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Review

LC–MS/MS for protein and peptide quantification in clinical chemistry☆

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ABSTRACT

The LC-triple quadrupole mass spectrometer (LC–MS/MS) is an increasingly common tool in the clinical laboratory. Established applications include routine assays for detecting inborn errors of metabolism, and for monitoring therapeutic drugs and steroids. Peptides and proteins in biological matrices have traditionally been quantified by immunological methods such as RIA or ELISA. These methods have the drawback of being insufficiently selective, often not allowing differentiation between the peptide and its derivatives or degradation fragments. The improved robustness and sensitivity of LC–MS-based techniques provide reliable alternatives for peptide quantification. Mass spectrometry does not require specific antibody reagents and is a powerful tool for the study of posttranslational modifications (PTM). In addition, several studies have demonstrated the utility of selected reaction monitoring (SRM) assays using stable-isotope-labelled (tryptic) peptides for quantifying proteins in human serum. Peptide-based MS/MS is a relatively new development in the measurement of clinically significant proteins, offering cost effectiveness, high throughput, multiplexed analysis and quantification, with the potential for combining the measurement of small molecules, peptides and proteins on a single technology platform. Quantitative analysis of proteins and peptides by LC–MS/MS is becoming a practical technique for clinical laboratories. To move from the laboratories of highly skilled analysts to routine clinical diagnostic laboratories requires that a number of technical hurdles be overcome in regard to sensitivity, imprecision, accuracy and the sample handling necessary for clinical use.

Contents

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1. Introduction

Tandem mass spectrometry is becoming an increasingly important analytical technology in the clinical laboratory environment [1,2]. Applications in toxicology and therapeutic drug monitoring have opened the door for tandem mass spectrometry and we are now seeing a vast array of new applications being developed. One of the first applications was the multiple analyte screening for inborn errors of metabolism [3]. This application embodied one of the seminal advantages of MS/MS, the ability to quantitate a large number of analytes in a single scalable measurement process. It is now used to screen for many other classes of metabolites. It has been shown to be effective for steroid metabolite profiling in the diagnosis of adrenal cortical dysfunction [4,5], and for multiple-analyte therapeutic drug monitoring [6].

The well-established selectivity and sensitivity offered by mass spectrometry for small molecule analysis would suggest that peptide quantification could also be optimally achieved by LC–MS/MS. Protein cleavage coupled with LC–MS/MS was described by Barr for quantifying apolipoprotein A-1as a reference material some years ago [7].

Various quantitative profiling approaches have been developed as the field of proteomics matures [8]. Different strategies have been reported for the study of post-translational modifications of proteins, quantitative peptidomic or quantitative proteomic studies, in which labels, or Isotopic Coded Affinity Tags (ICAT), are applied for comparisons between levels of peptides or proteins in biological samples (e.g., treated vs untreated). These conventional quantification procedures are generally employed on the basis of a relative quantification, as calculations are made from at least two different samples [9,10]. System biology requires an accurate quantification of a specified set of peptides/proteins across multiple samples. As a consequence, we see remarkable progress in the field of absolute quantification of peptides and proteins using mass spectrometry [11–13].

Many of these approaches, and the new technologies developed for proteomic analysis present substantial challenges for routine clinical application and for achieving the usual standards of clinical laboratory practice. Selected reaction monitoring (SRM) – also called multiple reaction monitoring – has emerged as a very promising technique for quantitative proteomics. It has the potential to overcome the shortcomings of current shotgun proteomic approaches. Moreover, SRM, using triple quadrupole mass spectrometers, is a proven clinical laboratory technique for the quantitative analysis of molecules in complex matrices such as serum and plasma. These properties have also established SRM as a tool for protein quantification for the clinical laboratory [14,15]. The limited range of a triple quadrupole mass spectrometer requires the proteolytic digestion of target protein followed by quantification of selected signature peptides. Recently, a number of LC–MS-based methods using different ion sources have been reported for the determination of proteins and peptides such as CRP [16], IGF [17] and different peptides [18].

The scope of this review will be to report on the advantages and challenges associated with the measurement of peptides and proteins in biological fluids from the standpoint of a clinical laboratory. After discussing the principles of SRM-based assays, new possibilities offered by this technique are illustrated. The possible practical impact in clinical laboratories, and points of concern, are discussed.

The focus will be on the SRM mass spectrometric approaches that offer potential opportunities for the future and fit most suitably in the clinical laboratory environment [19,20].

