New approaches for quantitative analysis in biological fluids using mass spectrometric detection

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Quantitative analysis with mass spectrometric detection is no longer limited to small molecules. A new focus of research is to understand biological processes at the molecular level and this requires more quantitative data for a large range of biomolecules, including low molecular weight compounds, peptides and proteins. On-line approaches such as liquid chromatography (LC) mass spectrometry are restricted to atmospheric pressure ionization (API) in combination with LC while off-line approaches can be performed either with API or matrix-assisted laser desorption ionization (MALDI). Selected reaction monitoring quantitation remains the favorite mode of detection for small molecules as well as for proteins and peptides. The potential of quantitative analysis without any chromatographic separation based on chip-based infusion or MALDI has been demonstrated. For the quantitation of small molecules, the use of an internal standard (IS) is well established with structural or isotopically labeled analogs. For proteins, the use of an IS remains more challenging and will need some further optimization. New ionization techniques, such as desorption electrospray ionization, may also open up some new opportunities for quantitative analysis.

Keywords: API, Atmospheric pressure ionization; Biomolecule; Desorption electrospray ionization; LC–MS, Liquid chromatography mass spectrometry; Mass spectrometric detection; MALDI, Matrix-assisted laser desorption ionization; Quantitative analysis; SRM, Selected reaction monitoring

1. Introduction

Over the last decade, there has been a shift in focus from liquid chromatography (LC) with ultraviolet (UV) or fluorescence detection towards LC with mass spectrometric (MS) detection (LC–MS) for the quantitative analysis of pharmaceutical compounds in biological matrices [1]. The success of LC–MS is in part related to the fact that the interfacing of LC with MS, using atmospheric pressure ionization (API), has become a very robust technique allowing the analysis of hundreds of samples every day. Another important aspect is that electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) [2] and photoionization [3] allow the ionization of a relatively large variety of analytes ranging from 100 to several thousand Daltons (Da). Many quantitative LC–MS/MS (LC–MS²) assays are performed routinely using triple quadrupole mass spectrometers operating in the selected reaction monitoring (SRM) mode with run cycles of less than 5 min and sensitivities in the pg/ml level. With biological matrices, ESI is prone to ionization suppression or enhancement, and good sample preparation remains the key to success [4]. Various sample-preparation strategies have been implemented, including: liquid–liquid extraction (LLE); and, off-line and on-line solid-phase extraction (SPE). Column switching using large particle, monolithic material or restricted-access material (RAM) has become particularly attractive, since it allows the direct injection of biological fluids, automatically [5].

LC–MS² has had a large impact in both pharmaceutical drug discovery and drug development, but for different reasons. In drug discovery, the throughput of LC–MS has allowed development of generic, quantitative assays for large libraries of compounds; this can provide pharmacokinetic data at an early stage in the drug-candidate selection process. For drug development, the sensitivity and the selectivity of LC–MS has contributed considerably to reducing the method-development time for assays. Faster run cycle times are always desirable, not only for increased sample throughput but also for rapid turn-around of sample analysis. This is particularly important for clinical studies requiring on-line analytical studies. One of the major differences between bioanalysis in drug discovery and drug
development is that, for drug development, the data generated with these methods are part of the submission dossiers and the assays have to be validated according to international guidelines. Despite the fact that LC–MS$^2$ is reaching its maturity, it will continue to play a very important role in bioanalysis.

There has been significant improvement in the performance of triple quadrupole mass spectrometers in the last decade. New hybrid instruments suitable for quantitation do not limit analysis to small molecules (below 1000 Da) and can quantify intact proteins or peptides in biological matrices that present a particular challenge for the pharmaceutical industry and academic research.

The aim of the present article is to review some novel approaches to quantitative analysis of small molecules as well as peptides and proteins in biological matrices.

