not uncommon. In this situation, isotopic cross-talk becomes a real issue. A little more than 1% of carbon is carbon 13. For an 'average' steroid, containing about 20 carbon molecules, this means that there is approximately a 17% chance that at least one of these is a carbon 13 and $17\%^2$ (~3%) chance that two are carbon 13, $17\%^3$ (~0.5%) that three are carbon 13, and so on. It is therefore not rare for a steroid, with an average MW of one or two Da less than another steroid, to cause very significant cross-talk in Q1 if the former is present at >10 fold higher concentration than the latter. If subsequent collision induced fragmentation in Q2 is different no harm is done. However, being closely related to each other, many steroids have very similar fragmentation patterns, an issue that is compounded, if, for reasons of convenience or increased sensitivity, a relatively non-specific Q3 ion is monitored (e.g. a 'water loss'). Monitoring more than one ion pair can help, but is not always successful.[86,87]

Some of these problems might also be minimised by new hybrid instrumentation that allows high-resolution Q3 scanning with comparable sensitivity to the usual single ion monitoring.[87] Data-dependent high-resolution precursor ion scans might then reveal the main isotopic peak of the high concentration interferent.

The problem of steroid interferences is further compounded by the fact that the extreme conditions in the source, high temperature and high ionisation state of all present molecules, is conducive towards formation of adjuncts, or conversely cleavage of side chains, which can occasionally convert even quite disparate steroid moieties into structurally very similar isobars/isomers of each other.

The most reliable way to avoid these issues is by meticulous chromatography. In most instances this will allow high specificity identification of compounds, even in difficult samples. However, for certain applications even this may not suffice. For example, we have observed artefactual creation of prednisolone in urine samples with very high cortisol concentrations (Figure 11). In this case, we traced the source of the problem to the solvents and conditions of extraction and/or chromatography, which caused chemical conversion of a small proportion of cortisol to prednisolone; the subsequent chromatography and MS/MS performed flawlessly, and the inadvertently *in vitro* created prednisolone was duly detected, causing clinical confusion/uncertainty.

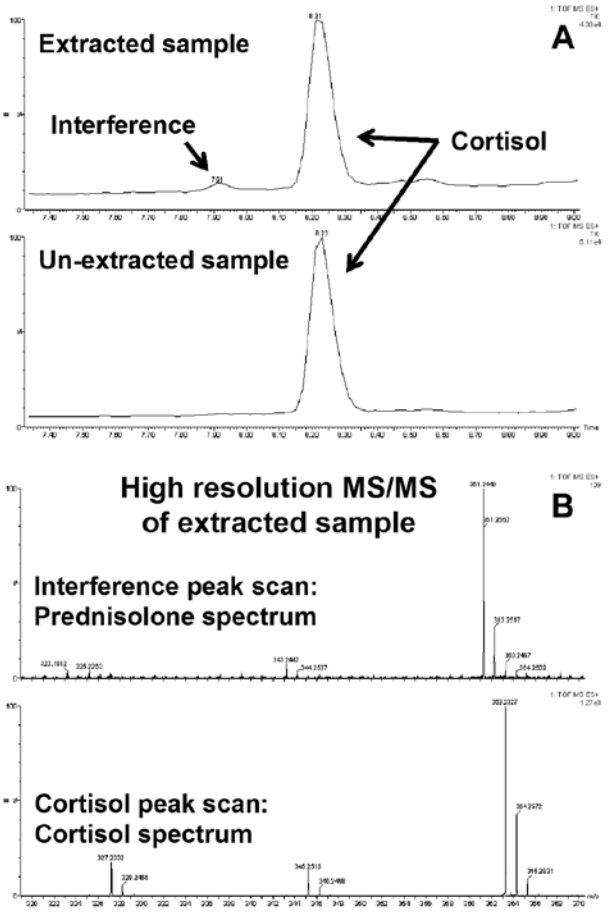


**Figure 11**. Prednisolone created by acid-base step during extraction of free cortisol from urine. (A) An interference can be detected in extracted samples, eluting just before the cortisol peak (top of panel A), but not in unextracted samples (bottom panel of panel A). (B) Subjecting the cortisol peak and the interference peak to a high resolution MS/MS scan on a quadrupole time-of-flight tandem mass spectrometer shows a spectrum consistent with prednisolone for the interference and a typical cortisol spectrum for the cortisol peak.