2. Selected reaction monitoring (SRM) for protein quantification

The SRM assay approach usually offers high specificity, even in complex sample matrices, through selection of a specific precursor ion in the first mass analyzer and selection of a specific fragment ion formed during passage of the precursor ion through a collision cell. In the case of proteins, a specific tryptic peptide (proteotypic peptide) can be selected as a stoichiometric representative of the protein from which it is cleaved. In principle, such an assay requires only knowledge of the masses of the selected peptide and its fragment ions. SRM modus allows the improvement of the limit of quantification and increased analytical speed and dynamic range. The linear response over a wide dynamic range enables the detection of low-abundance proteins in highly complex mixtures.

The great advantage of this approach is that it can be carried out using existing high-throughput LC–MS/MS platforms in the clinical laboratory. SRM exploits the unique targeting capabilities of the triple quadrupole mass analyser, the most commonly used instrument. It has the potential for combining the measurement of small molecules and proteins on a single technology platform, offering cost-effective, high-throughput, multiplexed validation and quantification. The technique promises high accuracy and analytical specificity, and should be readily adaptable to simultaneous multiplex analyses of many proteins.

However, the development of clinically useful protein and peptide SRM assays can be challenging for a variety of reasons. To examine the general strategies towards peptide quantification, some of the challenges presented to the analyst during protein/peptide analysis are discussed here.

2.1. Targeted peptide selection

The selection of proteotypic or signature peptides is the first step when designing a SRM assay. It is the most critical step in the quantitative analysis of target proteins [12,21,22]. The selections are predicted through modelling and proteomic data. The common databases such as Uniprot, Ensembl Genome Browser provide search tools and data on polymorphisms, post translational modifications and homologous sequences.

The surrogate peptide should have an amino acid sequence unique to the candidate protein and should be easily detectable by mass spectrometry. The aim is to select a few characteristic peptides which are unique and representative of the protein. Useful empirical rules can be found in the following reviews [22,23].

The peptide must have several important characteristics. First of all, it should be distinguishable from other more abundant proteins or from matrix ions. Secondly, the ionization must be sufficiently efficient to produce abundant ions in the mass spectrum. Finally, as the quantification is carried out on one single peptide for one protein, this peptide must uniquely identify the targeted protein

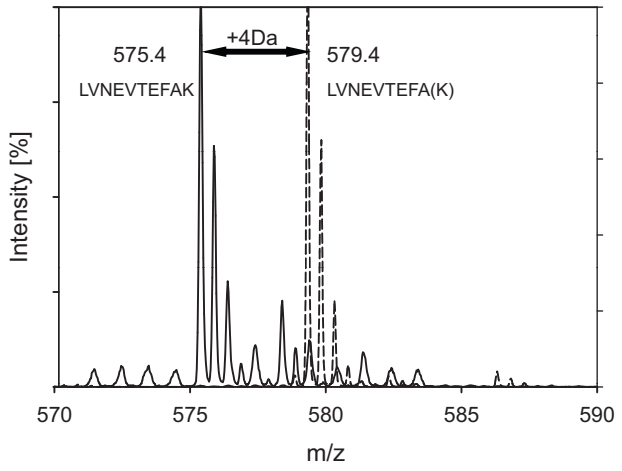

Fig. 1. MS spectra of the peptide LVNEVTEFAK and LVNEVTEFA(K). The peptide is proteotypic for albumin [19]. Doubly charged ions, mass difference: lysine/K +8 Da.

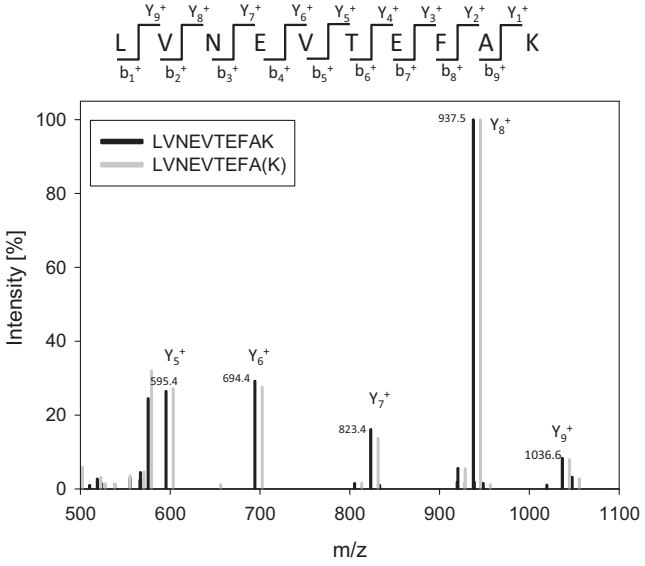

Fig. 2. MS/MS spectra of the unlabelled and labelled doubly charged precursor ions m/z = 575.4 and 579.4, collison energy 28 eV.

