Determination of Angiotensin-Converting Enzyme Inhibitory Peptide Leu-Lys-Pro-Asn-Met (LKPNM) in Bonito Muscle Hydrolysates by LC–MS/MS

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Proteolytic digestion of dried bonito muscle with thermolysin produces a hydrolysate with strong angiotensin-converting enzyme (ACE) inhibitory activity and is the basis of a dietary supplement with antihypertensive activity. A major portion of the ACE activity was shown previously to arise from the peptide Leu-Lys-Pro-Asn-Met (LKPNM). A straightforward method to quantify this peptide was developed using one-step C18 solid-phase extraction (SPE) followed by LC–MS/MS quantification. The SPE step resulted in a hydrolysate that was still crude, as illustrated by combined size-exclusion chromatography/multi-angle laser light scattering detection that showed that a major fraction of oligopeptides were in the 2–20 kDa range. This fraction has a weight-average molecular weight (M_w) of ~5.0 kDa. Method validation for specificity, linearity, accuracy, precision, and reproducibility showed that standard additions of synthetic LKPNM to bonito extract with SPE enrichment followed by LC–MS/MS is a suitably robust procedure for the determination of LKPNM content. The method was also successful for encapsulated powders in which the excipients used are insoluble in water and could be removed by centrifugation.

KEYWORDS: Angiotensin-converting enzyme (ACE); LC–MS/MS quantification; bonito muscle; Leu-Lys-Pro-Asn-Met (LKPNM)

INTRODUCTION

Angiotensin-converting enzyme (ACE) is a peptidyl-dipeptidase (EC3.4.15.1) that functions in the renin–angiotensin system to increase blood pressure (*1*). ACE catalyses the formation of Angiotensin II (Ang II), a potent vasoconstrictor, from Angiotensin I (Ang I) and inactivates bradykinin, a vasodilator. Inhibition of ACE is a first-line therapy for hypertension and congestive heart failure (*2*). The pharmacological ACE inhibitors (e.g., captopril and enalapril) are short peptide-like molecules that tightly bind to ACE at its active site and compete with Ang I for occupancy (*3*). For example, captopril, the first ACE inhibitor to be developed for pharmacological use, is an alanyl-proline derivative containing a sulfhydryl group to increase its affinity for the ACE active site (*3*, *4*). The normal regulation of ACE in vivo also may involve inhibition by naturally occurring peptides (*5*). Recent studies reveal that the angiotensin heptapeptide (Ang-(1-7)), which is produced from Ang I through the action of a neutral endopeptidase in the blood, is a competitive inhibitor of ACE and may, therefore, represent a physiological counterpart of the pharmacological compounds (*5*).

In addition to the synthetic compounds produced by the pharmaceutical industry, ACE inhibitory peptides can be generated from a variety of animal and plant protein sources through the action of proteolytic enzymes (*6*). Thus, protein digests containing ACE inhibitors have been produced from a variety of edible sources including milk, eggs, chicken, pork, beef, fish, and microalgae (*7*–*9*) and represent potential nutraceutical and functional food ingredients. Such digests are complex mixtures of peptide species containing one or more active constituents in low concentration. Active peptides are typically of 2–5 amino acids in length and often contain one or a combination of an aromatic amino acid (e.g., tyrosine), a branched-chain amino acid (e.g., isoleucine), and proline. Ensuring that an active peptide is present at the prescribed level within a digest, or within a formulation containing the digest, is not straightforward. Unlike synthetic ACE inhibitors that can be directly and accurately quantified by HPLC or CE (*10*), the analysis for active peptides in a nutraceutical product is a challenging analytical problem involving a combination of separation procedures such as HPLC with in vitro ACE activity measurements.

Fish protein is particularly suitable as a starting material for preparation of ACE inhibitory peptide hydrolysates (*11*, *12*). Proteolytic digestion of dried bonito muscle with thermolysin produces a hydrolysate with strong ACE inhibitory activity (*12*, *13*). Ingestion of 1.5–3.0 g/day of the hydrolysate results in significant blood pressure reduction in borderline and mildly

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hypertensive patients (*7, 14*). We are developing this hydrolysate for use as a nutraceutical and functional food ingredient. It contains a suite of at least eight ACE inhibitory peptides (*12*), with a significant fraction of the ACE inhibitory activity ascribed to a pentapeptide, Leu-Lys-Pro-Asn-Met (LKPNM). Because LKPNM is the major active component in the hydrolysate, quantifying it is essential for quality control monitoring. However, published methods to quantify LKPNM in the digest involve a series of semipreparative HPLC steps (*12*) involving a fractionation sequence on C18-, phenyl-, cyano-, and C18-phases. Because this purification is not practical as a quantitative tool, and because the ACE assay itself is not a substitute for a rigorous analytical protocol, another method is required to quantify LKPNM.

In this study we present a straightforward, quantitative method for measuring LKPNM in bonito muscle hydrolysate using one step of solid-phase extraction followed by quantification using LC−MS/MS. In addition, we summarize method validation presenting measures of specificity, linearity, accuracy, precision, and reproducibility.