**Novel Clinical Applications – Peptide and Protein Measurements by LC-MS/MS**

Just within a little more than a decade LC-MS/MS has transformed from an esoteric gold standard technology to an affordable, flexible and accessible technique for most clinical laboratories. LC-MS/MS has helped in developing routine methods of high sensitivity, high specificity, high throughput, and high cost effectiveness in biochemical genetics/newborn screening, drug and toxicology testing and endocrine testing of steroids and biogenic amines. These novel methods have made a positive impact on patient care on both economic and quality fronts.

The next goal and challenge for LC-MS/MS is to address limitations of immunoassays for proteins and peptides. MS technologies with diverse front-ends have already immeasurably enhanced our knowledge of basic protein science. However, translating this information to clinical practice has been challenging. There have been a number of translational attempts in the high profile fields of cancer and cardiovascular diseases. As a result of this work, several multi-marker profiles with qualitative patterns were developed. Unfortunately, during validation of these profiles, flawed signals were discovered, which were due to pre-analytical errors or inconsistent sample preparation between different sites.[88] Standardisation of sample handling and preparation loom as huge challenges that have to be conquered before biomarker profiles can come of age. Similarly, the bar for clinical validation of such multi-marker profiles is very high, by the very fact that they are multi-marker based. This increases the potential for error exponentially. We therefore have yet to see a successful proteomic LC-MS/MS multi-marker peptide/protein profile being implemented and offered for clinical testing.

By contrast, there has been success when LC-MS/MS has been applied to known, established peptides and proteins biomarkers i.e. targeted proteomics. LC-MS/MS methods to measure peptides/proteins in clinical practice can offer value in variety of scenarios, such as:

1. An immunoassay does not exist for the analyte.
2. An existing immunoassay does not answer some key clinical questions.
3. An existing immunoassay is subject to frequent interferences.
4. The analyte has multiple isoforms.
5. There is high result variability between different assays for the same analyte.
6. The workflows are very difficult for existing assays.

There are now many published examples of targeted clinical LC-MS/MS assays of peptides and proteins that address such situations, and we will discuss a few examples of such LC-MS/MS assays, which have been successfully developed and validated and can be implemented in clinical laboratories.

***Albumin***

Albumin is excreted in urine in relatively high concentration and is a moderately large protein. There are many established methods for urinary albumin measurement. Historically, albumin has been quantified using dye-binding methods or by immunoassays.[89] However, these assays do not necessarily give the same result and their accuracy has been questioned.[90] Consequently, there has been increased interest in alternative methods. In particular, HPLC-UV assays for urinary albumin measurement have gained in popularity, especially in diabetic patient populations, because it had been reported that urinary albumin concentrations measured by HPLC (size exclusion)-UV were about 40% higher in these patients than those measured by traditional methods. However, there has been considerable controversy, as to whether measurements of urinary albumin by HPLC-UV are truly more accurate than those obtained using immunoassays, or whether the higher concentrations found by HPLC-UV represent an analytical bias caused by some interference.[90,91]

We decided to investigate this question further by developing a gold-standard LC-MS/MS method for urinary albumin measurement. Intact albumin was infused, scanned across the whole mass range to verify that any peaks that were seen truly originated from albumin (or an N-terminal fragment of albumin; Figure 12A).[92] The scan spectrum showed N-terminal fragment ions, created by in-source breakage of intact albumin, corresponding to unlabelled $b_{24}^{4+}$ ($m/z$ = 685.1) and $b_{24}^{3+}$ ($m/z$ = 693.6) and these were used for quantification of human serum albumin (HSA) using in-house synthesised $^{15}$N-HSA as an internal standard: $^{15}N_{35}$-$b_{24}^{4+}$($m/z$ = 913.2) and $^{15}N_{35}$-$b_{24}^{3+}$ ($m/z$ = 924.1). The calibration curves for the LC-MS/MS method were linear ranging from .06-9.5 µmol/L (4 to 625 mg/L) and were highly reproducible - linear regression equation: y = 0.01 x + 0.20 ($r^2$ = 0.999). The intra-assay CVs (n = 20) were 12.6% (0.26 µmol/L), 10.1% (1.1 µmol/L), and 4.0% (3.2 µmol/L), and the inter-assay CVs were (n = 10) 12.2% (0.29 µmol/L), 11.0% (1.2 µmol/L), and 7.1% (3.5 µmol/L). The limit of quantification was set at 0.16 µmol/L (10.5 mg/L), the lowest analyte concentration that showed an inter-assay CV of <20%.