or one isoform thereof. Tryptic peptides containing amino acid residues with potential post-translational modifications should be avoided. Care should be taken to avoid targeting peptides which can undergo artifactual modification during sample preparation. Commonly observed mass changes are +16 or +32, reflecting methionine oxidation to sulfoxides, or sulfone, respectively, and can be observed for tryptophane, cysteine and histidine as well. Mass to charge ratio could be equal to another peptide, although the sequence is specific for the protein. Peptides with missed cleavages or non-tryptic cleavage sites should also be avoided. The preferred peptide length is approximately 10–20 amino acid residues. It is recommended to monitor at least three peptides for each targeted protein, in order to ensure assay specificity and detect any discrepancies related to interferences and the matrix effects of digested plasma [24]. SRM can also be used to quantify peptides with post-translational modifications such as phosphorylation [25].

2.2. SRM transition selection

The goal of the selected reaction monitoring procedure is to obtain the most sensitive, yet specific signal possible in the presence of a complex matrix. After selection of the peptides sensitive and robust MS/MS transitions (peptide precursor to product ion fragmentations) of target peptides must be identified. Proteins and peptides when protonated during ESI, form multiply charged ions, with mass to charge ratios (m/z) averaging 200–2000 amu. Fig. 1 shows a mass spectrum from the electrospray ionization of an albumin proteotypic peptide and its heavy isotope-labelled analog. Since species with different degrees of protonation will coexist, there will be multiple peaks observed for each peptide. To obtain a highly sensitive assay, it is therefore important to select transitions specific for the fragment which shows the most intense signal.

Because peptide bonds are relatively weak a sufficient number of fragments is formed from these charged peptides which are undergoing collision induced dissociation. Fragmentation usually results in the production of two complementary peptide ion series, termed the y-ions and b-ions. The y-ion retains a positive charge at its C-terminal end, while the b-ion retains the charge at the n-terminal end. The fragmentation pattern provides amino acid sequence information (see Fig. 2).

Since fragmentation patterns can vary from instrument to instrument, it is important to optimize each peptide individually, in order to determine the best balance of signal to noise [26]. After full MS scanning of the precursor ion is performed, collision energy and collision gas must be optimized. It is often necessary to choose between selecting the doubly charged or triply charged precursor ion. Both should be evaluated for sensitivity with the goal of monitoring a fragment ion at m/z ratio greater than that of the precursor ion. This can greatly reduce noise and improve the overall sensitivity of the assay. MS parameters require optimization and refinement. Multiplex assays require additional coordination and optimization of multiple sets of proteins and peptides. Using synthetic peptides or crude peptide arrays are advisable to optimize MS and chromatographic parameters [22] and can accelerate this step [27]. Many algorithms and methods have been reported to accelerate and simplify the design and implementation of SRM based methods.

Commonly, the best 2–4 transitions per peptide are selected for quantitative assays. The number of peptides which can be tested in a single analysis is limited by several factors like scan speed, chromatographic peak width and software limitations. The number can be increased by using scheduled SRM.

2.3. SRM transition validation

As for small molecules, inaccurate quantification may be caused by false positive identification, suppression caused by the matrix, interference in one or more monitored product ions, poor chromatography, instrument related signal attenuation and saturation. In complex mixtures, chromatographic signals from isobaric, or nearly -isobaric precursor peptides, might overlap with the specific precursor signals. It is important to ensure that the quantified signals do indeed derive from the targeted peptide. Similar to the approach described for small molecules, the relative intensities of the product ions for a given peptide can be used as an additional measure of selectivity (see Fig. 2). A set of coeluting peaks confirms that the detected SRM signals do derive from the targeted peptide, with constant ratios of the transitions throughout the linear range. Incorporation of a heavy isotope-labelled peptide adds an additional level of confidence to peptide identification by providing a reference to which the fragment ion ratios can be compared. Heavy isotope-labelled peptides (with incorporated ^{15}N and ^{13}C) will co-elute exactly with the non-labelled peptide and the intensity ratios of the transitions should be the same. Monitoring of at least 3 transitions of both analyte and the heavy isotope-labelled peptides is recommended [28]. Another possibility is the data

dependent acquisition of full scan MS spectra which allow the identification of the peptide.

