MASS SPECTROMETRY FOR THE QUANTIFICATION OF BIOACTIVE PEPTIDES IN BIOLOGICAL FLUIDS

Constantin Tamvakopoulos*
Foundation for Biomedical Research of the Academy of Athens (IIBEAA), Laboratory of Pharmacology-Pharmacotechnology, Athens, Greece 11527

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The study of pharmacologically active peptides is central to the understanding of disease and development of novel therapies. It would be advantageous to monitor the fate of bioactive peptides in biological fluids and tissues following their in vivo administration (exogenous administration) or the modulation of endogenous factors (e.g., peptide hormones) affected by the administration of a pharmacological agent. Measurement of administered compounds (small molecules) in plasma is a mature field. However, measurement of pharmacologically active peptides presents particular problems for quantitative mass spectrometry, including challenges from selectivity and sensitivity perspectives. Current approaches towards peptide quantification in biological fluids include immunoassays and mass spectrometric techniques. Immunoassays, although sensitive, lack the necessary selectivity for distinction between peptide and metabolites. Modified molecules induced by metabolic transformations (e.g., N- or C-terminal truncation of the peptide) might not be differentiated by the antibody used in the assay, leading to cross-reactivity. However, although it is generally accepted that mass spectrometry is an ideal technique for the quantification of trace levels of analytes in biological fluids, immunological techniques are still characterized by better limits of peptide detection. In this review article, novel mass spectrometric approaches and strategies on peptide quantification will be described. The current capabilities and prospects for advances in this critical area of research will be examined.

Keywords: *pharmacokinetics; mass spectrometry; drug discovery; target validation; bioactive peptides*

I. INTRODUCTION

Bioactive peptides are intriguing molecules that represent potent pharmacological tools that are often employed for the development of new animal models of disease, pharmacophores for the design of new drugs (Patchett et al., 1980; Nargund et al., 1998; Alexopoulos et al., 2004; Ye et al., 2005; Keramida et al., 2006), markers for disease (Chace, 2001; Dixon, Knopf, & Figg, 2001; Murphey et al., 2001; Frank & Hargreaves, 2003), or markers for substance abuse (De Kock, Rodgers, & Swanepoel, 2001). Although we have seen remarkable progress in protein analysis as a consequence of proteomic research, and small molecule analysis as a consequence of drug discovery/development initiatives, the field of absolute quantification of peptides by mass spectrometry has not grown as rapidly. We use the term ''absolute'' to define the scope of this review and to distinguish it from reports on the study of post-translational modifications of proteins (Smith, 2005), quantitative peptidomic, or quantitative proteomic studies (Lill, 2003), 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). Because of the vast complexity of the problem (a remarkably broad spectrum of peptides or proteins are monitored simultaneously), such studies typically provide relative quantification. An important distinction should be also be made at this point between analysis of exogenous peptides (e.g., determination of pharmacologically active peptides following administration in *in vivo* models) and analysis of endogenous peptides or proteins (e.g., determination of peptide hormones). Quantification of endogenous peptides or proteins is quite challenging from a purification, separation, and sensitivity perspective, particularly, if there is limited information on the specific peptide or protein in question. In the past few years, there has been significant emphasis on the role of proteomics in future therapies. Proteomic analysis is based on technological advances and modern research centers have invested heavily on mass spectrometry, which is the pivotal analytical tool by which this exciting field continues to evolve (Soloniev & Finch, 2005; Wysocki et al., 2005). Multiple platforms with distinct capabilities are employed for sample analysis from proteomic studies, such as matrix-assisted laser desorption ionization-time of flight (MALDI-TOF), electrospray ionization-liquid chromatography/mass spectrometry (ESI-LC/MS), ion trap instruments and Fourier transform-mass spectrometer (FT-MS) systems. Such tools help mass spectrometry experts to monitor up or downregulation of key proteins (often the signatures of human disease), and subsequently play a role in the deciphering of complex biological problems (Pan et al., 2005). Aebesold & Mann (2003) provide us with a valuable overview of the various mass spectrometer architectures available (shown in Fig. 1) and distinct possibilities offered by each platform as well as the potential impact of the technology in biology and medicine.

A similar explosion in mass spectrometry-based applications was observed in the past from industrial and academic

**Correspondence to:* Constantin Tamvakopoulos, Foundation for Biomedical Research of the Academy of Athens (IIBEAA), Laboratory of Pharmacology-Pharmacotechnology, Soranou Efesiou street 4, Athens, Greece 11527. E-mail: ctamvakop@bioacademy.gr

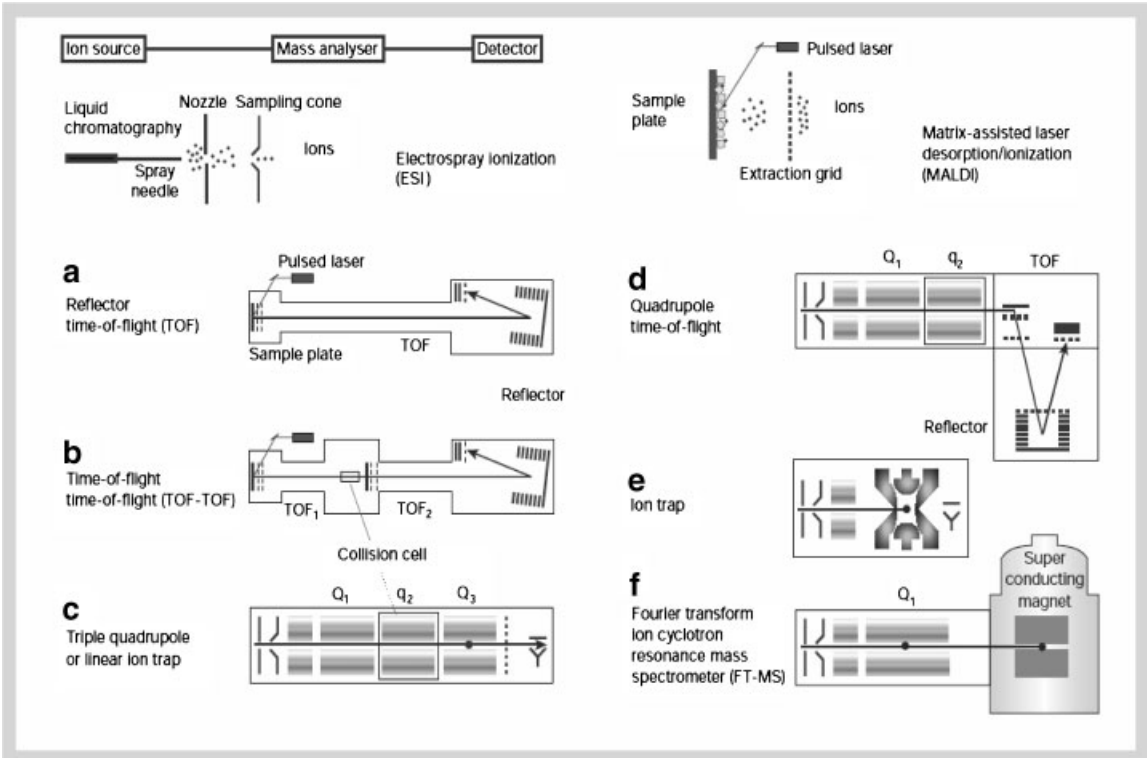

FIGURE 1. The left- and right-upper panels depict the ionization and sample introduction process in electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). The different instrumental configurations (**a–f**) are shown with their typical ion source. a: In reflector time-of-flight (TOF) instrument, b: The TOF-TOF instrument, c: Quadrupole mass spectrometers, d: The quadrupole TOF instrument, e: The (three-dimensional) ion trap, f: The FT-MS instrument. [Reprinted with permission, from Mcmillan Publishers Ltd: Nature, 422: 198–207, 2003; copyright 2003.]

laboratories, where small organic molecule analysis (MW < 1,000) was facilitated by new analytical tools (e.g., liquid chromatography coupled with triple quadrupole or ion trap instruments-LC-MS-MS). Mass spectrometry experts were charged with the important task of studying pharmacokinetic properties at the earliest phases of drug discovery (Olah, McLoughlin, & Gilbert, 1997; Tamvakopoulos et al., 2000; Colwell et al., 2002; Korfmacher, 2005). It is widely accepted that mass spectrometry-based early pharmacokinetic screening has been vital for modern drug discovery efforts, directly linked to the introduction of new medicines (Lin & Lu, 1997), and has contributed to the reduction of attrition rates of drugs in the development phase. The ability to monitor drug and/or metabolites in biological fluids following administration is crucial for our understanding of drug action and can only be achieved by sound mass spectrometric approaches. As with proteomic mass spectrometry facilities, drug discovery centers are well equipped with instruments that help scientists place emphasis on small molecule analysis and on quantitative aspects of mass spectrometry.