2. Quantitative analysis of small molecules

As more potent new chemical entities are tested as clinical candidates, there is a growing need to improve the limit of detection (LOD) of these compounds in biological matrices. Most of the quantitative assay development in the SRM mode is performed using a triple quadrupole with the first resolving quadrupole (Q1) and second resolving quadrupole (Q3) tuned at unit mass resolution [full width at half maximum (FWHM) of 0.7 Da]. Isobaric endogenous analytes can interfere with the signal of the analyte of interest and affect the limit of quantification (LOQ) of the assay. One way to remove these interferences would be to adapt either the sample-preparation method or the LC-separation strategy. Re-development of the assay is time-consuming and requires re-validation of the assay, which adds to the cost of sample throughput. These interferences may not have appeared with spiked plasma used to prepare calibration and quality-control (QC) samples, but with only a limited number of study samples.

Recently, a new triple quadrupole mass spectrometer system (Quantum, ThermoFinnigan) with enhanced resolution capabilities has become available. The enhanced resolution capability of the instrument allows the quadrupoles to be tuned at FWHM of 0.2 Da without significant loss in sensitivity. In most cases described, it is the Q1 resolution that is enhanced. Xu et al. [6] have investigated the enhanced resolution approach for discovery, bioanalytical applications. They found that, for the compounds investigated, the enhanced resolution mode improved the signal-to-noise ratio by a factor of 2. Another benefit was the improved dynamic range. The effect of enhanced resolution on selectivity is illustrated in Fig. 1. The gain in sensitivity (factor of 2) may not seem spectacular, but it is obtained at the assay LOQ. To achieve this LOQ, extensive sample preparation or improvement in chromatography would have been essential at unit mass resolution.

Jemal and Ouyang [7] also found that SRM bioanalytical methods, using better than unit mass resolution, can be developed as ruggedly as unit mass resolution methods, as demonstrated for the quantitative analysis of nefazodone (LOQ of 30 pg/ml) in human plasma. The same authors have also investigated the stability of the system and showed that a temperature fluctuation in the laboratory (e.g., 3–4°C) may cause a drift in the Q1 mass axis and change the absolute mass response. They therefore suggest implementing a QC procedure to monitor possible drift in mass accuracy. It may be easy to monitor the drift, but it is more challenging to establish procedures that compensate for this, on the fly, during batch analysis.

In bioanalysis, sample re-injection should be strictly avoided. Hughes et al. [8] reported a sensitive

Figure 1. Mass chromatograms of analyte (0.1 ng/ml) and internal standard: (a) at Q1 0.7 Da FWHM; (b) at Q1 0.2 Da FWHM (from [6], with permission).
determination of carbergoline in plasma. The enhanced resolution mode allowed them to reach an LOQ of 250 pg/ml using APCI compared to 500 pg/ml in the unit mass resolution mode. The enhanced resolution may be particularly interesting when running study samples. Method development is done in most cases with only a few different batches of plasma. Study samples may behave differently regarding their background interferences and, at the same time, the LOQ may not be maintained. This would normally require, especially for sensitive methods, extensive method re-development. The possibility of improving selectivity without having to modify the sample preparation or the chromatographic separation is of great interest.

With LC–MS², the role of chromatography has fundamentally changed compared to LC–UV methods. Due to the high selectivity of tandem MS (MS²), baseline separation of the analytes is no longer required. However, LC remains important for the concentration of the analyte during the injection step or to minimize matrix suppression. Separation of metabolites is required in selected cases only.