Both immunoassay and HPLC-UV correlated quite well with this new 'gold standard' LC-MS/MS method (Figure 12B and 12C). However, while the correlation was tighter for HPLC-UV, the bias against LC-MS/MS was much less for the immunoassay, suggesting that the HPLC-UV method does indeed systematically overestimate the albumin content

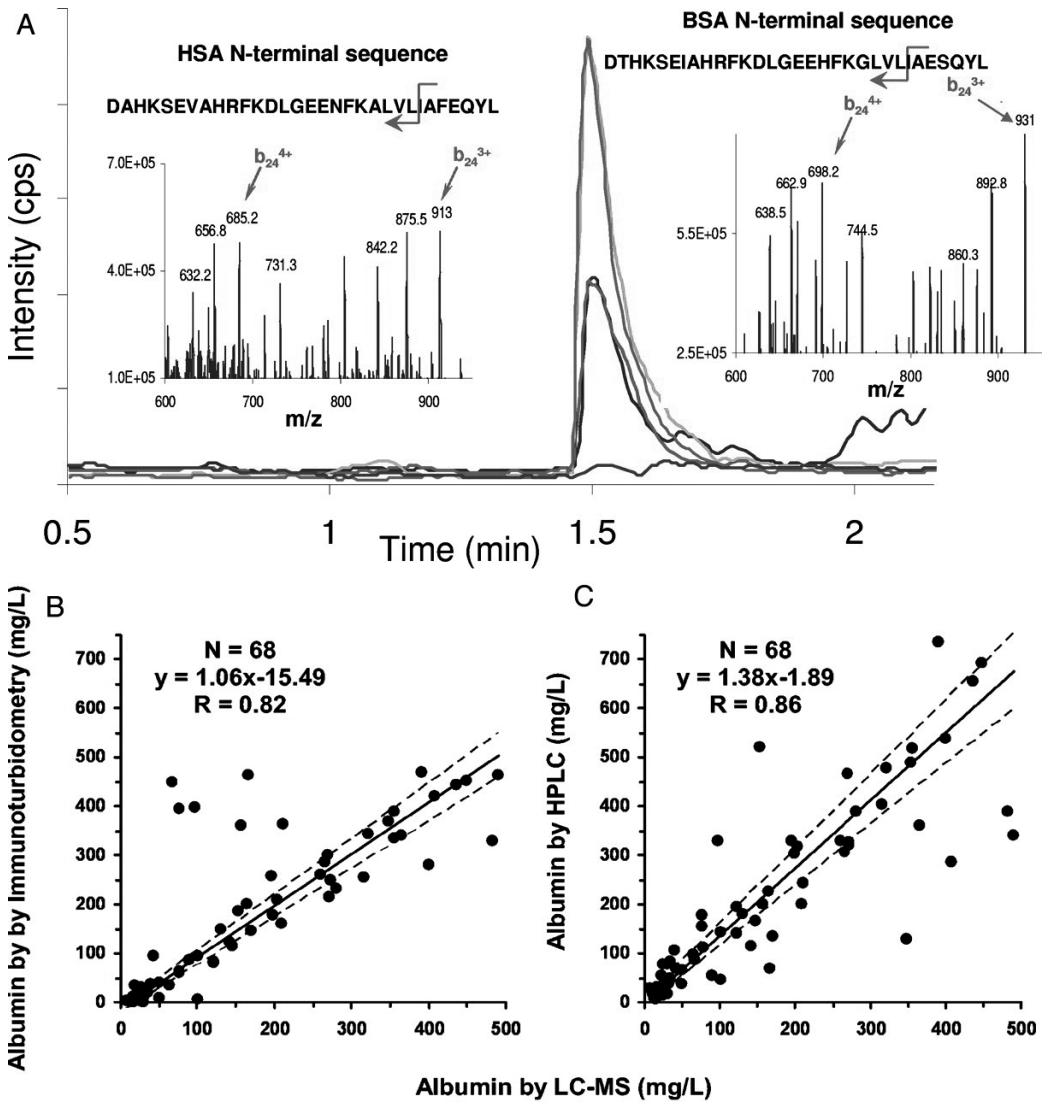


**Figure 12.** Urine albumin measurement by LC-MS/MS. (A) Spectra and chromatogram of human and bovine serum albumin urine subjected to LC-MS. There is in-source breakage of intact albumin and the 3+ and 4+ charge species of the N-terminal 24 amino acid fragment give a high intensity, proteotypic signal in both cases. (B) and (C) Method comparison of LC-MS with immunoturbidimetry (B) and HPLC-UV (C). Reprinted from Babic *et al.* (Clin Chem 2006;52:2155-7) with permission from Clinical Chemistry.

of urine, rather than giving a more accurate result than immunoassay.[92]

***Parathyroid Hormone***

Parathyroid hormone (PTH) measurement is an important part of the assessment of hypocalcaemia, hypercalcaemia, metabolic bone disease, and parathyroid gland tumours. PTH is synthesised in the parathyroid glands as a 115-amino acid precursor (prepro-PTH), cleaved to pro-PTH, and then to the bioactive 84-amino acid polypeptide (1–84 PTH or intact PTH). After secretion, 1–84 PTH has been reported to undergo rapid metabolism to form carboxyl-terminal, amino-terminal, and mid-molecule fragments.[93] Substantial variability and bias are commonly observed in the commercial automated sandwich immunoassays. Thorough understanding of the interferences and cross-reactivities with PTH fragments in these commercial assays is lacking,[94] and the variable biases between assays, as well as calibration drift over time, can lead to significant over- or underestimation of PTH concentrations in patients.