The well-established selectivity and sensitivity advantages offered by mass spectrometry for protein and small molecule analysis would suggest that peptide quantification could ideally be achieved by techniques such as LC-MS. However, it seems that immunological techniques are still widely employed for peptide quantification and that the potential of mass spectrometry has not been copiously explored for peptide quantification. This review will cover various types of peptides of biological interest and approaches where mass spectrometry-based techniques have been employed. Through the literature's review, we will attempt to identify the major challenges associated with the measurement of bioactive peptides in biological fluids, and focus on those mass spectrometric approaches that offer insight and potentially good opportunities for the future. We believe that as with proteomics and small molecule analysis, where extensive progress has been made because of the advantages offered by novel platforms and contribution of experts in the field, there will be a need for similar strives in the future to establish mass spectrometry-based selective, high sensitivity, robust assays for the measurement of endogenous or exogenous bioactive peptides in biological fluids.

II. BIOACTIVE PEPTIDES—PHYSICOCHEMICAL PROPERTIES

To examine the general strategies towards peptide quantification, we should consider some of the challenges presented to the analyst during peptide analysis as a consequence of the peptide's physicochemical properties. Table 1 summarizes a number of

TABLE 1. Peptide characteristics

Solubility
Polarity
Chemical stability
Stability in biological fluids
Adsorption to surfaces
Molecular weight
Ionization efficiency
Fragmentation
Endogenous versus exogenous peptide
Presence of endogenous peptide in control plasma

peptide attributes that should be taken into consideration prior to the development of a method for the quantification of a peptide in biological fluids. Peptide solubility and chromatographic behavior is influenced by the presence of basic or acidic amino acids. Chemical stability is also crucial for proper assay development since peptides can loose stability when placed in solution or in biological fluids. The significance of stability studies should not be underestimated otherwise the risk for unreliable results would be high. For example, we could have peptide alteration because of oxidation of methionine or tryptophan residues or disulfide bond cleavage. LC-MS appears to be a perfect tool for stability studies, since it can help in distinguishing between the peptide of interest and its degradation products. Inhibitor cocktails are often used when peptides are analyzed in plasma samples. In a study reported by Wolf et al. (2001) the quantification of the endogenous incretin hormones, glucose-dependent insulinotropic polypeptide GIP_{1-42} and GIP_{3-42} was accomplished in plasma by LC-MS. During sample preparation, a cocktail inhibitor mixture previously described by Pietzsch et al., (1996) containing aprotinine, glutathione, sodium azide, streptomycine sulfate, EDTA, dithiothreitol, phenylmethanesulfonic acid fluoride, and chloramphenicol, was added to plasma to ensure stability of the peptides of interest. An important addition to the cocktail mixture, an inhibitor to the enzyme dipeptidyl peptidase IV (DP IV), valine-pyrrolilide, was added to the plasma to protect the circulating peptide (GIP_{1-42}) from DP IV-catalyzed *N*-terminal degradation (Kieffer, McIntosh, & Pederson, 1995). As with any molecule in question, such stability considerations are compound-specific and it is up to the analyst to resort to the appropriate methods to further ensure a reliable assay. Given that, it is not always possible to know what enzymes must be inhibited, it would be advantageous to consider alternatives to cocktail inhibitor mixtures, such as addition of acid prior to extraction and very fast temperature lowering. What is also commonly observed with peptides, particularly at low levels, is adsorption to solid surfaces. John et al. (2004) provide an example of how transfer loss of a model peptide (MP1) from a polypropylene vial can be minimized with the addition of acetonitrile, or detergent and how loss is more extensive in low concentration solutions. Although experiments such as those described by John et al. require extra effort they seem to be a rather crucial component of successful assay development.

A critical consideration in peptide analysis by LC-MS is the molecular weight (MW). This is important as it impacts the choice of extraction procedure from biological fluid, chromatographic separation, and mass spectral analysis. For the purposes of this review, we will mostly focus on peptides with molecular weights below 5 kDa. With peptides of lower molecular weights (~1,000) the approach for development of a quantitative assay in biological fluids is similar as procedures followed for small organic molecules. However, an inherent feature of peptide ionization under electrospray conditions, is that typically multiple-charged species are formed. Formation of different charged states is also observed with lower molecular weight peptides. This characteristic tends to complicate analysis since the formation of multiple-charged species and intensity of corresponding ions can depend on the solvent system that is used for chromatography, on the peptide's amino acid sequence, and on parameters employed for ionization (e.g., declustering potential, electrospray voltage). Under MS/MS conditions, these multiple-charged species also tend to yield product ions (fragmentation spectra) with different transmission efficiencies than singly charged species do, often obligating the analyst to consider multiple options (e.g., numerous multiple reaction monitoring pairs) before decisions on the most appropriate detection system can be made. Such product ion transmission efficiencies tend to vary also from platform to platform used for the analysis (e.g., ion trap instruments versus triple quadrupole instruments), thus making instrument selection a rather important component in the process of assay development. Mock, Shen, and Tamvakopoulos (2002) provide an example of how quantification of melanotan-II (MT-II), a cyclic low molecular weight (MW: 1,024 Da) analog of endogenously expressed melanotan stimulating hormone (α-MSH), was achieved in rat plasma. The most intense peak (*m/z*: 513), corresponding to the $[M+H]^{2+}$ ion, was optimized in full scan (FS) mode and transitions *m/z*: $513.1 \rightarrow 437.7$ and *m/z*: $513.1 \rightarrow 413.2$ were optimized in multiple reaction monitoring (MRM) mode for quantitative measurements. The specific methodology yielded sensitivity of 5 ng/mL in rat plasma and the applicability of the approach was demonstrated with a pharmacokinetic study following exogenous intravenous administration (shown in Fig. 2). Concentrations of MT-II in rat plasma were measured up to 4 h following a 1 mg/kg intravenous dose, and pharmacokinetic parameters such as half-life ($t_{1/2}$) and plasma clearance (Cl_p) were determined. With regard to higher molecular weight peptides (e.g., MW: 4,000–5,000 Da), the challenges are significant because multiple charged states (often up to five) tend to impact the intensity of the various peaks (less intensity in comparison to singly charged molecules) and consequently the overall sensitivity of the assay. Often, we encounter approaches that focus on optimization of one or two of the charged states that become the basis of detection. Some examples of assays developed with peptides in this molecular weight range (4,000–5,000 Da) will be covered in this review. Finally, reported studies on the development of assays on high molecular weight peptides or proteins (MW: 25,000–30,000 Da), indicate a great deal of challenges with regard to sample preparation and detection. What has been tested