MATERIALS AND METHODS

Sample Preparation. The peptide LKPNM was synthesized by the Advanced Protein Technology Centre (Hospital for Sick Children, Toronto, ON) and analyzed for purity (>95%) by LC/MS. A stock solution of 0.8 mg/mL LKPNM (**A1**) was prepared by dissolving an accurately known weight in Milli-Q deionized water. Dried bonito peptide hydrolysate was obtained from Nippon Supplement (Osaka, Japan). The hydrolysate was prepared by suspending dried, ground bonito in water, boiling it to deactivate endogenous enzymes followed by digestion with thermolysin for 3 h at 70 °C. Finally, centrifugation and filtering of the supernatant produced a solution of the hydrolysate. In some cases, this was further purified by ultrafiltration. A 250 mg/mL stock of bonito hydrolysate solution (**C1**) was prepared by adding Milli-Q deionized water to bonito and sonicating for 5 min. Standard additions of LKPNM (**A1**) and water to **C1** gave 125 mg/mL bonito hydrolysate solutions with spike levels covering the range 0.05−0.3 mg/mL (**D2**−**D6**) plus the unspiked solution **D1**.

Preconcentration of the LKPNM content and partial cleanup of the hydrolysate solutions was obtained using 6-mL (1 g) C18 solid-phase extraction cartridges (Waters Sep-Pak, Waters Corp., Milford, MA) on a vacuum-assisted extraction manifold. Each cartridge was conditioned by rinsing with 6 mL of MeOH and equilibrated by rinsing with 6 mL of H₂O. One mL of each of the solutions **D1**−**D6** was transferred onto separate cartridges that were then rinsed with 20 mL of Milli-Q deionized water followed by 10 mL of 20% aq MeOH to remove unwanted, unretained compounds. The samples were eluted with 10 mL of 40% aq MeOH and collected inside the extraction manifold chamber into 20-mL scintillation vials. The solvent was removed and each sample, **D1a**−**D6a**, was re-suspended in exactly 1.0 mL of Milli-Q deionized water. All samples were filtered through 0.2-μm polyvinylidine fluoride (PVDF) centrifugal filters (Fisher Scientific, Nepean, ON) and divided into two autosampler vials for subsequent LC/MS analysis.

LC−MS and LC−MS/MS Analysis. All chromatography employed a Waters 2690 HPLC (Milford, MA) and Waters 996 photodiode array controlled via the Mass Lynx data system (Micromass, Manchester, U.K.). A 150 × 20 mm i.d. Waters Symmetry C18 column with 5-μm particles was used at 25 °C at a flow-rate of 0.3 mL/min. The column effluent was split 1/20 using an LC Packings (Amsterdam, The Netherlands) Accurate splitter so that 15 μL/min flowed to the electrospray source and the remainder went to the photodiode array. The solvent system was A, MilliQ water/0.05% trifluoroacetic acid (TFA); and B, acetonitrile/0.05% TFA. The gradient used was 90%A (*t* = 0 min) going to 70%A (*t* = 8 min) and 20%A (*t* = 10 min), held at 20%A for 4 min, then back to 90%A (*t* = 15 min), and reequilibrated for 5 min. The photodiode array was scanned from 200 to 400 nm with 1-s scan times. All analyses were performed using an injection volume of 10 μL and triplicate injections for each point.

A Micromass Q-Tof I mass spectrometer with Z-spray electrospray source was optimized for the analysis in positive ion mode (cone voltage 40V, source block 120 °C, desolvation heater 400 °C, collision energy 25 eV, and Ar collision gas). A critical factor was found to be the position of the electrospray probe, which was optimally placed such that a stable spray was aimed as far from the sampling cone as possible without loss of more than 50% of the signal intensity. This positioning, in addition to the low flow-rate to the source, allowed the experiment to be performed with very high concentrations of hydrolysate (125 mg/mL prior to cleanup) without loss of sensitivity of the mass spectrometer for well in excess of 100 injections. For LC−MS the Q-Tof was operated using an acquisition range *m/z* 100−1400 and 1-s integration time. For MS/MS experiments, the resolution of MS-1 (i.e., the quadrupole mass filter) was set to a mass window of 1 Da. The Q-Tof was operated in positive ion MS/MS mode for quantification of LKPNM using the precursor *m/z* 602.4 (MH⁺ for LKPNM), an acquisition range *m/z* 100−650, and 1-s integration time. All chromatograms were smoothed (two three-point smooths) and integrated using the Mass Lynx quantification package. Some experiments were performed by direct infusion of a standard solution into the electrospray source from a KD Scientific syringe driver (Fisher Scientific, Nepean, ON) at a flow rate of 1 mL/hr.

Data Analysis. Plots were made of the LC−MS/MS peak area for the transition *m/z* 602.4→361.3 + 212.2 or *m/z* 602.4→361.3 versus spike concentration. The actual concentration for LKPNM (C_{LKPNM}) in the bonito extract was determined from the intercept on the *x*-axis. As the concentration of bonito powder in **D1**−**D6** was 125 mg/mL, the actual LKPNM content of the dry bonito powder in mg/g was C_{LKPNM}/0.125. The amount of a known batch of hydrolysate added to a formulation was determined by directly comparing the average response (duplicate extractions, triplicate injections) for zero spike addition in the formulation (solution **D1**) with that for the same batch of hydrolysate alone, or from the absolute concentrations obtained from the standard additions curves.

Combined Size-Exclusion Chromatography/Multi-Angle Laser Light Scattering Detection/Refractive Index Detection (SEC/MALS/RI). Bonito hydrolysate was investigated by size-exclusion chromatography using a TSK gel G3000PWxl column (300 × 7.8 i.d mm, 6-μm particles) with a TSK gel PWxl guard (40 × 6 mm i.d., 12-μm particles) (Tosoh Biosep, Montgomeryville, PA). The mobile phase was 0.088 M sodium acetate with 40 ppm sodium azide, adjusted to pH 4.5 using acetic acid and filtered through a 0.1-μm membrane using vacuum filtration. The flow rate was constant at 0.75 mL/min with a 0.1-μm inline filter located between the pump and injector. Bonito hydrolysate solution was redissolved in the mobile phase at a concentration of ∼10 mg/mL after the C18 SPE cleanup. The sample was sonicated and filtered through a 0.2-μm Anopore aluminum oxide membrane syringe filter (Whatman, Clifton, NJ). A 100-μL aliquot of this solution was injected using an Agilent 1100 autosampler and isocratic pump.