To address these issues we decided to develop and validate a reference LC-MS/MS method for 1-84 PTH, which might facilitate immunoassay harmonisation. Such a method may also prove useful in selected individual patients with confusing immunoassay results.

Because of the very low concentrations of circulating PTH (healthy population reference range: 2.1-6.4 pmol/L (20-60 ng/L), our approach was to enrich and clean up the sample before LC-MS/MS, using immunoaffinity beads. Next we digested the intact PTH into peptides and optimised the LC-MS/MS conditions for best signal to noise ratio. We verified the purity of the calibrators 1-84 PTH (>95% pure, confirmed by time-of-flight mass spectrometry). We realised early on that using an intact PTH internal standard, rather than simply labelled internal standard peptides, was critical for the normalisation of the extraction and the digestion steps. $^{15}$N labelled PTH internal standard was therefore produced in bacteria transfected with a PTH expression construct and grown in $^{15}$N-containing media. The LC-MS/MS method for 1–84 PTH requires 1 mL serum. For quantification of 1-84, the SRM response from the N-terminal tryptic peptide 1–13 PTH ($^{1}$SVSEIQLMHNLGK$^{13}$) was used (Figure 13). The LC-MS/MS assay proved linear from 4.1-484 pmol/L (39.1–4560 ng/L), and the limits of detection and quantification were 1.5-4.1 pmol/L (14.5 ng/L and 39.1 ng/L), respectively, using highly sensitive triple quadrupole instruments. The intra-assay CVs ranged from 6% to 11%, and the inter-assay CVs ranged from 7% to 17%. Interference by PTH fragments 1–44 PTH, 7–84 PTH, 43–68 PTH, 52–84 PTH, 64–84 PTH, and PTH-related protein was between <1% and <0.001%. This LC-MS/MS method is therefore much less prone to cross-reactivity than immunoassays, owing to the fact that the LC-MS/MS method was specifically optimised for the N-terminal tryptic 1–13 PTH fragment.