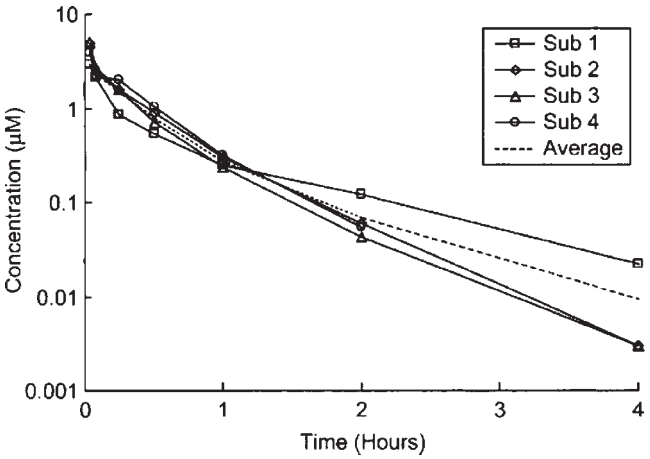

FIGURE 2. Concentration-time profile of MT-II in rat, following a 1 mg/kg intravenous. administration. [Reprinted with permission, from Rapid Commun Mass Spectrom 22: 2142–2147, Mock S, Shen X, and Tamvakopoulos C. 2002. Determination of Melanotan II in rat plasma by liquid chromatography-tandem mass spectrometry. Determination of pharmacokinetic parameters in rat following intravenous administration, with permission from John Wiley & Sons copyright 2002.]

with reasonable success as a feasible alternative to the intact detection of protein is cleavage of protein by chemical or enzymatic means followed by the quantification of peptide fragments that serve as a representation of the concentration of intact protein (the strategy is shown in Fig. 3). Such approaches are summarized by Desiderio and co-workers (Dass, Kusmierz, & Desiderio, 1991), Barnidge et al. (2003, 2004), and Kuhn et al. (2004) for assays of beta-endorphin, rhodopsin, prostate-specific antigen (PSA), and *C*-reactive protein (CRP), respectively. Gygi and colleagues (Gerber et al., 2003; Kirkpatrick, Gerber, & Gygi, 2005) have developed the absolute quantification (AQUA) strategy for quantification of differences in protein (and post-translational modification) expression levels in cell lysates. Applicability of the AQUA approach has been demonstrated with quantification of horse heart myoglobin (used for validation) in yeast background and by other applications such as quantitative determination of the phosphorylation of Ser-1126 of human separase protein.

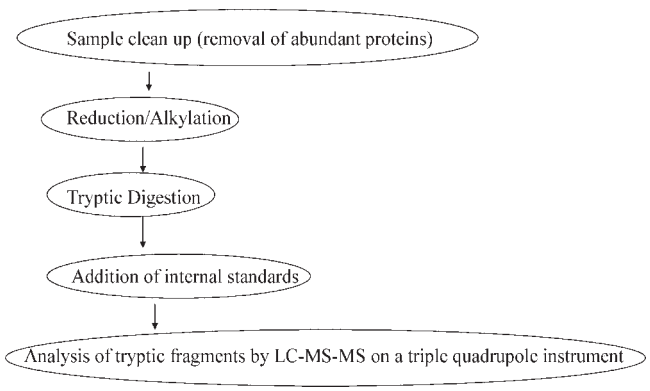

FIGURE 3. Procedure for the analysis of peptides, following tryptic digestion.

III. SAMPLE PREPARATION

A. Extraction from Plasma (Serum)

The general procedures employed for peptide extraction from biological fluids have been summarized by John et al. (2004). As with small organic molecules, protein precipitation with acetonitrile, methanol, ethanol, or trichloroacetic acid is considered a relatively simple extraction technique, and it can be used with reasonable expectations, particularly for low volumes of biological fluid (<200 μL). However, it should be noted that protein precipitation is more likely to perform better with peptides that are non-polar, because peptides of high polarity would be recovered poorly by protein precipitation. Protein precipitation would be less selective in comparison to other extraction procedures, and thus some more consideration should be involved in chromatographic separation of the peptide from possible interfering components. Liquid–liquid extraction from human plasma or serum has been reported in the literature with a limited number of low molecular weight peptides. As an example, a depsipeptide can be extracted into ethyl acetate from rat or human plasma with reported recovery of 60–70% (Chan, Bakhtiar, & Jiang, 1997). Alternatively, *n*-butyl chloride was selected for the extraction of rennin inhibitor, CP-80,794 (MW: 620 Da) from human serum (Fouda et al., 1991). Peptides with charged groups would be less suitable candidates for liquid–liquid extraction because they would have a higher affinity for the aqueous phase.

Solid-phase extraction (SPE) has been widely employed for peptide extraction mainly from human or animal plasma. Because manufacturers of SPE technology offer many options in terms of the available chemistries (reversed-phase, strong cation, or anion-exchange), SPE represents a rather practical method for peptide extraction from biological fluids. The ability to use 96-well plates is also attractive, because higher throughputs and automation can be achieved.

Finally, one of the techniques with noteworthy selectivity for peptide extraction and pre-concentration prior to analysis was reported by Wolf et al. (2001) for the analysis of endogenous levels of GIP_{1-42} and GIP_{3-42} in human plasma. A *C*-terminal-directed antibody (rabbit-anti-C-GIP) was coupled to magnetic beads for immunoprecipitation of total GIP. Following a pre-concentration step, the two peptides of interest, GIP_{1-42} and GIP_{3-42}, were chromatographically separated and analyzed by MS. The principles used for the immunoprecipitation method are depicted in Figure 4. Magnetic beads were covalently bound to secondary anti-rabbit IgG antibodies. Primary anti-C-GIP antibodies (antibodies that recognize the *C*-terminus of the peptide) were incubated with the beads and then human plasma, containing GIP_{1-42} and GIP_{3-42} and the conservative solution was added and incubated for 4 h by the magnet. Supernatant removal allowed sample purification and pre-concentration was achieved with elution from the beads using a low volume of

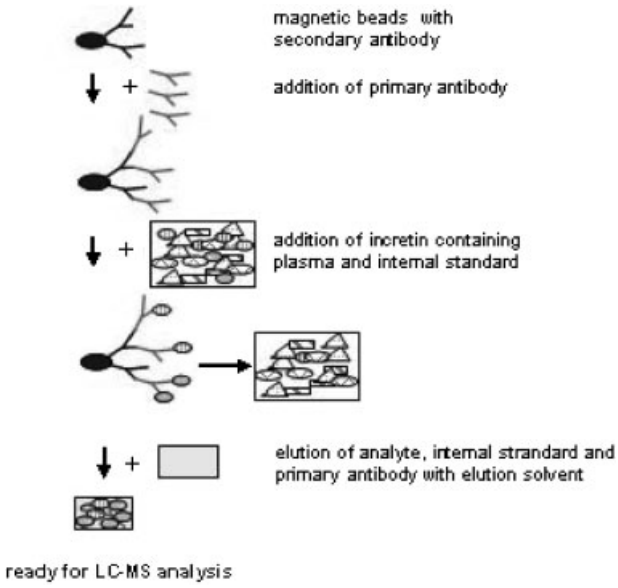

FIGURE 4. Principle of immunoprecipitation of peptides and preconcentration prior to analysis. [Reprinted with permission, from Journal of Chromatography A, 926, Wolf R., Rosche F., Hoffmann T., Demuth HU; Immunoprecipitation and liquid chromatographic-mass spectrometric detection of the peptide glucose-dependent insulinotropic polypeptides GIP_{1-42} and GIP_{3-42} from human plasma samples. New sensitive method to analyze physiological concentrations of peptide hormones. 21–27, 2001, with permission from Elsevier copyright 2001.]

eluting solvent (50 μL of 80% methanol containing 0.5% formic acid) prior to LC-MS analysis. Using the described approach, concentrations for the two peptides were determined in the 5–250 pM range, using 1.9 mL of human plasma. The applicability of this approach was demonstrated by measuring plasma levels of the two peptides in a clinical study in the presence and absence of a DP-IV inhibitor following meal stimulation (shown in Fig. 5). Following meal stimulation, there was an increase of GIP concentrations. The formation of inactive GIP_{3-42} was blocked by the DP IV inhibitor. This study by Wolf et al. offers an excellent example of the usefulness of immunological approaches for the achievement of selectivity (e.g., the antibody recognizes the *C*-terminal of the peptide in question). However, for detection and distinction between the two peptides that are different at the *N*-terminus (GIP_{1-42} and GIP_{3-42}), it is necessary to employ mass spectrometry, where mass differences can readily be distinguished. Wolf et al. (2004) reported improvements of the assay with determination of GIP levels in 1 mL of human plasma (vs. 1.9 mL of plasma in the original studies) and expansion of the assay to include the quantitative measurement of endogenous glucagon-like peptides $GLP\text{-}1_{7-36}$ and $GLP\text{-}1_{9-36}$ in plasma. Although immunoprecipitation is potentially a costly technique that requires a fair amount of method development work, it is extremely powerful for the simultaneous quantification of related peptides at the pM level and thus we envision that more of those sample preparation approaches will be essential to enhance detection limits of bioactive peptides in biological fluids. The technique's applicability will also largely depend on the peptide in question, since preparation of antibodies for certain peptides might not be a trivial task.