A DAWN DSP laser photometer (Wyatt Technology Corp., Santa Barbara, CA) was used to measure laser light scattered by the sample at multiple angles around a glass flow cell (MALS) (*15*). The Wyatt Astra software calculated the molar mass distribution of the peptides in the bonito hydrolysate from the MALS response, the concentration-dependent Optilab RI detector (Wyatt) response, and an estimated specific refractive index increment d*n*/d*c* value of 0.185 typical for the protein bovine serum albumin (*16*).

RESULTS AND DISCUSSION

Development of the LC−MS/MS Method. Initial attempts at using semipreparative HPLC methods (*12*) to isolate fractions enriched in ACE-inhibitory peptides in general, and LKPNM in particular, indicated that this would be impractical for a routine quantitative assay. The latter was also true of a separation method based on a combination of C18 and Sephadex LH-20 and/or Sephadex G-25 in open columns. A major limitation of these two experiments was the difficulty of reliably making recovery measurements because isotopically labeled LKPNM
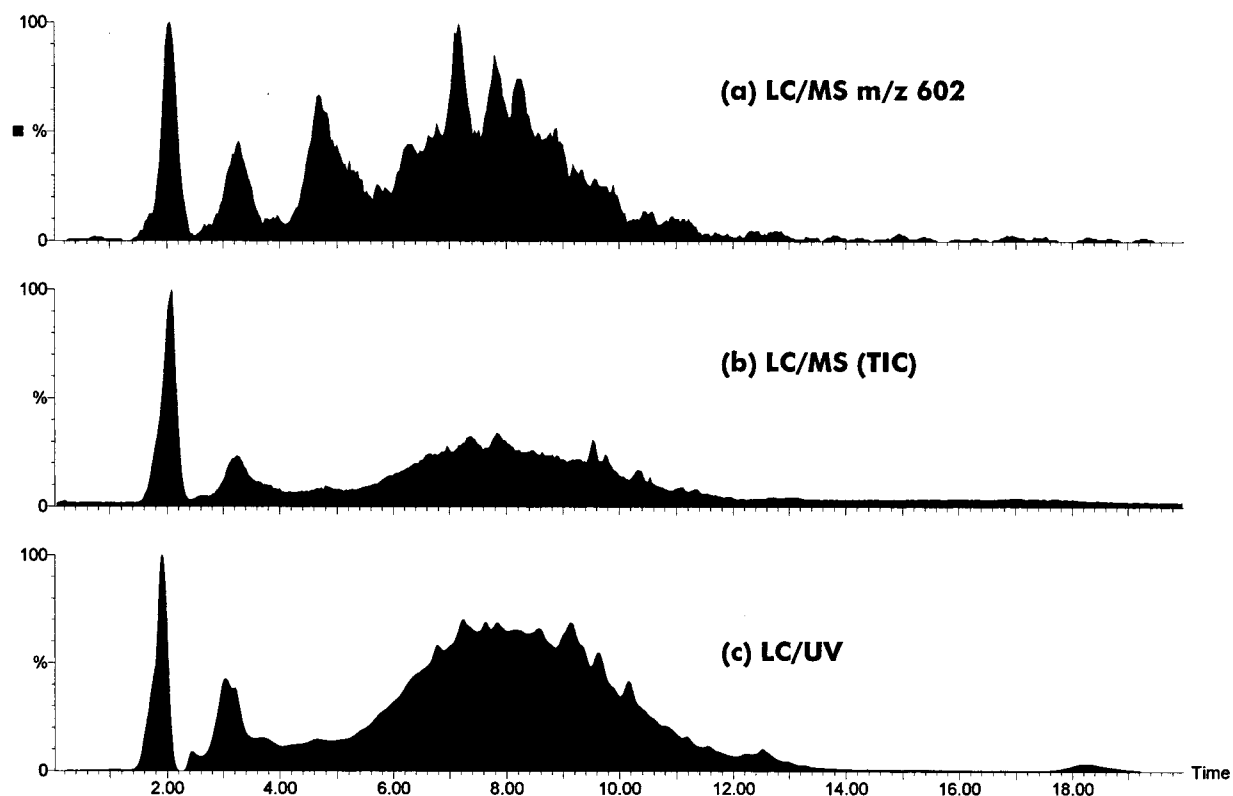
Figure 1. Bonito hydrolysate (125 mg/mL) solution **D1** after SPE cleanup. (a) LC/MS mass chromatogram of *m/z* 602.4; (b) LC/MS (total ion current) trace; (c) LC/UV chromatogram.