For routine assays, where ultimate sensitivity is not essential and metabolite cross-talk is not an issue, quantification should be possible with flow-injection analysis (FIA) or direct infusion. Dethy et al. [9] have demonstrated the direct bioanalysis of verapamil and its metabolite norverapamil in plasma using nanoelectrospray infusion from a silicon chip. This chip-based infusion system (NanoMate, Advion BioSciences), made of an array of 100 or 400 nozzles, enables the rapid infusion of the sample at a flow rate of approximately 250 nl/min. For each analysis, a new pipette tip and a new nozzle are used to eliminate the carry-over issues often encountered with LC–MS² analysis. Detection is performed on a triple quadrupole mass spectrometer operated in SRM mode. With chip-based infusion, the software no longer integrates chromatographic peaks but rather an “infusiogram” or square shaped “peak” created by the extracted ion chromatogram from the infusion process, as illustrated in Fig. 2. Construction of the calibration curve is performed in a similar way to LC–MS² – by plotting the “peak” area ratio of the analyte over the internal standard (IS). Using a plasma aliquot (100 µl), the assay was found to be linear in the range 2.5–500 ng/ml. An infusion time of approximately 1 min was long enough to collect sufficient data. Using chip-based infusion samples, carry-over, evident during LC–MS, was completely eliminated and sample throughput was improved. A batch of 96 samples could be analyzed in less than 90 min. Method development is also simplified because:

(i) no chromatographic separation means that optimization is unnecessary; and,
(ii) the analytes and the IS always co-elute and non-labeled ISs can be used.

Another advantage is that deterioration of chromatographic separation during analyses of very large batches is no longer an issue. It was anticipated that, due to infusion at nl/min flow rates resulting in reduced ionization suppression, sample preparation could be limited to protein precipitation. However, attempts to quantify midazolam in human plasma with a chip-based infusion strategy using only protein precipitation as sample preparation resulted in low signal abundance [10]. The addition of a desalting step after protein precipitation significantly improved the signal and allowed assay validation in the range 1.5–500 ng/ml.

![Figure 2. Chip-based infusion ion current profiles representing a single standard curve for verapamil and norverapamil (2.5, 5, 10, 25, 50, 100, 250 and 500 ng/ml) (from [9], with permission).](http://www.elsevier.com/locate/trac)
Wickremsinhe et al. [11] recently compared the performance of chip-based infusion and LC–MS$^2$ for the validation of assays that conform with regulatory guidelines and good laboratory practice (GLP) requirements. They reported that the lack of carry-over enabled development and validation of a 500,000-fold dynamic range assay for reboxetine with good precision and accuracy. The ability to extend the dynamic range of an assay significantly reduces the need to repeat analysis and therefore increases sample throughput.

In quantitative LC–MS$^2$ bioanalysis, validation of the assay is performed with spiked samples using targeted SRM analysis. Study samples may contain co-eluting metabolites or co-medication, which may affect the selectivity of the assay. The use of a confirmatory SRM transition is one way to check the selectivity of the assay. However, the confirmatory transition needs to have the same sensitivity as the quantitation transition, which limits its application. Confirmatory analysis becomes an even more important issue when little or no chromatographic separation is performed.

Triple quadrupole linear ion trap (QqQ$_{LIT}$) technology allows simultaneous qualitative and quantitative analysis [12]. In the same LC–MS run, it is possible to perform precise, accurate quantitation and to record a full scan product-ion spectrum for confirmation analysis. Leuthold et al. [13] have extended this concept with chip-based infusion by performing simultaneous SRM and MS$^3$ quantitation in the same run, thus increasing analytical specificity. One limitation of the approach is that the phase II metabolites of the parent drug can fragment in the source and affect quantitation. With chip-based infusion, good sample preparation such as LLE was found to be very important to minimize matrix suppression and to develop rugged analytical methods.

Matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF-MS) [14,15] is commonly used for the qualitative analysis of high molecular weight compounds, such as peptides and proteins [16], synthetic polymers [17], DNA [18] and lipids [19]. MALDI was originally developed for the analysis of peptides and proteins on TOF instruments. MALDI has the intrinsic advantage that it can achieve a high sample throughput compared to electrospray LC–MS. Sample preparation and separation can also be decoupled from the MS analysis. The MALDI target plate can be easily archived, allowing simple re-analysis of selected samples. The high-throughput capability of MALDI and the different ionization mechanism also makes it an attractive alternative to ESI for the analysis of low molecular weight (LMW) compounds [20]. Interferences of matrix ions and the ionization of the LMW compounds are the challenges of this technique [21,22] especially as only one stage of MS analysis is used, unlike MS$^2$ on triple quadrupoles where SRM is performed. Desorption/ionization on silicon (DIOS) has been described for the analysis of LMW compounds with no chemical background [23,24].