B. Extraction from Tissues

Most of the applications reported in the literature, involve analysis of bioactive peptides in plasma or serum (animal or human) with limited insights as to what would be needed to detect and quantify bioactive peptides in tissues. As is often the case with analysis of small organic molecules, in addition to analysis in plasma, it is necessary to quantify drugs in tissues to address issues of brain penetration or distribution into other important organs and tissues following administration (e.g., tumors for anti-cancer activity). The main focus of tissue peptide analysis in the literature has been on measurements of endogenous neuropeptides such as β-endorphin in the human pituitary (Dass et al., 1989; Dass, Kusmierz, & Desiderio, 1991). Those procedures are among the first reports where comprehensive approaches were described for the detection and identification of β-endorphin-containing peptides in the human pituitary. Following tissue homogenization, peptides were purified by SPE and reverse-phase HPLC. Collected fractions were treated with trypsin, and tryptic peptides were analyzed by liquid secondary ion mass spectrometry (Yan et al., 1997). Such approaches were directed mainly towards new peptide identification. Because peptides of interest had to be analyzed quantitatively, a number of studies on quantification of neuropeptides in human pituitary were reported in the literature (Desiderio, 1992, 1996).

In a recent review article on identification and quantification of endogenous peptides (peptidomics) in neuroendocrine tissues, Fricker et al. (2006) provided valuable information on the types of complexities faced with the analysis of endogenous neuropeptides in neuroendocrine tissues. Handling of the specific tissue could greatly impact peptide recovery and proper detection. As the review article indicates, proteolytic fragments of abundant proteins can often adversely impact the analysis of low-level peptide hormones (lower recovery, poor identification or ion suppression). Tissue collection and treatment (also part of the extraction procedure) in the postmortem period under specific conditions (e.g., microwaving of the tissue) is advised to limit the degradation of proteins. In a study intended to evaluate the brain penetration of MT-II, Trivedi et al. (2003) reported the use of LC-MS-MS for the determination of MT-II in rat plasma and brain homogenate following intravenous administration. MT-II was extracted from rat brain following homogenization by solid-phase extraction, and 0.25 mL aliquots of homogenate were loaded onto Oasis HLB 12 cc (500 mg) cartridges along with internal standard and 0.5 mL of water. Following washes of the cartridge with water, the peptide was eluted with 2 mL of 90% acetonitrile, 0.1% trifluoro acetic acid (TFA). Using the described extraction procedure, measurements in brain homogenate were achieved down to 20 nM levels (20 ng/g). The low brain penetration observed by the LC-MS-MS measurements (brain to plasma ratios of ∼0.02) was consistent with the autoradiographic results of the study that indicated no significant radioactivity in rat brain regions, following intravenous administration of ^{125}I-MT-II. Because we expect that there will be a need

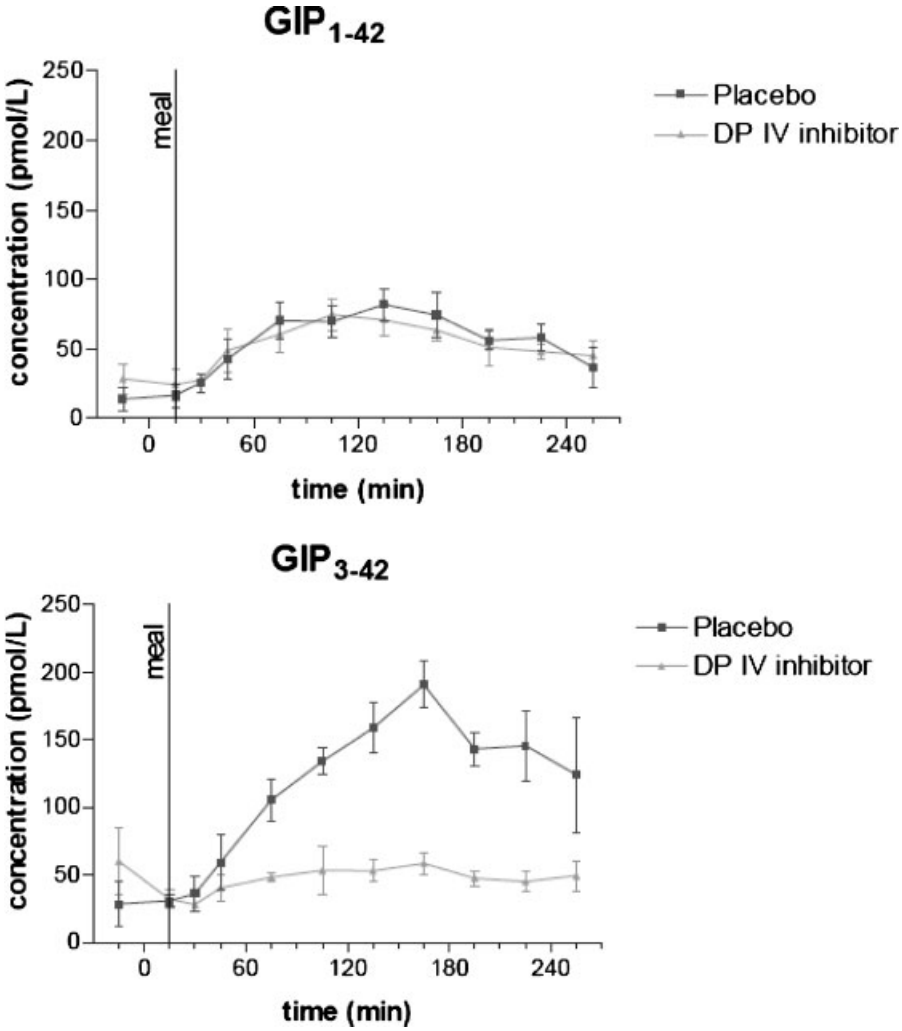

FIGURE 5. Determination of GIP_{1-42} and GIP_{3-42} during the course of a clinical study in the presence and absence of DP IV inhibitor. [Reprinted with permission, from Journal of Chromatography A, 926, Wolf R.,Rosche F.,Hoffmann T., Demuth HU; Immunoprecipitation and liquid chromatographic-mass spectrometric detection of the peptide glucose-dependent insulinotropic polypeptides GIP_{1-42} and GIP_{3-42} from human plasma samples. New sensitive method to analyze physiological concentrations of peptide hormones. 21–27, 2001, with permission from Elsevier copyright 2001.]

for similar studies in the future, it will be important to have more information on available methods (advantages and disadvantages) so that extraction and analysis of peptides can be achieved and optimized in tissues such as the lung, liver, or tumors.

IV. METHODS OF DETECTION

A. LC-MS in Full Scan (FS) or Single Ion Monitoring (SIM) Modes

Full scan or SIM modes have been applied to a number of studies for quantification of peptides in biological fluids. Most assays involve electrospray ionization (ESI). The FS modes tend to offer limited sensitivity in comparison to other options, particularly when quadrupole instruments are used. Most often, if quadrupole (single quadrupole) instruments are available, SIM modes are the methods of choice for quantitative analysis because specific ions characteristic of the analyte of interest can be monitored. With ion trap instruments, FS sensitivity is improved in comparison to quadrupole instruments. The potential benefits for peptide quantification with the recently introduced linear ion traps (LIT) are significant from the view point of sensitivity and dynamic range enhancements, mainly because of the ion capacity of the linear traps; however, applications on peptide quantification have not been reported in the literature yet. It is generally accepted that such new tools (linear ion traps) should be valuable for quantification of bioactive peptides or small organic molecules

in FS modes. Applicability of this technology (LIT) for metabolite identification and bioanalysis with emphasis on small molecule drugs was recently reported by Xia et al. (2003); Hopfgartner, Husser, & Zell (2003); and Mauriala et al. (2005)).