Method comparison of LC-MS/MS and the Roche Cobas® PTH immunoassay yielded a Deming fit of LC-MS/MS = 1.01*x* immunoassay – 13.21. The mean bias by Bland-Altman plot was –9.4%. In patients with renal failure, which leads to PTH fragment accumulation, the LC-MS/MS method allowed clear identification of some individuals who had apparently inaccurate immunoassay measurements (Figure 14).

***LC-MS/MS Assessment of Renin-Angiotensin System Function***

The renin-angiotensin system generates angiotensin II (Ang II), which is a potent vasoconstrictor, important for regulating blood pressure. There are three crucial components to this system: 1) renin, 2) angiotensin, and 3) aldosterone. Renin is primarily released by the kidneys by high aldosterone levels and converts angiotensinogen to angiotensin I (Ang I),

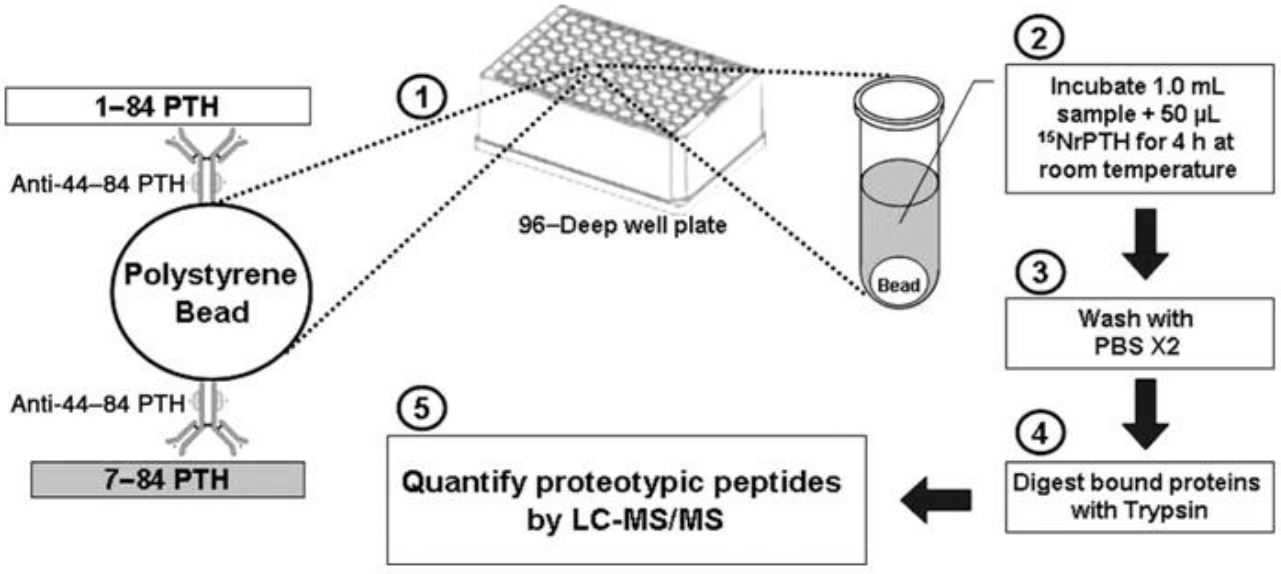


**Figure 13.** Workflow of measurement of intact parathyroid hormone (1-84 PTH) by LC-MS/MS in serum and plasma. PTH from patient samples and 15N-labelled recombinant 1-84 PTH are captured with antibodies on polystyrene beads (1 and 2), washed (3) and trypsin digested (4). The digest is then subjected to LC-MS/MS with SRM settings to detect the amino acid 1-13 proteotypic trypsin fragment. Reprinted from Kumar *et al.* (Clin Chem 2010;56:306-13) with permission from Clinical Chemistry.