Several papers have reported the use of MALDI for the characterization of small molecules but the potential of the technique has not yet been investigated fully. Pharmacokinetic data for the synthetic opioid peptide analog (DAMGO, MW = 514 Da) in ovine plasma was determined by MALDI-TOF-MS [25]. A plasma aliquot of 300 μl was used. Calibration in the range 5–50 ng/ml used a d5 deuterated analog of DAMGO. The analysis of post-source decay fragments lowered the LOD, and the use of the precursor-product ion relationship improved the specificity.

Mims and Hercules [26,27] developed an assay for the quantitation of six bile acids from urine and plasma on a TOF instrument in negative mode using an 9-aminoacridine matrix. SPE was applied as sample preparation and N-1-naphthylphthalamic acid was used as the IS.

Triple quadrupole instruments combined with electrospray LC–MS are the primary choice for the quantitative analysis of pharmaceutical compounds in biological matrices operated in the SRM mode, so the combination of orthogonal MALDI with a triple quadrupole system for quantitative analysis is very promising. Hatsis et al. [28] have demonstrated the quantitative analysis of benzodiazepines in pill formulations on a prototype orthogonal MALDI triple quadrupole mass spectrometer. The instrument was equipped with a high-repetition nitrogen laser (1000 Hz) for higher sample throughput; traditional MALDI sources are typically operated in the range 5–50 Hz. A calibration range of three orders of magnitude with good reproducibility was obtained. The reproducibility was found to be better at a laser frequency of 1000 Hz than at 20 Hz with or without the IS.

Cole et al. [29] investigated MALDI–SRM using a 1500-Hz nitrogen laser for the analysis of 225 pharmaceutical compounds (MW range 188–890 Da). They observed that 83% of the compounds were successfully detected with sensitivity better than 50 nM. The matrix was not found to be crucial and the use of an IS compensated for experimental variables.

The application of MALDI–SRM has also been demonstrated for the analysis of talinolol in human plasma without any LC separation [30]. The assay was found to be linear in the range 1–1000 ng/ml using a 200-μl plasma aliquot. Precision and accuracy were found to be acceptable for performing quantitative analysis. LLE was selected as sample preparation to minimize matrix suppression, because direct protein precipitation with perchloric acid failed due to severe matrix suppression. However, an in situ wash of the spot with an aqueous solution permitted restoration of the signal, allowing quantitative analysis with a simplified sample-preparation procedure. With a high-repetition laser, the analysis...
of one spot lasts less than 2 s, allowing the analysis of 96 samples in approximately 4 min. As demonstrated previously with chip-based infusion, LC separation is not always necessary for quantitative analysis. MALDI offers an ionization approach complementary to chip-based infusion. In both cases, the decoupling of the separation step should allow simpler multiplexing of these approaches.

MALDI can be performed in the atmospheric pressure (AP) mode [31,32] as well as in the vacuum mode. The quantitative analysis of lysergic acid in urine by AP–MALDI–SRM on an ion trap (IT) has been reported by Cui et al. [33]. The results obtained in the range 1–100 ng/ml were found to be comparable to the LC–MS results. Interfacing of AP–MALDI is much simpler than that of vacuum MALDI. AP–MALDI appears to be a softer ionization technique, although ion transport from atmosphere to vacuum may also affect the analytical sensitivity of the technique [34]. The differences between these approaches need to be further evaluated with particular attention to sensitivity and spectra quality.