A substantial number of quantitative peptide applications have been reported in the SIM modes. This mode typically yields some benefits with regard to the signal to noise ratio (s/n), but certainly lacks the selectivity that would be offered in comparison to data acquired by tandem mass spectrometry (MS-MS). With adequate sample preparation and chromatographic separation, it is possible to achieve analysis in biological fluids even in pM concentrations as was demonstrated by Wolf et al. (2001) in the analysis of GIP. It should be noted that, conceivably, use of low flow rates (20–40 μL/min), coupled with the pre-concentration approach previously described, have played a role in enhancing sensitivity for the particular study. Yamaguchi et al. (2000) developed an LC-ESI-MS method for the determination of KW-5139 (leu^{13}-motilin) in rat plasma. Leu^{13}-motilin, a genetically engineered variant of the 22-amino acid peptide motilin, is of interest for the treatment of post-operative intestinal paralysis. For the analysis of the peptide (intended for exogenous administration), experimental conditions such as pH and mobile phase salt concentration (ammonium formate) were optimized for the $[M+3H]^3$ ion of KW-5139, and analysis in the SIM mode resulted in a limit of quantification of 0.5 ng/mL. Figure 6 represents a positive ion electrospray ionization mass spectrum for KW-5139 and for the internal standard used for the study. The effects of pH on the peak intensity of the $[M+3H]^{3+}$ ion are depicted in Figure 7. The method's applicability was demonstrated by measuring rat plasma concentrations of this peptide following a 1 μg/kg intravenous administration. A concept previously described for the analysis of small organic molecules (Jemal et al., 2003), where analysis in LC-MS in the SIM or SRM modes is enhanced

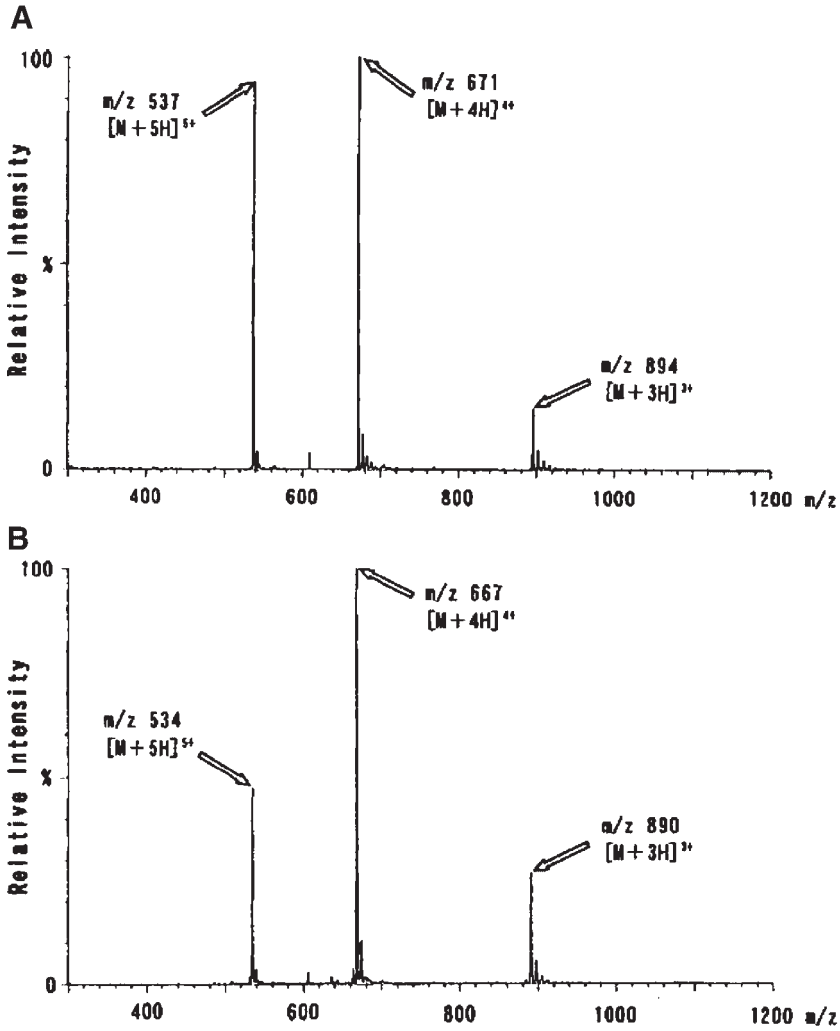

FIGURE 6. Positive ion electrospray ionization mass spectra of (**A**) KW-5139 and (**B**) IS. [Reprinted with permission, from Biomed Chromatogr 14: 77–81, 2000, Yamaguchi K, Takashima M, Uchimura T, Kobayashi S., Development of a sensitive liquid chromatography-electrospray ionization mass spectrometry method for the measurement of KW-5139 in rat plasma, with permission from John Wiley & Sons copyright 2000.]

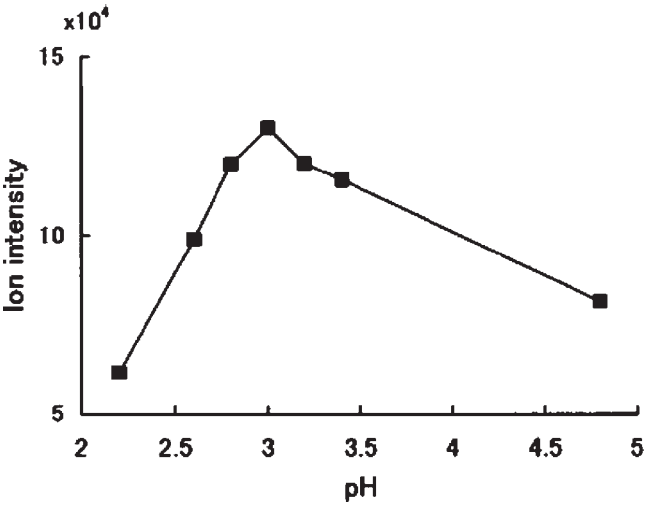

FIGURE 7. Effect of pH on the peak intensity of the $[M + 3H]^{3+}$ ion of KW-5139 infused into LC-MS. [Reprinted with permission, from Biomed Chromatogr 14: 77–81, 2000, Yamaguchi K, Takashima M, Uchimura T, Kobayashi S., Development of a sensitive liquid chromatography-electrospray ionization mass spectrometry method for the measurement of KW-5139 in rat plasma, with permission from John Wiley & Sons copyright 2000.]

by data acquisition with enhanced resolution, should be applicable for quantification of peptides. The higher resolution might help to exclude any interfering components from the biological matrix, and to yield improved s/n ratios and limits of detection.

B. LC-MS-MS Using Ion Trap Instruments

Three-dimensional trap instruments are mostly suited for qualitative analysis and offer good sensitivity in the FS mode and the capability for fragmentation (MS^n) by which a molecule's structural characteristics can be determined (Zhong et al., 2001; Samuel et al., 2003). In their review article, John et al. (2004) provide a list of LC-MS peptide assays categorized by the type of platform used for detection. Almost 50% of the applications listed the review article by John et al., involve ion-trap instruments (three-dimensional traps), with higher limits of detection in comparison to triple quadrupole instruments (10–200 ng/mL vs. 0.005–5 ng/mL). A study of Oosterkamp, Gelpi, and Abian (1998) is especially noteworthy because excellent sensitivity was achieved for the quantification of endogenous endothelin peptides (ETs) in cell supernatants (limits of quantification ~0.1 ng/mL, 1.5 fmol on column). The high sensitivity was achieved by using column-switching nano liquid chromatography and a micro-electrospray interface (nanoLC/μESI-MS). Besides the advantage of pre-concentration of sample by column switching, the authors demonstrated that, consistent with the theory of ESI operation, low flow rates will yield high sensitivity (flow rates were optimized at 0.55 μL/min for this application). In this particular study, unsatisfactory reproducibility (RSD > 50% on repeated on column injections of endothelin peptides) could be overcome by incorporating a synthetic endothelin peptide, [3, 11 Ala]-ET-1, as an internal standard in the analysis. Zhu et al. (2003) used an ion trap instrument to study the role of DP IV on a number of bioactive peptides. Finally, it should be noted that fragmentation efficiencies (relative formation of product ions relative to the precursor ion) differ between MS/MS instrument configurations. Ion trap instruments tend to have higher collision induced dissociation (CID) fragmentation efficiencies in comparison to triple quadrupole instruments (Johnson et al., 1990; Thomson et al., 1995) and presumably this characteristic could be beneficial when peptides are analyzed by ion trap instruments in MS/MS modes.