is prohibitively expensive to obtain in the required amounts for its use as an internal standard. In addition, these multi-column sample preparations are time-consuming, result in poor reproducibility, and permit only a single sample to be collected at a time. The most practical approach to the extraction and cleanup of materials for QC and stability monitoring purposes was to adapt the separations to disposable SPE cartridges which would then allow simultaneous rapid extraction of multiple samples. A straightforward adaptation of the published HPLC method gave poor recoveries, and the use of multiple SPE cartridges proved to be labor-intensive. However, from our earlier work using bonito and other fish hydrolysates, it was known that the use of C18 chromatography alone could result in retention of ACE inhibitory activity along with a very significant reduction in the mass collected. Because similar results were obtained using C18 cartridges, we used this knowledge to develop a method to ensure quantitative recovery of LKPNM while maximizing the removal of other material. This produced partially purified samples with concentrations suitable for analysis by LC/MS. To develop the method, a synthetic pentapeptide standard for LKPNM was used to assess recoveries from SPE as measured by flow-injection electrospray mass spectrometry. It was found that washing the column with up to 20−25% methanol could be used without loss of LKPNM whereas quantitative recovery of LKPNM spikes was achieved by subsequent elution of 10 mL of 40% methanol solution. This procedure resulted in removal of around 68% of the mass of the hydrolysate. At this level of cleanup, the LC/UV and LC/MS (total ion current) traces (**Figure 1c** and **b**) showed a complex mixture which was virtually unresolved. A greater level of specificity was achievable by taking advantage of the selectivity of the mass spectrometer, extracting from the LC/MS trace a mass chromatogram for the MH$^+$ ion of LKPNM (**Figure 1a**). In this case several distinct peaks were seen, including one at about 3.0 min not seen at all in the LC/UV or

LC/MS (total ion current) traces, and later assigned to LKPNM. However, the poor signal-to-noise and interference evident from other isobaric species, suggested that even greater selectivity and sensitivity was required for quantification.

To investigate whether the species seen as the intense broad feature in the chromatogram between 5 and 11 min were a result of a complex mixture of hydrolysis products or residual protein/oligopeptides, we used a combination of aqueous size-exclusion chromatography coupled to a multi-angle laser light scattering detector with simultaneous refractive index detection (SEC/MALS/RI) (*15, 16*). These experiments allowed the molar mass distribution of peptides of greater than about 1 kDa to be measured, which is not possible by electrospray mass spectrometry for such a crude mixture. It can be seen from **Figure 2** that the hydrolysate solution contained a significant amount of oligopeptide in the range 2 to 20 kDa over elution volumes of 8.5 to 12.0 mL, this particular fraction having a weight-average molecular weight (M_w) of ∼5.0 kDa. It should be noted that the MALS detector is insensitive to low masses, so beyond the linear region of the molar mass plot at >11 mL there is no meaningful molar mass information, even though there is still a large response from the refractive index detector.

It was concluded that the hydrolysate still contained a significant amount of partially digested peptides, and hence, without further fractionation, an improvement in the chromatography illustrated in **Figure 1** was unlikely. We therefore pursued tandem mass spectrometry as an alternative to further cleanup of the sample. The MS/MS spectrum of the MH$^+$ ion of LKPNM standard (*m/z* 602.4) was obtained by infusion of the sample into the electrospray source, thereby allowing optimization of the collision conditions. **Figure 3a** shows the resulting MS/MS spectrum which was dominated by the y_3'' fragment ion (*17, 18*) at *m/z* 361.3 plus smaller contributions from *m/z* 212.2 (a fragment ion containing proline and asparagine residues resulting from internal cleavage) and the c_1 ion,
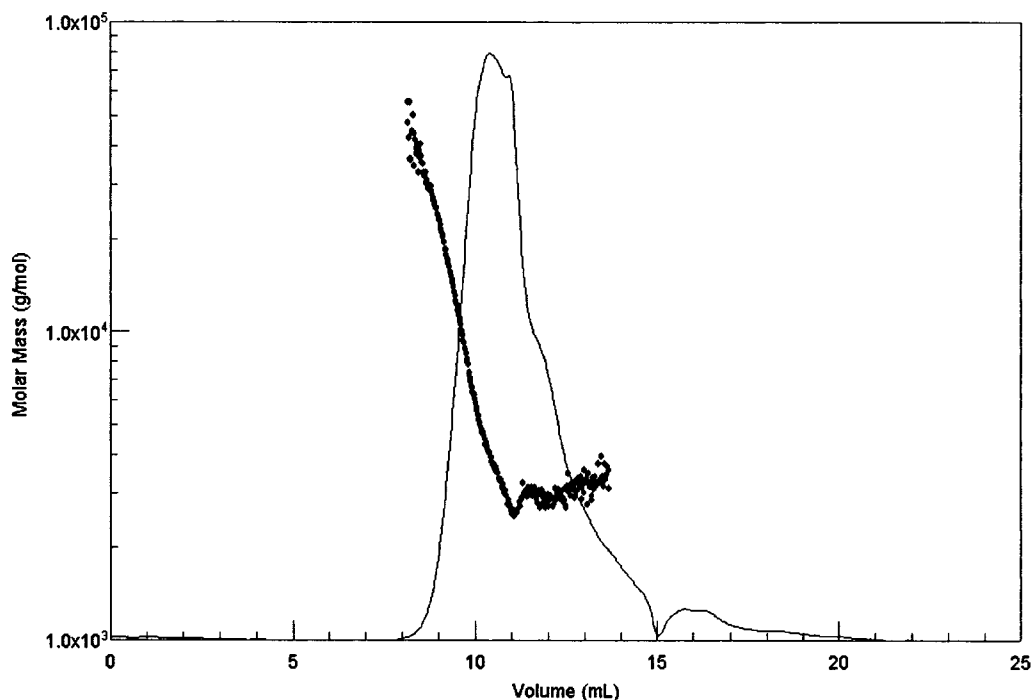
Figure 2. Size-exclusion chromatography coupled to a multi-angle laser light scattering detector with simultaneous refractive index detection (SEC/MALS/RI) of bonito muscle hydrolysate solution. Solid line (peak) indicates the RI trace; dotted line is the molar mass distribution calculated from the MALS detector.
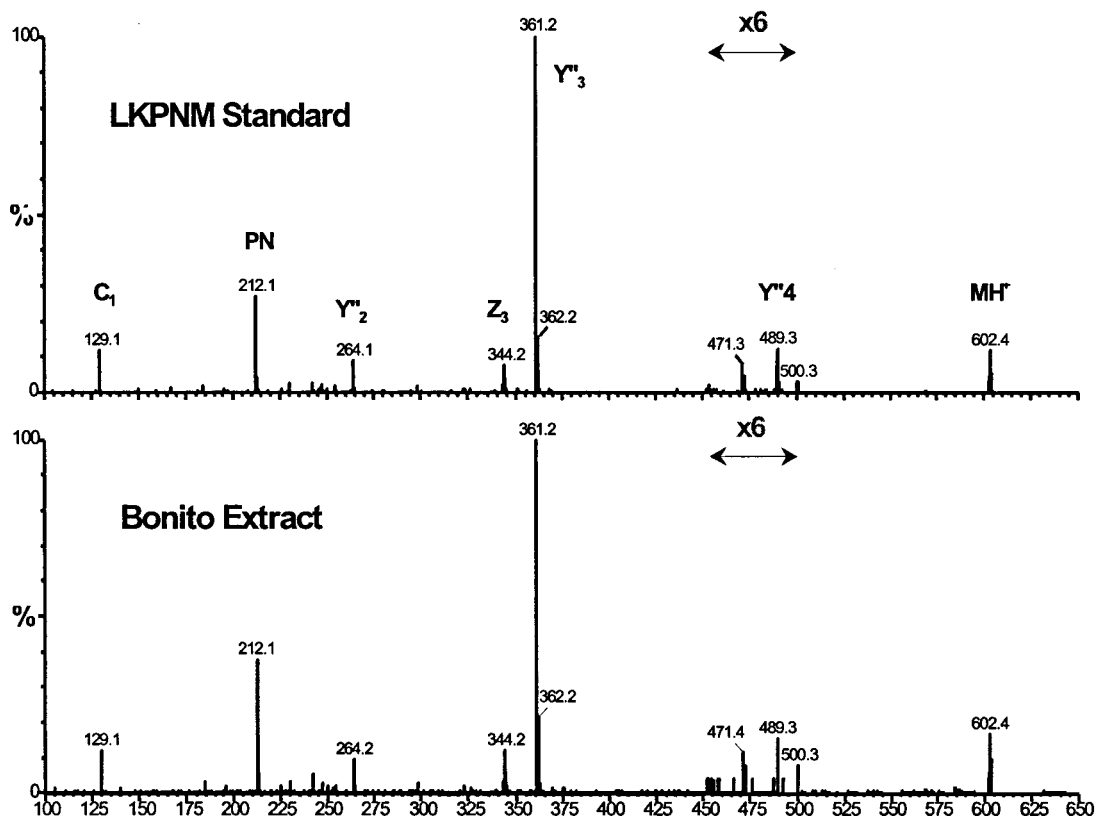