2.4. Quantitative analysis

Proteins are digested with a protease such as trypsin and proteotypic peptides are used as the stoichiometric surrogate. For absolute protein quantification, the sample could be spiked with isotopically labelled protein (e.g., the absolute quantification [AQUA] approach) [12,29]. In the example (see Fig. 1) the lysine residue is substituted with heavy lysine residues containing seven ^{13}C and one ^{15}N. Both peptides have the same physicochemical properties including chromatographic coelution, ionization efficiency, and relative distribution of fragment ion intensities. Due to their 8 Da mass difference the peptides can differentially detected. A sufficiently large mass difference between the precursor and the fragment ions prevents cross talk. Dependent on the resolution, the mass difference should be at least 5–6 Da. Each peptide is quantified relative to the matching heavy labelled peptide. Isotope labelling increases the complexity and cost of the assay but compensates matrix effects and increases the dynamic range. Isotope labelled standards are a prerequisite for exact quantification.

In contrast to the relative quantification approach, which is sufficient for many proteomic issues, most clinical problems require precise and absolute quantification. Therefore, accurately quantified isotopically labelled peptides or proteins must be added to the samples. Custom-specified peptides with incorporated heavy labelled amino acids can be ordered from several suppliers. Accurate determination of the peptide concentrations which is done mostly by amino acid analysis is vital. Loss of the peptides due to degradation or modification during storage, or to adsorption can interfere with the results. Proteins with different isoforms, chemical, co- and posttranslational modification, and partial digestion may be a hazard.

In most of the reported assays external calibrator mixtures are used which contain purified protein added to normal serum/plasma matrix. The calibrators are digested in parallel with unknown samples. Although the samples are spiked with peptides before any kind of separation or enrichment, the variability of digestion cannot be controlled by this approach, incomplete or unspecific digestion can corrupt the results. In the case of labelled proteins this can be avoided. Except for mass, this recombinant internal standard is identical to the native protein, and can account for variations in both digestion and LC–MS/MS processes [30].

An example for this approach is the determination of urinary albumin by Seegmiller et al. who used a full-length recombinant ^{15}N-labelled human serum albumin as the internal standard [19]. The advantage of a labelled internal protein standard for sufficient recovery tracking during the entire sample processing, was shown in a multisite assessment by Addona et al. [24].

Isotopically labelled proteins are obtained by cloning the appropriate genes into expression vectors or by metabolic labelling in *Escherichia coli* or yeast [31]. The use of peptides, or proteins of other species, as analog internal standards could be a promising alternative [32,33]. They often differ by only a few amino acids and may be commercially available.

2.5. Software tools

Surrogate peptides can be predicted through modelling. Several databases of tandem mass spectra from proteomic experimental data (www.peptideatlas.org, [34]) can be used for selection of potentially useful tryptic peptides and fragments. In silico processes can predict the peptide sequence and charge state of the signature peptide and product ions. Several software tools have been announced which support the setup of SRM assays. Besides platform-specific tools such as MRMPilot (ABI Sciex), Pinpoint (Thermo Scientific), Verify (Waters) and Optimizer (Agilent Technologies) several software packages are available (commercially or free download) (e.g., PeptideAtlas [34], Skyline [26], mProphet [35], MaRiMba [36] or MRMaid [37]). Cham et al. provide an excellent overview of the state-of-the-art in automated SRM transition design tools in the public domain, explaining how the systems work and how to use them [38].

Recently, different tools have been reported for the validation process of SRM measurement data, which is especially important for large data sets [35,39]. The frequent occurrence of interferences requires that SRM-MS data be manually reviewed. Abbatiello et al. [28] replaced this time-intensive process by an algorithm which objectively evaluates SRM-MS data for inaccurate and imprecise transitions. It works with results exported from SRM-MS data-processing software and may be implemented within such software packages.

2.6. Outlook: further developments

So far, setting up SRM assays remains challenging and requires substantial effort despite the recently developed software solutions supporting the process. To avoid redundant transition selection in several different laboratories, validated transitions should be stored in centralized databases, together with experimental parameters [40], as in for example the MRMAtlas (http://www.mrmatleas.org). Also in other projects such as the Human Proteome Detection and Quantification project, the goal is to quantify all known proteins in the human proteome by means of the production of stable isotope-labelled peptide standards and corresponding antipeptide antibodies [41]. Isotopically labelled peptides should become routinely available at reasonable prices, as panels of isotopically labelled peptides in combination with optimized SRM parameters.

3. Experimental procedures

The quantitative analysis of peptides and proteins in biological matrices, however, continues to be a demanding task, due to the complexity of both the matrix and the analytical characteristics of these large molecules. There are several published LC–MS applications for the quantification of peptides and proteins (see Table 1). Developing SRM-based assays of proteins in a clinical environment according to the exacting Food and Drug Administration (FDA) quality standard, still remains a challenge. Peptide degradation due to contaminating enzymes, peptide aggregation, non specific association with plastic tubes, and peptide oxidation can influence peptide responses.