C. LC-MS-MS (Using Triple Quadrupole Instruments)

Liquid chromatography coupled with tandem mass spectrometry on a triple quadrupole instrument (Fig. 1c) offers a good choice for the development of selective and sensitive assays for the measurement of peptides in biological fluids. As it is evident from numerous reports in the literature (Stokvis et al., 2002, 2004; Jemal, 2000; Jemal & Ouyang, 2000), triple quadrupole instruments are well tested for quantification, particularly when known molecules are monitored in MRM or selected reaction monitoring (SRM) modes. In this case, following ionization, specific ions unique to the analyte in question are filtered through the first quadrupole (Q1) and their product ions generated in the collision cell, are filtered through the third quadrupole (Q3) prior to detection. Following elution from the HPLC system, molecules are detected as specific MRM transitions (precursor → product ion) with effective duty cycles, resulting in assays of high sensitivity and maximum specificity (peaks with optimum signal/noise ratios). As stated earlier, peptides can be quantified with triple quadrupole instruments often with limits of quantification to the low ng/mL or even pg/mL levels. One of the main differences between analysis of peptides and small organic molecules is that the generation of multiple-charged states with peptides, necessitates optimization of precursor ion (e.g., one- or two-charged states with optimum signal intensity should be monitored) and optimization of fragmentation of those precursor ions in the MS/MS modes. Although, in general, the literature reports suggest that highest sensitivity of quantification of bioactive peptides in biological fluids is achieved with triple quadrupole instruments, it should be noted that fragmentation efficiencies are typically low (5–10%) with triple quadrupole instruments, in comparison to other platforms (e.g., ion traps). Most peptides in MS/MS modes undergo extensive fragmentation typical of their amino acid sequences, often generating a spectrum of multiple peaks that results in weaker signal intensity of the specific MRM transitions. Therefore, using the sum of the most intense signals is suggested for the enhancement of signal. As an example of peptide analysis on a triple quadrupole instrument, we refer to the study of Yin et al. (2003) in which the development of an LC-MS-MS method for the quantification of Aplidin® in human plasma was described. Aplidin (dehydrodidemin), a cyclic depsipeptide isolated from a Mediterranean marine tunicate, possesses *in vitro* activity against tumor cell lines. Aplidin has recently entered phase I clinical trials;

therefore, a sensitive assay is required for the analysis of this peptide in human plasma. Yin et al. used didemnin B, a structural analog of Aplidin, as an internal standard and a triple quadrupole mass spectrometer equipped with a TurboIonSpray interface for the development of an assay in the 0.05–50 ng/mL range, using 100 μL of human plasma. The described approach resulted into a rather sensitive assay with a good dynamic range (1,000 fold) and potential for high throughput analysis of this peptide in clinical studies (analysis time per run of 3 min). Aplidin with a molecular weight of ∼1,110 Da appears to have an intense peak of the MH^+ ion at *m/z* 1,110.7 (the most abundant peak) that fragments in the MS/MS mode to give primarily an ion at *m/z* 295.3. The simplicity of the spectra (lack of multiple-charged ions and lack of extensive fragmentation), which is atypical for many peptides, as well as optimization of HPLC conditions (column temperature at 60°C to merge two conformers of Aplidin into one peak), presumably provided the high sensitivity in this assay. As stated earlier, the ability to develop sensitive assays (low nM or pM) in biological fluids for peptides of higher molecular weights (1,500–5,000 Da) remains a significant challenge. This challenge becomes even more pronounced when mass spectrometry is employed for the analysis of important endogenous proteins of higher molecular weights (MW ∼25,000 Da) such as prostate-specific antigen (PSA) or *C*-reactive protein (CRP). Reports by Barnidge et al. (2004) and Kuhn et al. (2004), describe mass spectrometric-based approaches for the absolute quantification of PSA and CRP in human serum. In both reports, the approach is similar and it is based on cleavage of the intact protein (PSA or CRP) with trypsin and subsequent detection of low molecular weight peptide fragments by LC-MS-MS on a triple quadrupole instrument. The general strategy is represented in Figure 3. Optimization of the cleavage step, extraction of protein from serum (e.g., selective removal of human serum albumin, immunoglobulin), incorporation and selection of internal standards are all important factors for the analysis, but overall use of a triple quadrupole instrument appears to be a good choice to achieve the highest sensitivity and dynamic range. In their study of PSA quantification in serum, Barnidge et al. demonstrated that the digestion of PSA by trypsin was complete by evaluation of denatured serum samples containing PSA by Western blot analysis. The results are shown in Figure 8. A comparison between the bands that represented serum samples contained PSA before digestion and absence of bands after digestion, indicated that PSA was digested below the levels of detection for the antibody (low μg/L range). Although the studies by Barnidge et al. and Kuhn et al. are novel, valuable, and represent good examples of how to achieve absolute quantification in serum with proteins of this size, both authors raise valid concerns about issues such as sensitivity and reproducibility of the described assays, suggesting that further work is required to improve the current state of the art.

The strategies the employ digestion of proteins have been extended by Gygi and colleagues towards measurement of precise amounts of endogenously expressed phosphorylated proteins from whole-cell lysates following separation by SDS/PAGE. The AQUA strategy relies on the use of synthetic internal standard peptides (stable isotopes) that are introduced at fixed concentrations to cell lysates during digestion. The amounts of peptide produced by digestion are reflective of the concentration

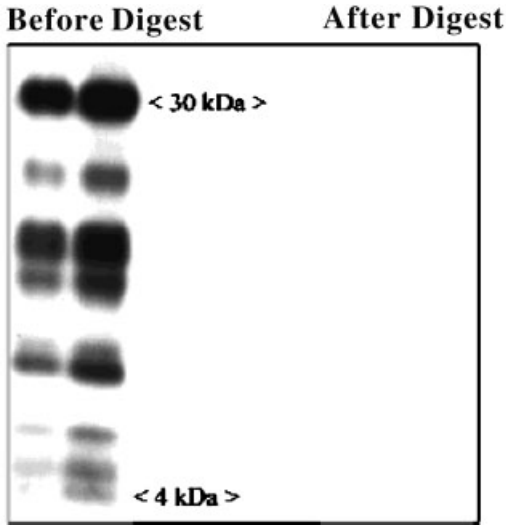

FIGURE 8. Western blot of PSA added to denatured serum before and after digestion with trypsin. The polyclonal antibody recognized intact PSA at 30 kDa as well as fragments of PSA found in the standard. Fragments of PSA > 4 kDa were not detected after digestion using this antibody that has μg/L sensitivity for PSA and its fragments. [Reprinted with permission, from Journal of Proteome Research, 2004, 3, 644–652. Barnidge DR, Goodmanson MK, Klee GC, Muddiman DC. 2004. Absolute quantification of the model biomarker prostate-specific antigen in serum by LC-MS/MS using protein cleavage and isotope dilution mass spectrometry, with permission from The American Chemical Society copyright 2004.]