Another interesting application of MALDI is MALDI–MS imaging (MALDI–MSI), which allows the direct analysis of tissue samples [35,36]. Typically with MALDI–MSI, a thin slice of frozen tissue sample is attached onto a sample plate and matrix is deposited onto the tissue. MALDI spectra can be then obtained from any position on the tissue in profiling mode (low resolution) or in imaging mode (high resolution). The resulting spectra contain information from peptides, proteins, endogenous metabolites or from drugs and their metabolites. By using MALDI-QqTOF-MS, an anti-tumor drug was unambiguously determined in mouse-tumor tissue by monitoring the dissociation of the protonated drug to its predominant fragment [37]. The traditional approach of electrospray LC–MS2 for tissue analysis is significantly more time-consuming and does not provide a visual image locating the presence of the species.

3. Quantitative analysis of peptides and proteins

Protein and peptide drugs cover a broad range of clinical application, such as bacterial infections, inflammatory and rheumatic diseases, pain, hypertension, AIDS and cancer [38]. During drug development, assays need to be developed to follow plasma concentration of these compounds. The absolute quantitation of endogenous peptides and proteins in biological fluids as disease markers, in clinical diagnosis or in proteome research, is also a topic of growing importance. Protein and peptide quantitation is most often performed using immunoassays. Despite the great sensitivity of this approach (i.e., picomolar to femtomolar concentrations), antibodies are required and the presence of endogenous analytes can severely affect the selectivity of the assay. In the cases of peptides and proteins, immunoassays would then quantify the total amount of related analytes. MS, with either ESI or MALDI, has great potential because it can differentiate between the various peptide analogs based on their collision-induced dissociation (CID) spectra or the protein isoforms based on their molecular weight or product-ion spectra.

The combination of LC and MS that is particularly interesting for peptide quantitation has been reviewed recently [39]. Various separation mechanisms can be applied with LC, including ion-exchange and reversed-phase chromatography. As for small molecules, peptide detection with LC–MS is performed using MS2 in the SRM mode, with triple quadrupole or using IT systems using MS2. With LC–MS, obtaining a LOQ below 1 ng/ml is difficult to achieve because ESI generates multiply charged ions. The charge distribution cannot always be tuned to a single charge state and overall sensitivity of the assay is affected. The second point is that the CID of peptides generates many fragments and so affects the overall sensitivity.

To achieve high detection sensitivity, the combination of nanoscale LC (nLC) with MS becomes necessary. Practically, to maintain the benefit of high sensitivity, due to the reduced inner diameter of the column, the same volume of sample should be injected on the miniaturized column as on conventional columns. This can be achieved with column switching that combines a sample clean-up and sample-concentration step. Varesio et al. [40] have applied the column-switching approach for the detection of amyloid–β peptide in plasma using MS detection in the SIM mode.

The active and inactive glucose-dependent insulinotropic polypeptides (GIPs), GIP1–42 and GIP3–42, can be differentiated by MS. Selective sample preparation and pre-concentration remain very important steps for achieving good LODs. Wolf et al. [41,42] reported the use of immunoprecipitation with magnetic beads for the simultaneous LC–MS determination of the GIP hormones in human plasma (Fig. 3). Despite the fact that proteins can be detected by ESI, direct protein quantitation with LC–MS using stable isotope dilution MS (IDMS) is not currently a very practical approach.

An interesting strategy for absolute protein quantitation is protein cleavage-IDMS (PC-IDMS) [43,44]. With this technique, the IS is a different molecule from the analyte, and is generally an isotope-labeled analogue of a peptide fragment. Various types of IS including peptides with and without cleavable sites have been used. The protein in the presence of the IS undergoes proteolytic enzyme digestion and both the selected peptide fragment and the IS are detected by LC–MS2 in the SRM mode. Absolute quantitation of a prostate-specific antigen biomarker in serum has been reported by Barnidge et al. [45]. Their results showed that currently with PC-IDMS
the accuracy, precision and sensitivity for the assay were not as good as those observed with immunoassays. However, they demonstrated the ability to quantify a protein in the presence of a broad dynamic range of background proteins. This approach can be further improved in terms of sample preparation and chromatography. The approach has potential and improvements in sample preparation and chromatography would boost it. Also, the anticipated improvement in MS sensitivity should benefit the technique.