3.1. Sample processing

Many low-abundance proteins are available to SRM analysis without up-front purification or enrichment [19]. The direct assay approach involves quantification based on the ratio of an isotope labelled peptide internal standard and the proteotypic peptide analysed. After denaturation and reduction with dithiothreitol to prevent intramolecular and intermolecular disulphide bonds, alkylation (modifying sulfhydryl groups to prevent re-formation of disulphide bonds) and trypsin digestion is performed.

Target protein purification prior to enzymatic digestion will often be necessary to improve quantification limits. Various purification methods (see Table 1) have been described, such as solid phase extraction, protein precipitation, size exclusion, ultrafiltration and liquid–liquid extraction [42]. The potential of immunodepletion was shown by Anderson et al. [43]. A comparison

Table 1
Examples of SRM assays for protein quantification.

Protein	Sample processing	Digestion	LOQ	HPLC column	Reference
IGF-1, IGFBP3	–	Tryptic, NH_4HCO_3 16 h, 37 °C	4 mg/L, 3 mg/L	2.1 × 150 mm C18 400 μl/min	Kirsch [30]
Ceruloplasmin dry blood spot	–	Tryptic overnight, 37 °C	7 mg/L	2.1 × 50 mm C18 100 μl/min	deWilde [83]
Albumin in urine[a]	–	Tryptic 1 h, 37 °C	3 mg/L	2.1 × 50 mm C18 250 μl/min	Seegmiller [19]
Apolipoprotein A1, Apolipoprotein B	–	Tryptic/trifluorethanol 21 h, 37 °C	90 mg/L, 40 mg/L	3.2 × 100 mm C18 400 μl/min	Agger [47]
hGH	Fractionation 10 × 250 mm C18	Tryptic 24 h, 37 °C	1.7 μg/L	10 × 250 mm SCX	Arsene [66]
CRP	Depletion + size exclusion chromatography	Tryptic 12 h, 37 °C		10 cm × 75 μm C18 0.2 μl/min	Kuhn [16]
IGF	SPE	Tryptic overnight, 37 °C	125 μg/L	2.1 × 100 mm C18 0.5 ml/min	Barton [17]
PSA	MS/MS/MS MCX SPE	Tryptic/urea overnight, 37 °C	4 μg/L	2.1 × 100 mm C18 0.3 ml/min	Fortin [63]
CRP	Immunocapture protein	Tryptic/Rapi-gest 45-48 h, 37 °C		2.1 × 150 mm C18 220 μl/min	Kilpatrick [67]
1–84 PTH[a]	Immunocapture protein	Tryptic 30 min	14.5/39.1 μg/L	2.1 × 50 mm C18 250 μl/min	Kumar [31]
Zn-α2 glycoprotein	SISCAPA	Tryptic/urea overnight 37 °C	320 μg/L	50 × 2.1 mm C18 250 μl/min	Bondar [51]
Thyroglobulin	SISCAPA	Tryptic/Tween 20 h, 37 °C	4 μg/L	0.15 mm, C18 1 μl/min	Hoofnagle [54]
cTnI, IL-33	SISCAPA	Tryptic/urea 18–22 h, 37 °C	3 μg/L	10 cm × 75 μm C18 3 μm 300 nL/min	Kuhn [55]
Pepsin/pepsinogen saliva[b]	SISCAPA online immunoaffinity	Asp-N/heat 16 h, 37 °C	0.17 μg/L	5 cm × 75 μm C18 400 nl/min	Neubert [59]

[a] Labelled protein as internal standard.
[b] Digestion with AspN.

of different immunodepletion strategies was reported in a recent review [44].

3.2. Enzymatic digestion

The method relies on the denaturation of all proteins in a sample and the proteolytic digestion of proteins into peptides. Trypsin digestion can be quite variable from laboratory to laboratory. The digestion process depends on the individual protein structure, such as disulfide bridges, specific folds, solubility, glycosylation etc. Peptides could be generated at varying rates during trypsin digestion.

The assay is based on the assumption that a protein is reproducibly and efficiently converted to its proteotypic peptides [45]. The digestion step is the most important contributor to variability for the whole assay. To improve interlaboratory reproducibility and the robustness of assay procedures, practical methods are necessary for the proteolytic digestion step, and for monitoring that digestion.

Addona et al. established and tested a standard protocol across several laboratories to assess the reproducibility of SRM for protein quantification [24]. Highly reproducible quantitative results were obtained after selecting a single transition per protein. The main variation across laboratories was from the sample preparation process.