of the target protein and the synthetic internal standard peptides used for quantification are designed to mimic the tryptic peptide fragment (e.g., a peptide with a heavy leucine residue containing six ^{13}C and one ^{15}N that differs from the non-labeled peptide by a 7-Da mass difference). It should be noted that the use of appropriate internal standards is extremely critical for quantitative studies, particularly for endogenous peptides or proteins that are typically present in low concentrations (pM). Most papers published on quantification of endogenous proteins by tryptic digestion and subsequent analysis of tryptic fragments, place great emphasis on the choice of internal standard. Similar to the analysis of small molecules, isotopically labeled peptide internal standards offer the advantage of similarity in extraction efficiency with the target peptide, chromatographic behavior, ionization efficiency, and fragmentation characteristics. Because of the fact that peptides have multiple charges, it is ideal to choose internal standards that differ from the peptide (analyte) by a minimum of 6 amu, to prevent the overlap of isotope peaks in the case of triply charged precursor ion. For quantification of Rhodopsin in rod outer segments, Barnidge et al. (2003), used two forms of peptide standards (TETSQVAPA), an unlabeled form (MW 902.22 Da) and a labeled form with three deuterium atoms on the methyl group of each alanine (MW 908.43 Da). Stokvis et al. (2004), reported on the improvements observed in quantification of Kahalalide F in human plasma by switching from an analog to a stable isotopically labeled internal standard. The authors claimed that the replacement of a butyric acid-derivative analog of Kahalalide F to $^2H_8$-Kahalalide F lead to

improvements in terms of precision and accuracy and advantages in terms of prolongation of stability of the processed samples, because of a more rapid degradation of the butyric acid derivative internal standard in comparison to Kahalalide F. The study by Kirkpatrick, Gerber, and Gygi (2005) provides us with a thorough examination of internal standard selection for the AQUA strategy. The authors propose the use of heavy stable isotopes at only one position because of cost. The authors also suggest that it is preferable to use ^{13}C and ^{15}N-labeled amino acids because they do not show chromatographic retention shifts as deuterium-containing compounds. In some cases, it is preferred to introduce the isotopic label in such a position that the fragment (product ion) that is monitored in MRM mode contains the heavy residue so that the internal standard is distinct from the analyte based on precursor and product ion.

D. Liquid Chromatography/Quadrupole Time-of-Flight (LC-Q-TOF) Mass Spectrometry

Wan and Desiderio (2003) employed liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (represented in Fig. 1d) to quantify the mu opioid-receptor agonist, [Dmt^1]DALDA (Dmt-D-Arg-Phe-Lys-NH_2;Dmt = 2′,6′-dimethyltyrosine;'super-DALDA'), in ovine plasma. The quantification method combined capillary HPLC, a nanospray source, and a quadrupole time-of-flight mass spectrometer. A high level of detection sensitivity and specificity was achieved in the 4–64 ng/mL range, and the methodology was applied towards an *in vivo* study in which a sheep had been injected with [Dmt^1]DALDA at 0.06 mg/kg (exogenous peptide administration). Although the study demonstrated that the assay's dynamic range was adequate for analysis of samples, the low dynamic range, characteristic of Q-TOF instrumentation, is not ideal for quantification studies and thus not frequently used for peptide quantification.

E. Detection by Matrix-Assisted Laser Desorption/Ionization MALDI

The work of Tholey, Zabet-Moghaddam, and Heinzle (2006) demonstrates that matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) can be applied towards quantification of peptides provided that ionic liquid matrices (ILM) are used for the preparation of homogeneous samples. ILMs are mixtures of typical MALDI matrices such as α-cyano-4-hydroxycinnamic acid (CCA) with equimolar amounts of organic bases such as 3-(dimethylamino)-1-propylamine (DMAPA) that, due to high sample homogeneity, enable the improved relative quantification of amino acids (Zabet-Moghaddam, Heinzle, & Tholey, 2004). Single peptides or mixtures of peptides such as angiotensin II, neurotensin, substance P, and their trypsin-catalyzed digests were quantified by this approach with a dynamic range of linearity of ∼1 order of magnitude. The authors suggest that this type of approach could be applicable for fast screening of new enzymes or the search for substrates or inhibitors. Nelson et al. (2004)) described the mass spectrometry immunoassay (MSIA) technique for quantification of IGF-1 in human plasma. Prior to extraction of IGF-1 and internal standard (rat IGF-1 mass shifted from human IGF-1), the authors of the study indicated the need for disruption of IGF/IGFBP complexes by addition of SDS to the plasma sample. Affinity isolation for the extraction of IGF-1 and internal standard was achieved from human plasma by use of activated affinity pipet tips derivatized with rabbit anti-human IGF-1 antibody. IGFs were eluted and prepared for MALDI-TOF analysis by drawing 4 μL of MALDI matrix (α-cyano-4-hydroxycinnamic acid, in 33% acetonitrile, and 0.4% TFA) and subsequent deposition onto a target plate. A quantitative MSIA working curve, constructed from serially diluted IGF-1 standards was presented in the study described, in the 7.7 μg/L–1,000 μg/L range. The study demonstrated a linear dynamic range of at least two orders of magnitude and applicability of the described approach was demonstrated for measurement of IGF-1 in human plasma from eight healthy individuals.

The studies describe above, indicate that applicability of MALDI-MS for quantification of peptides in biological fluids is feasible, but improvements in the dynamic ranges and homogenous distribution of the sample in the matrix will be needed for this technique to become of practical significance.

V. CONCLUSIONS—FUTURE PERSPECTIVES

In this article, an overview of mass spectrometric methods for the analysis of peptides is provided. At this stage, I would like to direct the readers to those techniques that have shown promise and offer a high degree of expectation. Once a thorough consideration has been given to the items described in Table 1, selectivity and sensitivity enhancements can be achieved as follows: (a) selective extraction of peptide from biological tissue combined with sample pre-concentration prior to analysis, (b) enhancement of chromatographic conditions, and (c) enhanced detection by mass spectrometry. It should be clear from the emphasis, we have placed on immunoprecipitation in this manuscript that we consider it a rather powerful way of ''isolating'' low quantities of peptides from biological fluids in a quantitative way (approximately 75% recovery of GIP_{1-42} and GIP_{3-42} was reported by Wolf et al.) and subsequently preconcentrating the samples for maximum sensitivity. We recognize the potential difficulties with obtaining specific antibodies for peptides of interest and the costs associated with the technique. Our laboratory is currently involved in using antibodies for bioactive peptides of interest and working out the conditions required for optimum peptide recovery. We are interested in expanding this approach towards the measurement of peptides in relevant tissues for pharmacologic action (e.g., tumors, brain) and anticipate that, through such projects, we will be able to demonstrate that tissue distribution is critical for pharmacologic action. What has been given attention in the literature and is widely used from proteomic laboratories, is ionization on nanospray sources because the lower flow rates offer significant advantages with respect to sensitivity (Giorgianni et al., 2004). However, it is rather uncommon for mass spectrometry laboratories that focus on routine quantitative analysis of small molecules to use nanospray ionization because of the lower throughput (long runs, long equilibration times), the potential for pressure buildup on the system because of