Figure 3. (a) MS/MS spectrum of MH+ ion by direct infusion of synthetic LKPNM; (b) LC–MS/MS spectrum of MH+ ion of LKPNM from bonito muscle hydrolysate solution D1.

m/z 129.1. Thus, LC−MS/MS of the transitions m/z 602→361, 602→212, and 602→129 should result in chromatograms of high selectivity and good sensitivity.

In this study we used a hybrid quadrupole/time-of-flight mass spectrometer (Micromass Q-Tof), rather than a triple quadrupole mass spectrometer which is more typically used for quantitative LC−MS/MS. The great advantages of the latter are the abilities to dwell for most of the sampling time on the selected transition of interest thereby maximizing sensitivity, to switch rapidly between transitions, and to scan rapidly without loss of chromatographic definition. On the other hand, in addition to much higher fragment ion resolution, the Q-Tof has the
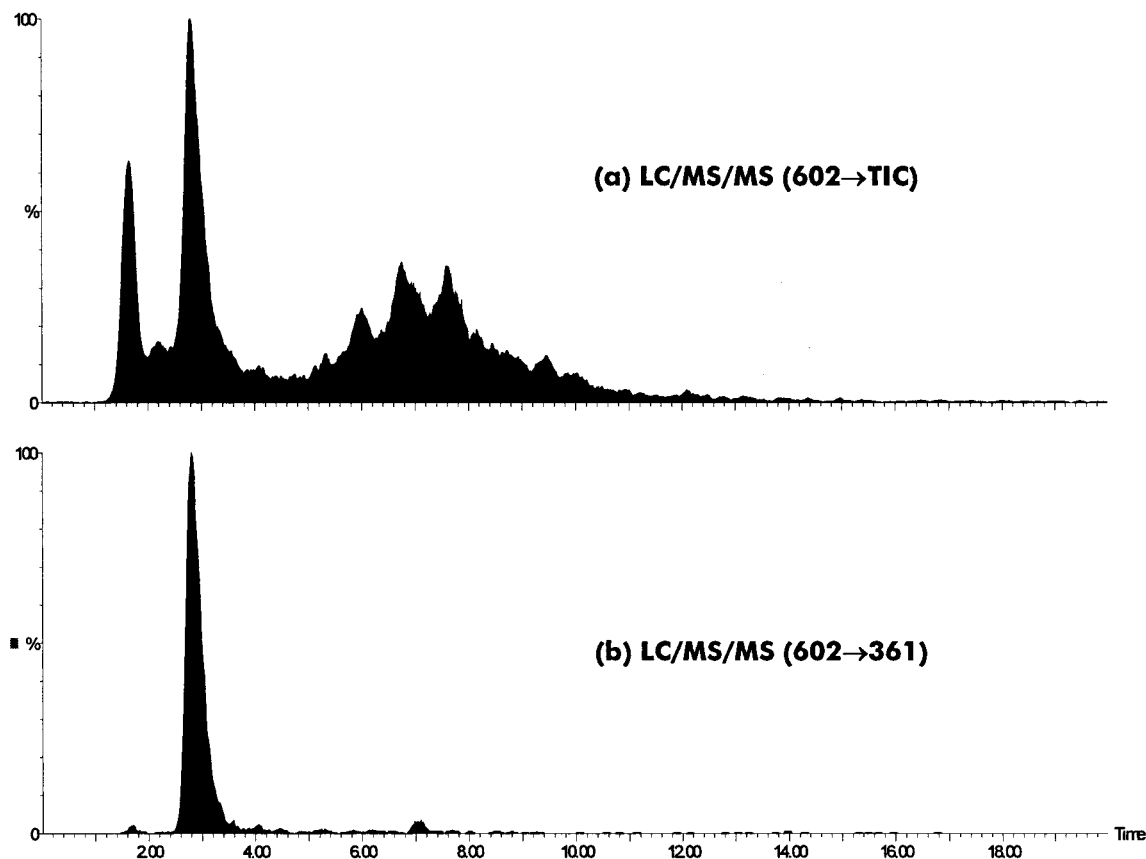
LKPNM Peptide Content in Fish Muscle Hydrolysates