Therefore, robust protocols in those small changes in digestion time and conditions are negligible, are required for a high reproducibility. Ideally, complete digestion of the protein occurs in a short time period, resulting in the maximum observed peptide signal, followed by a "steady state" condition [46,47]. In the literature, different protocols with different denaturation agents, reduction reagents and digestion conditions are reported, but only a few reports have compared different protocols with regard to absolute quantification requirements. One such the study is that of Proc et al. who recommend sodium deoxycholate with a 9 h digestion procedure as the optimum protocol [48].

Other caveats are that also truncated forms might also be measured, posttranslational modifications could change the masses, or modifications could inhibit digestion by trypsin. For clinical applications, digestion protocols should be selected which are specific to the target proteins [49]. Sample preparation must still be carefully defined and rigorous standardized operating protocols must be used to obtain sufficient reproducibility.

3.3. Enrichment strategies

Though many low-abundance proteins are still amenable to SRM analysis without up-front purification [50,51] or enrichment, the targeted, clinically relevant limits of quantification in the low nanogram/milliliter range are often not accessible without additional sample processing steps. This may involve sample fractionation, immunodepletion of the most abundant proteins, solid phase extraction or antibody enrichment of the targeted protein or peptide.

Keshishian et al. lowered the identification detection limit down to 1–10 ng/ml after the depletion of highly abundant plasma proteins and peptide separation by strong cationic-exchange chromatography [52].

There are two approaches to the enrichment of low-abundance proteins before quantification. The "bottom up" approach incubates the digested sample with antibodies generated against specific proteolytic peptides before analysis. Immunoaffinity enrichment of peptides after digestion of serum/plasma is termed SISCAPA (stable isotope standards and capture by anti-peptide antibodies) and was successfully used in some recent experiments [53]. The main advantage is that synthetic peptides can be more readily manufactured than proteins. Utilizing immunoaffinity to selectively concentrate the target peptides has been shown to extend the sensitivity of a peptide assay by at least two orders of magnitude and with further development, would seem capable of extending the SRM method to cover the full known dynamic range of plasma.

Hoofnagle could detect thyroglobulin at picomolar concentrations [54] and SISCAPA was used to quantify plasma cardiac troponin I [55,56].

The top-down approach uses antibodies to capture the intact protein before digestion. The digestion of the protein is performed after elution or in situ attached to the antibody. Superparamagnetic and agarose beads are used as solid phase support material for immunoprecipitation. The need for antibodies and the laborious optimization of immobilization, capture and elution conditions are limiting factors.

Technological advances for the purification of proteins and peptides such as the use of online immunoaffinity columns or the implementation of a trap–wash–elute system for magnetic beads coated with the immunoaffinity reagents, will further improve sensitivity and sample processing in fully integrated systems [53,57–59].

3.4. Chromatography

Most proteomics procedures use nano- or microflow liquid chromatography ($\leq$1 μl/min) for improved limits of detection. These systems are not as robust as normal flow systems and would be difficult to deploy in a clinical setting. The results of Agger et al. [47] who evaluated whether normal flow rates could be used to detect peptides from the apolipoproteins, suggest that the normal flow system is capable of reliability detecting target analytes in the tryptic digests.

Barton used fused core particle for the separation of proteotypic peptide from protein digests [17]. UPLC enables the use of small beads and extended column lengths which increases chromatographic resolution. Higher separation capability and a reduction in run time could also be achieved by monolithic columns, without a high backpressure [32].

The decrease in signal intensity moving from nanospray to electrospray conditions is partially cancelled out by the higher loading capacity of a conventional bore 2.1 mm inner diameter chromatography in comparison to a capillary column. Sample volume is not the limiting factor in clinical studies as in proteomic studies.

Most of the applications employ one dimension reversed phase chromatography for the separation of signature peptides. A promising alternative could be online extraction which would minimize matrix effects and improve sensitivity [32,60].

3.5. Mass spectrometry

In the last few years the resolution, mass accuracy and scanning speed of mass spectrometers have increased dramatically [61,62]. A promising strategy to improve sensitivity, is the MRM^3 approach using a hybrid triple quadrupole-linear-ion trap instrument. This technology allows for secondary fragmentation in the third quadrupole by trapping the product ion formed, which is then sent to the detector. Fortin et al. show that MRM^3, coupled to conventional chromatography resulted in a 3- to 5 fold improvement of the limits of detection and quantification for determining PSA levels in serum [63]. SRM interferences are removed by two stage collision-induced decay fragmentation.