Recently, a combination of nanoelectrospray, tandem MS and an $^{18}$O-labeled peptide IS was applied to the quantitation of proteins from in-solution digests [46]. During digestion, $^{18}$O atoms are incorporated into C-terminal carboxyl groups of tryptic peptides (shift of 2 or 4 Da). The ISs ($^{18}$O-labeled peptides) can be obtained from a stock solution of the protein of interest, so any detectable peptides by ESI–MS2 will have their IS counterpart. The absolute concentrations of the proteins can then be calculated from the ratios of the intensities of the peptides and their respective ISs.

MALDI has also been explored for the quantitation of biomolecules in biological tissue or fluids [47]. Advantages of MALDI are the high-throughput capability of the system and that sample preparation or chromatographic separation can be decoupled from the MS detection. MALDI in combination with TOF-MS also allows analysis of mixtures of proteins in the form of protonated molecular ions. Bucknall et al. [47] have analyzed growth hormone (MW = 18,363 Da) in rat pituitary tissue. Linear calibration curves (1–21 μmol/g tissue) could be obtained using β-lactoglobuline despite the IS having no homology with the analyte.

Surface enhanced laser-desorption/ionization (SELDI) is a distinctive form of laser-desorption/ionization where the target plays an active role in the sample-preparation procedure and ionization process [48]. Depending on the chemical or biochemical treatment, the SELDI surface acts as SPE or an affinity probe. The target itself is used for sample fractionation and purification of biological samples prior to direct analysis by laser-desorption/ionization. SELDI is mainly applied to protein profiling and biomarker discovery by comparing protein profiles from control and patient groups.

4. Desorption electrospray ionization

Desorption ESI (DESI) is a new API technique introduced by Cooks and co-workers [49] for the ionization of organic compounds, peptides and proteins from metal, polymer and mineral surfaces without any matrix addition. The analytes deposited on the surface of the target are ionized by the impact of charged particles formed by ESI. Typically, an acidic mixture methanol/water (1/1) is sprayed at a flow rate of 3–15 μl/min with a high voltage of approximately 4 kV. The linear velocity of the nebulizing gas is higher than for pneumatically assisted electrospray. Atmospheric gas-phase ions are transferred into the IT mass spectrometer through a 30-cm transfer line. Extensive comparisons of sensitivity of DESI with ESI or MALDI have not been performed yet, but lysozyme in the range 10–50 pg could be detected with DESI. High-throughput and in vivo sampling of living tissue surfaces have also been demonstrated. Quantitative analysis can be obtained by adding an
appropriate amount of IS onto the surface of the target. Recently, DESI has also been applied to couple thin-layer chromatography with MS [50].

5. Conclusions

Quantitative analysis with MS detection is no longer limited to small molecules. A new focus of research is to understand biological processes at the molecular level and this requires more quantitative data for a large range of biomolecules. High sensitivity and selectivity of MS remain important issues because they allow more efficient method development and validation. On-line approaches, such as LC–MS, are restricted to API in combination with LC, while off-line approaches can be performed with either API or MALDI. SRM quantitation remains the favorite mode of detection for small molecules as well as for proteins and peptides. The potential of quantitative analysis without any chromatographic separation, based on chip-based infusion or MALDI, have been demonstrated. However, to minimize matrix suppression, sample preparation remains a very important step. Immunoaffinity sample preparation in combination with MS detection is not used to its full potential. For quantification of small molecules, the use of an IS is a well-established procedure with structural or isotopically labeled analogs. In the case of proteins, the use of an IS remains more challenging and will need some further optimization. With the various new strategies, the issue of assay validation needs to be addressed because the concepts used with classical LC–MS² may not be adequate. Finally, expected improvements in MS sensitivity and resolution or new ionization techniques will strongly affect the way future quantitative analysis is performed.

References