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Figure 4. (a) Total ion current LC–MS/MS trace with precursor ion *m/z* 602.4 for bonito muscle hydrolysate; (b) LC–MS/MS trace for *m/z* 602.4→361.3 for bonito muscle hydrolysate solution D4.

advantage of recording the entire fragment ion spectrum every scan with much higher sensitivity than can be achieved by scanning instruments such as a quadrupole mass spectrometer (reference or data). Thus, in the development of a quantitative LC−MS/MS method, once the spectra are recorded on the Q-Tof, any or all of the fragment ions present can be chosen for subsequent data processing. The recorded responses of the selected fragment ions can then be used for quantification or to increase the specificity of the analysis by further confirmation of the peptide structure.

The result is illustrated in **Figure 4** which compares the complex total ion current LC−MS/MS chromatogram with precursor ion *m/z* 602.4 for the bonito muscle hydrolysate D4 (**Figure 4a**) with the LC−MS/MS trace for *m/z* 602.4→361.3 where a single peak was observed at a retention time of 3.0 min indicating the dramatic improvement in specificity. Comparison of the MS/MS spectrum averaged across this peak for bonito solution **D1** (**Figure 3b**) with that obtained directly from the standard solution (**Figure 3a**) showed an excellent spectral match, which again demonstrates the specificity of LC−MS/MS.

An ideal approach to developing a quantitative LC−MS/MS method would be to use LKPNM labeled with stable isotopes as an internal standard. This could reliably correct for matrix effects and other recovery losses. As this was not available, and because it would be difficult to obtain an alternative internal standard with chemical properties similar to those of the analyte that could be chromatographically resolved in the hydrolysate, quantification by means of standard additions was used. An advantage of this method is that recovery losses can be corrected for by spiking the hydrolysate prior to SPE, and that quantification can be based upon a standard addition curve to ensure

linearity. Furthermore, synthetic peptides of sufficient quantity are available at modest cost.

It was recognized that robustness would be a key attribute of the method to be developed because the objective was to develop a method involving minimal pre-fractionation of the hydrolysate. Hence, a high total concentration of sample (125 mg/mL bonito powder prior to SPE) would be required, resulting in potential contamination of the column and electrospray source. In fact, it was found that after the C18 SPE, highly reproducible chromatography could be achieved with the Waters Symmetry C18. However, when the entire 0.3 mL/min column flow was passed to the electrospray source, a deposit soon formed on the sample cone resulting in steady deterioration in response, a condition not conducive to quantitative analysis. This problem was overcome by two modifications. First, reducing the flow to the detector to around 15 μL/min eliminated the deposits and resulted in little loss of sensitivity because electrospray sources are sensitive to concentration rather than amount (*19*, *20*). Second, the spray from the electrospray needle (which is perpendicular to the sampling cone in the Micromass Z-Spray source) was directed as far away from the tip of the sampling cone as possible, without loss of more than 50% of the signal intensity. This resulted in the formation of a solid brown spot on the rear baffle plate several millimeters off-axis to the tip of the sample cone. This positioning, in addition to the low flow rate to the source, allowed the experiment to be performed with very high concentrations of hydrolysate for at least 100 injections without excessive loss of sensitivity of the mass spectrometer. As a demonstration, a recent run list of 80 injections showed an 11% decrease in response between the average of the first and last 3 injections of a particular spiked sample. However, by running triplicate injections of a complete

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standard additions experiment sequentially (as opposed to running replicates at different times during the run list), the effect of decreasing sensitivity is greatly reduced.

In the case of encapsulated powder formulations, this method has been successfully used to determine the amount of hydrolysate present by selecting only excipients that are insoluble in water. In this way, the entire contents of the capsule can be dispersed in water and the excipients are simply removed by centrifugation. The amount of LKPNM is determined both in the capsule powder and in a known amount of the hydrolysate batch used to make the capsule. For greatest accuracy, the LC−MS/MS experiments used to determine these two standard additions curves must be run at the same time in order to ensure that the slopes are very similar and there is no change in LC−MS response. The two results can then be compared to determine the amount (weight of hydrolysate) of powder in one capsule.