3.6. Outlook: technical developments

As faster mass spectrometers will enable increased sampling speed while maintaining adequate sensitivity, the quantitative protein analysis of large sets of clinical samples could become a reality. The performance, and ease of use of tandem mass spectrometers will continue to improve. Over the past decade each new generation of triple quadrupole tandem mass spectrometers has demonstrated order of magnitude increases in sensitivity compared to the previous generation. The resulting substantial increase in sensitivity and mass resolution will further expand the range of analytical applications. Improvements of the electrospray design, the least efficient component of the ESI MS/MS, will provide opportunities for further progress in analytical sensitivity. Improvements in the work flow using automation and shorter chromatographic run times will enable efficient sample throughput and increase precision.

4. Outlook: clinical applications

4.1. SRM assay as reference method

One application of this technology is the development of new reference methods to standardize protein assays. For many immunoassays, there is an urgent need for reliable reference methodology to verify calibrators and assay performance [31,32,64–68]. SRM assays could represent a reference measurement procedure which allows measurements of highest metrological quality.

Two recent examples are haemoglobin A1c [69–74] and C-peptide [75]. HbA1c is the stable glucose adduct to the N-terminal group of the beta-chain of HbA0. The measurement of HbA1c in human blood is most important for the long-term control of the glycemic state in diabetic patients. Because there was no internationally agreed reference method, the IFCC Working Group on HbA1c Standardization developed a reference method for HbA1c. In a first step haemoglobin is cleaved into peptides by the enzyme endoproteinase Glu-C, and in a second step the glycated and non-glycated N-terminal hexapeptides of the beta-chain obtained, are separated and quantified, either by HPLC and electrospray ionisation mass spectrometry, or with a two-dimensional approach using HPLC and capillary electrophoresis with UV-detection. HbA1c is measured as a ratio between the glycated and non-glycated hexapeptides. The analytical performance of the reference method has been evaluated by an international network of reference laboratories comprising laboratories from Europe, Japan and the USA. The intercomparison studies of the network showed excellent results with intra-laboratory CVs of 0.5–2% and inter-laboratory CVs of 1.4–2.3%. The new reference method has been approved by the member societies of the International Federation of Clinical Chemistry and Laboratory Medicine and is the basis for uniform standardization of HbA1c routine assays worldwide.

4.2. SRM assay vs immunoassay

So far, immunoassays are used most commonly for the monitoring of proteins and peptides. Immunoassays are among the most sensitive and precise analytical methods. They can be many orders of magnitude more sensitive than MS/MS and can be very easily automated.

However, immunoassays have the disadvantage that, on the one hand, the antibodies used cross-react to a varying extent with metabolites, and on the other hand posttranslational modifications could often not be differentiated. This is a particular problem in protein therapeutics assays with drug metabolites which may or may not be active, but are structurally very similar to the target peptide or protein [18].

Due to a lack of standardization, different assay results are often not comparable.

For several assays the dynamic range is low. Although there is intense interest in miniaturizing sets of such assays in array format, significant problems remain in the production of suitable antibodies and in the simultaneous optimization of multiple assays in one fluid.

Nevertheless, there is a consensus among experts that immunoassays will most likely not be replaced by MS-based methods in the clinical laboratory [20]. In particular, the lower limit of quantification prevents the replacement of traditional immunoassays, although further research and improvement is expected. Furthermore, advances in automated sample preparation, clean up, and online fractionation, and improvements in mass accuracy and resolving power would be required. For the immediate future, the main focus will be on assays for which immunoassays of good quality do not exist, or assays for the determination of heterogeneous proteins whose isoforms respond similarly in the immunoassay but whose diagnostic value differs greatly [76,77].

Niederkofler et al. reported an assay for various isoforms of B-type natriuretic peptide, which differentiates well between the inactive and biologically active forms for example of BNP which are important for therapeutic treatment [78,79].

PTH immunoassays can be susceptible to interference by cross reacting PTH fragments which show different physiological activity and have different half-lives. Very high concentrations of C-terminal fragments could be found in particular in renal failure. An assay using immunocapture purification with LC–MS/MS detection provide accurate and precise PTH results [31,80].

4.3. SRM assay as a screening method

Another use of SRM assay which appears extremely promising, is the application as a screening method, for example for the determination of hemoglobinopathies [81–83]. These rare disorders are actually quite common in certain populations. Newborn screening permits early pre-symptomatic interventions that would improve the outcome. In contrast to existing techniques of Hb analysis measuring intact tetramers, SRM assay measures individual denatured globin proteins or, after tryptic digestion, specific peptides. Using tryptic peptides it was possible to screen for the clinically important Hb variants and β-thalassemia. The new methodology enables high sensitivity and high specificity for the detection of a wide variety of Hb variants as well as the ability to differentiate heterozygous genotypes.