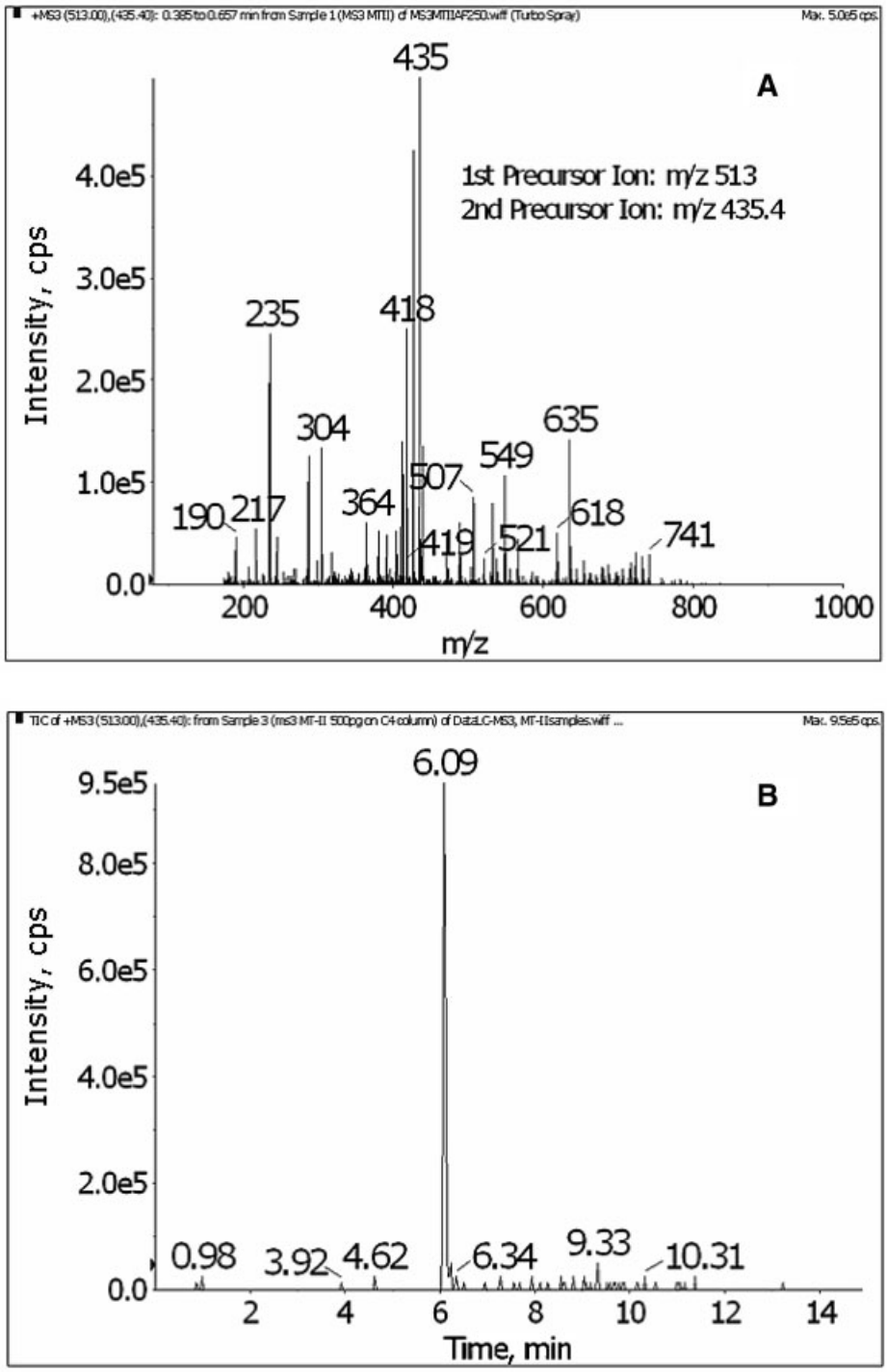

FIGURE 9. **A**: MS/MS/MS spectrum following the infusion of MT-II into a Triple Quadrupole—Linear Ion Trap system (1st Precursor ion: *m/z* 513, 2nd Precursor ion: *m/z* 435.4). **B**: Total ion chromatogram generated following the injection of 500 pg of MT-II on a LC/MS/MS/MS system, using the MS^3 scan mode for detection.

endogenous components from the biological fluid, and overall signal stability. I consider that, given the advances in chromatographic systems (HPLC systems that can deliver low flow rates reproducibly) and availability of HPLC columns (various solid-phase options with low internal diameters), it would be wise for investigators that focus on peptide quantification in biological fluids to consider the benefits of low flow rates and investigate if analysis on nanospray sources is practically feasible.

Another technique discussed in this review is protein or peptide cleavage by trypsin, followed by analysis of the lower molecular weight fragments on a triple quadrupole instrument. The shorter peptides that resulted from the cleavage are typically less complex molecules in comparison to the precursor higher molecular weight peptides. The lower molecular weight peptide fragments can be detected with higher sensitivity. Although digestion is a common practice in proteomic laboratories, information on optimization of the digestion step is not widely available; something that is extremely critical for quantification studies. The availability of internal standards, strategies for their selection, and a proper method of detection is also of importance for a successful outcome. The digestion approach has resulted in some promising reports with high molecular weight peptides such as PSA, CRP, and the applications of the AQUA method that have been mentioned in this review. However, it seems that some work will be required before this technique can be routinely used for peptide quantification in biological fluids.

In our discussion of instrument options available for quantification studies, the potential of linear ion traps for peptide analysis was considered. The potential benefits of the larger capacity linear traps (enhanced sensitivity, larger dynamic range) have not been demonstrated yet with the quantification of peptides. However, as discussed earlier, we expect that there will be some breakthroughs in the field using this relatively new technology. Of great interest remains the prospect of utilizing MS/MS/MS for the quantification of peptides, an approach that currently has no practical implications with the three-dimensional traps because of significant losses in sensitivity observed past the second stages of MS (MS/MS). We are currently testing such approaches in our laboratory for the quantification of MT-II (MW: 1,024 Da). Figure 9A represents the MS/MS/MS spectra of MT-II (1st Precursor ion: *m/z* 513, 2nd Precursor ion: *m/z* 435.4) and Figure 9B a chromatogram of 500 pg of MT-II on column injection, using the MS^3 scan mode for detection. Data were generated from a hybrid triple quadrupole linear ion trap system. We are currently evaluating the limits of sensitivity, precision and accuracy, and linearity for the detection of MT-II in plasma.

In this review article, I have attempted to demonstrate the importance of employing mass spectrometric techniques for the quantification of bioactive peptides. It is critical to develop sensitive, selective, and cost-effective approaches for the quantification of bioactive peptides to maximize substantial achievements from the field of proteomics and peptidomics (e.g., discovery of peptide biomarkers), to play a significant role in the advancement of medicine, and the development of future therapies. Peptides are complex molecules with diverse physicochemical characteristics, and although progress has been made, the development of ''generic'' methodologies will not be as easy as it has been perhaps for the analysis of small organic molecules.

VI. ABBREVIATIONS

anti-C-GIP	C-terminal directed antibody
AQUA	absolute quantification
CCA	α-cyano-4-hydroxycinnamic acid
GLP-1	glucagon-like peptide
DMAPA	3-(dimethylamino)-1-propylamine
CRP	*C*-reactive protein
DP IV	dipeptidyl peptidase IV
ETs	endothelin peptides
ESI	electrospray ionization
ESI-LC/MS	electrospray ionization-liquid chromatography/mass spectrometry
FS	full scan
FT-MS	fourier transform-mass spectrometer
GIP	glucose-dependent insulinotropic polypeptide
HPLC	high performance liquid chromatography
ICAT	isotopic coded affinity tag
ILM	ionic liquid matrices
LC-MS-MS	liquid chromatography coupled with triple quadrupole or ion trap instruments
LC-Q-TOF	liquid chromatography/quadrupole time-of-flight
LIT	linear ion trap
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
MALDI-TOF	matrix-assisted laser desorption ionization-time of flight
MP1	model peptide
MRM	multiple reaction monitoring
MS-MS	tandem mass spectrometry
MSIA	mass spectrometry immunoassay
MT-II	melanotan-II
MW	molecular weight
nanoLC/μESI	nano liquid chromatography and a micro-electrospray interface
PSA	prostate-specific antigen
RSD	relative standard deviation
SIM	single ion monitoring
SPE	Solid-phase extraction
SRM	selected reaction monitoring
TFA	trifluoro acetic acid

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Dr. Tamvakopoulos received a B.A. in Chemistry from the University of Chicago in 1986 and a Ph.D. in Chemistry/Biochemistry from Brown University in 1992. He joined the department of Pharmacokinetics and Drug Metabolism at Purdue Pharma as a Senior Scientist and then as a Principal Scientist until 1997. In 1997, he joined the Chemistry department at Merck & Co as a Senior Research Chemist and a Research Fellow. Since 2004, he accepted a tenure track position at the Department of Pharmacology, Institute for Biomedical Research, Academy of Athens. His research revolves around the development and application of novel mass spectrometric techniques with significance in biomedical research.