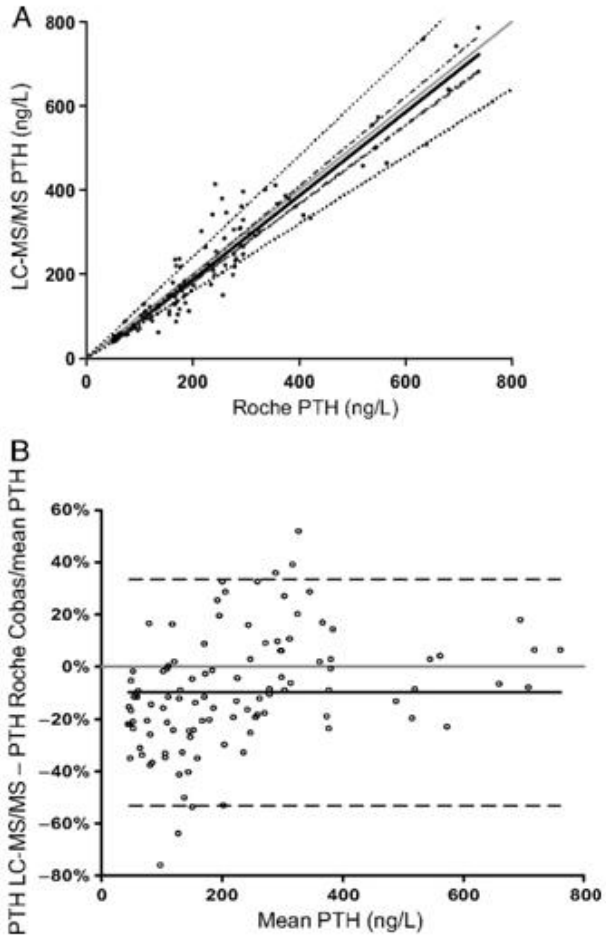


**Figure 14.** Method comparison between LC-MS/MS and immunoassay (Roche Cobas) measurement of PTH. (A) Scatterplot with regression fit and confidence intervals for slope and fit. (B) Bland-Altman plot showing percentage difference between methods plotted against the mean PTH of both methods. There is evidence for non-linearity of the difference, with the difference between the two assays first declining from a high percentage low bias for the immunoassay to a lesser percentage bias, and then to increasingly higher percentage biases for the immunoassays at very high PTH concentrations. Reprinted from Kumar *et al.* (Clin Chem 2010;56:306-13) with permission from Clinical Chemistry.

which is then in turn converted to angiotensin II (Ang II) by angiotensin converting enzyme (ACE). For Ang II, no commercial clinical assay is available. This is due to the fact that the bioactive hormone Ang II circulates in very low concentrations and has a short half life. Therefore, plasma renin activity (PRA) is used as a surrogate marker and has become an essential diagnostic tool. In this assay, the renin in patient plasma is allowed to act on the plasma's endogenous substrate, angiotensinogen, at 37 °C for a defined period of time, producing Ang I, which is measured by radioimmunoassay. The result is then compared to results obtained from the same sample at 4°C (at which renin is inactive). Conversion of Ang I to Ang II by ACE is inhibited with dimercaprol and 8-hydroxyquinoline. Renin activity is expressed in ng of Ang I produced per mL of plasma per hour of incubation. Limitations of the immunoassay for Ang I include the usual limitations discussed for RIAs in the previous section.

We decided to investigate whether we could replace the immunoassay for Ang I in PRA measurements with an LC-MS/MS assay, and have succeeded in developing and validating this assay.[95] It uses online extraction with an easy workflow and offers high throughput. A recent LC-MS/MS method for Ang I published by another group also uses automated online sample extraction and requires only 150 µL of plasma.[96] The MRM transitions reported for Ang I, were *m/z* 433 to 619 and *m/z* 433 to 641; IS, *m/z* 435 to 625 and *m/z* 435 to 653; internal degradation standard *m/z* 437 to 638 and

*m/z* 437 to 660. The later LC-MS/MS method involved two different internal standards and resulted in the discovery that 2–5% of the patient samples contain proteases and peptidases that degrade Ang I produced during renin-induced generation. This problem is mitigated by use of the LC-MS/MS method, as this method has improved analytical sensitivity for Ang I compared with RIAs. The required incubation time for renin-induced generation of Ang I is therefore shorter, minimising protease/peptidase induced loss of Ang I.