The final method for the identification of sickle protein involves tryptic digestion of whole blood, automated injection of the digest, two MRM acquisitions, and an injection to injecton time of approximately 1 min. Unequivocal sequence information can still be obtained for haemoglobin C, D, O and E. This method offers considerable advantages for newborn screening. It is faster and more specific than conventional methods. It requires no chromatography and uses the same solvent for amino acid and acylcarnitine screening, allowing the two methods to be run sequentially on the same MS/MS instrument.

The validated biomarker for ß-thalassemia trait detection is HbA_2. HbA_2 is a tetramer of equal α- and δ-globins, suggesting the possibility that measuring specific δ-protein peptides by MS/MS might provide a useful surrogate biomarker. Daniel et al. extended the MS/MS approach to include the quantitative measurement of the relative amounts of δ- and ß-globins, in order to determine the utility of the ratio as a surrogate biomarker of HbA_2 in the detection of ß-thalassemia trait [84].

4.4. Biomarker validation

Proteomics has enabled an impressive identification of the proteins circulating in plasma. However, despite the large investment and the effort involved in finding biomarker candidates, only a few protein biomarkers are currently used routinely in a clinical setting. So, there is a growing need to validate new biomarkers accurately, rapidly, and cost effectively [85,86]. Unfortunately, antibodies are not readily available for many newly discovered potential markers. The discovery and application of new proteins for diagnostic applications would be accelerated if specific antibodies were not necessary. Protein quantification, with high reproducibility and throughput, could improve the validation process of newly discovered putative biomarkers and the success rate of approved biomarkers [27,87,88]. Immunological assays can be costly and time-consuming, and the development of specific antibodies against candidate biomarkers may be limited. By contrast, SRM assays allow the rapid and cost effective identification and quantification of biomarkers. Picotti et al. could monitor more than 100 assays in a single 1 h analysis [22].

4.5. Posttranslational modifications

Mass spectrometry has become a powerful tool for investigating the posttranslational status of proteins. By taking advantage of the characteristic fragmentation patterns, it is possible to identify phosphopeptides [29,89]. SRM has great potential for the reliable identification and quantification of post translational modifications (PTMs) and other chemical modifications [69]. Its unique ability to characterize post translational modifications increases the value of SRM as an alternative to antibody-based approaches as has been shown for ghrelin and deacyl ghrelin [32].

4.6. Preanalytical quality control and normalization

In addition to the precise quantification of proteins, the multichannel nature of the tandem mass spectrometry can help to check analyte degradation processes during sample collection and analysis. Stable isotope-labelled internal standard peptides, stable isotope-labelled internal standard polypeptides or stable isotope-labelled protein analogs which could be included in blood draw tubes could enable measurement of proteolytic variability and analyte degradation [90].

During the clinical validation of a renin activity assay, Bystrom et al. detected a surprising analyte-degradation phenomenon. By means of an additional second internal standard peptide they could monitor the sample specific angiotensin I degradation during analysis and could identify a subpopulation of plasma samples with substantial peptidase activity [91].

Sample amount normalization is difficult, especially for tissue assays. The sample amount is commonly based on the total protein mass as determined by assays such as Lowry or Bradford, which are often not particularly accurate. Normalization on the basis of proteins expected to be constant through out the experiment, would be a interesting alternative. These proteins could be included in the SRM assay and quantified together with the target protein set.

5. Conclusions

Selected (Multiple) reaction monitoring mass spectrometry of peptides with stable isotope-labelled internal standards is increasingly being used to develop quantitative assays for proteins in complex biological matrices. Several studies have demonstrated the potential clinical utility of LC–MS/MS quantification of proteins. A key advantage is the ability to easily quantify multiple proteins and enable highly multiplexed analysis. SRM has great potential for the reliable identification and quantification of PTMs and other chemical modifications. Rapid assay development is possible. Specific analyte detection reagent is not necessarily required, saving time and expense in development and validation of immunoassays. High analytical selectivity allows the precise quantification of individual protein isoforms associated with protein sequence micro heterogeneity and many clinically relevant variants. It provides a reference measurement procedure which allows measurements of

highest metrological quality. This new technology promises the large-scale preclinical verification of putative biomarkers.

To move from the laboratories of highly skilled analysts to routine clinical diagnostic laboratories requires that a number of technical barriers be overcome in regard to the imprecision, accuracy and sample handling necessary for clinical use. The technology is still in the early stages for protein analysis and challenges such as the development of simplified procedures and equipment will have to be addressed in the near future to increase widespread application in the clinical setting. New mass analyzers will provide higher resolution, increased sensitivity and signal-to-noise ratios.

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