Validation of the LC−MS/MS Method. As the purpose of our investigation was to establish a method for determining the LKPNM content in hydrolysates produced as dietary supplements, a method validation to confirm the suitability and performance of this procedure was considered essential.

Quantification was achieved by standard additions of the synthetic peptide to the hydrolysate prior to solid-phase extraction and LC−MS/MS. The MS/MS experiments monitor the transitions m/z 602.4 (MH$^+$ ion of LKPNM) → 361.3, 212.2, 129.1 which are specific to LKPNM and thus allow its determination in a highly complex mixture. The validation used a Micromass Q-Tof mass spectrometer and hence the entire fragment ion spectrum was recorded in every scan. The method applies equally to recording specific fragment ions (metastable reaction monitoring, MRM) such as would be performed using a triple quadrupole mass spectrometer and would be expected to result in improved signal-to-noise. The parameters investigated include specificity, linearity, accuracy, precision, and reproducibility.

Specificity. An improvement in specificity may be observed by comparing the UV and TIC (MS only) traces (**Figure 1c** and **b**) with the traces observed during the LC−MS/MS acquisitions (**Figure 4a** and **b**). Ultimate specificity is obtained by selecting the transition for m/z 602.4→361.3 from the LC−MS/MS total ion current trace. The transition to the m/z 361.3 daughter ion is a major fragmentation pathway of the MH$^+$ parent ion arising from the LKPNM peptide. Using LC−MS/MS, the broad, diffuse humps observed in **Figure 1c** and **b** are reduced to a single peak attributable to LKPNM, observed with a retention time of 3.0 min (**Figure 4b**).

Comparison of the MS/MS spectrum averaged across this peak for bonito solution **D1a** (**Figure 3b**) with that obtained directly from the standard solution **B3b** (**Figure 3a**) showed an excellent spectral match, which again demonstrates the specificity of LC−MS/MS.

The LC−MS/MS trace of the m/z 602.4→[361.3 + 212.2 + 129.1] (the three most intense fragment ions) for six **D1** solutions showed an average area percentage of 95% for the LKPNM peak, slightly worse than the target specificity which was set at >95%. This is due to the fact that m/z 129 is a common fragment ion distributed over many peptides. However, in the traces of m/z 602.4→[361.3 + 212.2] and m/z 602.4→361.3 the LKPNM peak had average area percentages of 97 and 96%, respectively, demonstrating good specificity. One advantage of recording the entire MS/MS spectrum is that it is possible to further investigate any additional peaks that are present in these chromatograms. In this case, it was observed that the MS/MS
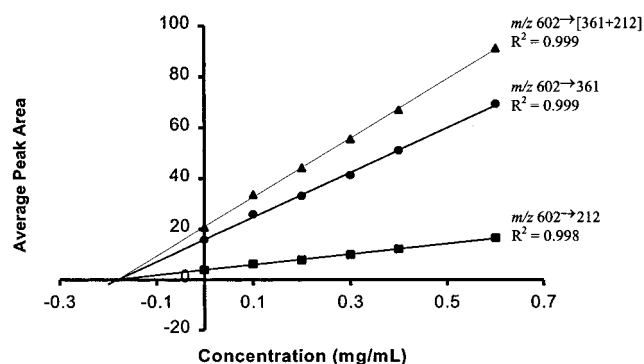

Figure 5. Standard additions of synthetic LKPNM spiked into bonito extract. LC–MS/MS peak area vs spike concentration for m/z 602.4→361.3, m/z 602.4→212.2, and m/z 602.4→[361.3 + 212.2].

spectra of all such peaks were quite distinct from that of LKPNM, again demonstrating the specificity of the method.

Linearity. The results of standard additions of synthetic LKPNM spiked into bonito extract are plotted in **Figure 5**. The graph shows peak areas versus spike concentration for m/z 602.4→361.3, m/z 602.4→212.2, and m/z 602.4→[361.3 + 212.2], each point representing the average of triplicate injections.

Excellent linearity was observed with both m/z 602.4→361.3 and m/z 602.4→[361.3 + 212.2] which had $r^2 = 0.999$, and good linearity was observed for m/z 602.4→212.2 which had $r^2 = 0.998$. However, there was significant scatter in the data points, giving rise to a relative standard deviation ranging from 2% to 10% (average RSD = 7%) and, hence, it is essential that triplicate injections be used. This scatter would be expected to improve if selected reaction monitoring were available.

Accuracy. *Prediction of Zero Additions Intercept.* Not surprisingly, changes were observed in the accuracy of the prediction of the response for zero additions with increasing number of standard additions. Thus, with 5 additions the experimentally determined zero addition response could be predicted within 3%, but for 4 and 3 additions this accuracy decreased to 8% and 14%, respectively. This translated into a prediction of the zero addition concentration (negative intercept at zero peak area) changing by up to 3% or 8% for the m/z 602.4→361.3 and m/z 602.4→[361.3 + 212.2] transitions with 3 or 4 additions as compared to 5 additions. In summary, it is preferable to use 5 additions for greatest accuracy, but 4 additions would be expected to produce acceptable (<5%) accuracy.

Recovery Measurement. To determine recovery, duplicate solutions of three concentrations of LKPNM standard (0.05, 0.15, and 0.3 mg/mL) were analyzed by LC−MS/MS at the same nominal concentrations both before and after passing through SPE. Triplicate injections of each were performed in order to reduce the uncertainty in the measurements.