We have also optimised a LC-MS/MS method for direct Ang II measurement with a LOQ of 20 pg/mL (Figure 15). The sample requirement is 1 mL of plasma with offline SPE extraction. If it can be shown that this assay of the endpoint-peptide Ang II, without PRA measurement, correlates with clinical parameters as well as PRA measurements do, then this will represent a much simpler, preferable method for assessment of the renin-angiotensin system.

***Thyroglobulin – Thyroid Cancer Marker***

Thyroglobulin (Tg), because of its high organ-specificity, is an excellent tumour marker for thyroid cancer recurrence in patients who have undergone complete removal of their thyroid gland for thyroid carcinoma. Unfortunately, about 20% of these patients have anti-thyroglobulin autoantibodies (TgABs), which can interfere with the accurate detection and quantification of Tg.[97]

In a recent study, immuno-affinity enrichment of tryptic peptides was used in concert with LC-MS/MS to quantify thyroglobulin accurately in patients with TgABs.[98] Trypsin digests both Tg and TgABs, removing any TgAB interference. The tryptic Tg fragments are then captured with fragment-specific antibodies, allowing detection of Tg at picomolar concentrations. The detection limit for endogenous Tg in serum was 2.6 µg/L (4 pmol/L). Direct comparison with a Tg immunoassay revealed good correlation ($r^2 = 0.81$). While the analytical sensitivity is not quite sufficient for clinical practice, which now uses a serum Tg concentration of <0.1 µg/L as the yardstick for cure,[99] more recent results presented at the 2010 USA Endocrine Society meeting indicate that this methodology can achieve the required analytical sensitivity of 0.1 µg/L.[100]

**Summary and Conclusions**

Clinical MS/MS, in its relatively straightforward configuration of triple quadrupole LC-MS/MS, has seen incredible success and growth for a wide range of low molecular weight analytes during the last 10-15 years. From initial use in biochemical genetics/newborn screening and drug and toxicology testing, it has quickly expanded into endocrine testing, where it now forms the mainstay of referral/reference steroid and biogenic amine testing, capable of high sensitivity and specificity quantification of these analytes in large numbers of patient samples.

However, with the exponential growth in use, limitations of LC-MS/MS have become apparent. These centre on the interacting triangle of **sensitivity – specificity – throughput** and will continue to demand our attention.

Expansion into targeted peptides and protein detection has started more recently and is **'Here, now'**. We can expect this field will grow in scope and importance exponentially, not unlike what happened with low molecular weight analytes. By contrast, the much-heralded LC-MS/MS biomarker bonanza remains on the distant horizon for now.

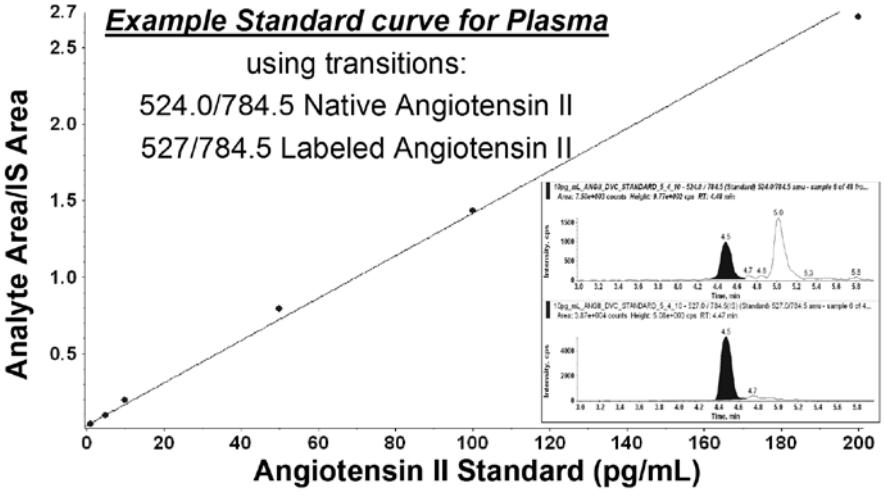


**Figure 15.** Example of a standard curve of LC-MS/MS measurement of angiotensin II in plasma.

**Competing Interests:** None declared.

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