Taking the average of all results for each solution (duplicate preparation, triplicate injections) gave recoveries of 96.3%, 100.6%, and 103.7% for 0.05, 0.15, and 0.3 mg/mL solutions, respectively, and an overall average of 100.2 ± 3.7%. This clearly illustrates that quantitative recovery of the LKPNM standard is achieved. It should also be noted that the excellent linearity seen above for standard additions to bonito extracts (solutions **D1a**−**D6a**) indicates consistent recoveries over multiple extractions.

Precision. Precision was estimated by five analyses of a single **D1** solution. The result was obtained with relative standard deviations of 3%, 3%, and 2% for m/z 602.4→361.3,

m/z 602.4→212.2, and m/z 602.4→[361.3 + 212.2] transitions, respectively. This was considered an acceptable level of precision in all cases, although it is likely that better precision could be obtained using an instrument capable of selected ion recording.

Reproducibility. Reproducibility was estimated by duplicate injections of five separate **D1** solutions prepared by a second analyst. This resulted in relative standard deviations of 8%, 7%, and 8% for the m/z 602.4→361.3, m/z 602.4→212.2, and m/z 602.4→[361.3 + 212.2] transitions, respectively. When these results are compared to the average values for **D1** determined by the first analyst, they differed by −3%, 4%, and −4% for m/z 602.4→361.3, m/z 602.4→212.2, and m/z 602.4→[361.3 + 212.2] transitions, respectively. Overall, these results demonstrate an acceptable level of reproducibility, although the relative standard deviations were higher than anticipated. In practice, any effect of this scatter is somewhat offset by the procedure which effectively repeats the extraction on a given sample several times, albeit at different spike levels.

All of the above data indicate that standard additions to bonito extract with measurement by LC−MS/MS of either the areas for m/z 602.4→361.3 or m/z 602.4→[361.3 + 212.2] determined using the procedure developed is suitable for the determination of LKPNM content. A typical result is an LKPNM concentration of around 0.2 mg/mL in solution **D1**, which corresponds to about 2 mg LKPNM/g dried bonito peptide hydrolysate. In the case of encapsulated powders, selection of excipients that are water-insoluble and that can be removed by centrifugation allows for the successful use of this method. It would be relatively simple to extend the method to simultaneously quantify other small peptides that are present at sufficiently high abundance. In summary, this method is a straightforward and robust procedure, which, in conjunction with activity measurements, allows stringent measurement of product consistency for an extremely complex mixture produced from the proteolytic digestion of dried bonito muscle.

LITERATURE CITED

(1) Laragh, J. H.; Bear, L.; Brunner, H. R.; Buhler, F. G.; Ealey, J. E.; Vaughan, E. D. Renin, angiotensin and aldosterone system in pathogenesis and management of hypertensive vascular disease. *Am. J. Med.* **1972**, *52*, 633−652.

(2) Gavras, H.; Gavras, I. Bioactive peptides in the treatment of hypertension and heart failure. *Biomed. Health Res.* **1999**, *22*, 41−50.

(3) Cushman, D. W.; Ondetti, M. A.; Gordon, E. M.; Natarajan, S.; Karanewsky, D. S.; Krapcho, J.; Petrillo, E. W., Jr. Rational design and biochemical utility of specific inhibitors of angiotensin-converting enzyme. *J. Cardiovasc. Pharmacol.* **1987**, *10 Supp 7*, S17−30.

(4) Ondetti, M. A.; Rubin, B.; Cushman, D. W. Design of specific inhibitors of angiotensin-converting enzyme: A new class of orally active hypertensive agents. *Science* **1977**, *196*, 441−447.

(5) Roks, A. J. M.; van Geel, P. P.; Pinto, Y. M.; Buikema, H.; Henning, R. H.; de Zeeuw, D.; van Gilst, W. H. Angiotensin-(1-7) is a modulator of the human renin-angiotensin system. *Hypertension* **1999**, *34*, 296−301.

(6) Fujita, H.; Yokoyama, K.; Yoshikawa, M. Classification and antihypertensive activity of angiotensin I-converting enzyme inhibitory peptides derived from food proteins. *J. Food Sci.* **2000**, *65*, 564−569.

(7) Fujita, H.; Yamagami, T.; Ohshima, K. Effects of an ace-inhibitory agent, katsuobushi oligopeptide, in the spontaneously hypertensive rat and in borderline and mildly hypertensive subjects. *Nutr. Res.* **2001**, *21*, 1149−1158.

(8) Kim, S.-K.; Byun, H.-G.; Park, P.-J.; Shahidi, F. Angiotensin I converting enzyme inhibitory peptides purified from bovine skin gelatin hydrolysate. *J. Agric. Food Chem.* **2001**, *49*, 2992−2997.

(9) Suetsuna, K.; Chen, J.-R. Identification of antihypertensive peptides from peptic digest of two microalgae, *Chlorella vulgaris* and *Spirulina platenis*. *Mar. Biotechnol.* **2001**, *3*, 305−309.

(10) Hillaert, S.; Van den Bossche, W. The quantitative determination of several inhibitors of the angiotensin-converting enzyme by CE. *J. Pharm. Biomed. Anal.* **2001**, *25*, 775−783.

(11) Matsufuji, H.; Matsui, T.; Ohshige, S.; Kawasaki, T.; Osajima, K.; Osajima, Y. Antihypertensive effects of angiotensin fragments in SHR. *Biosci. Biotechnol. Biochem.* **1995**, *59*, 1398−1401.

(12) Yokoyama, K.; Chiba, H.; Yoshikawa, M. Peptide inhibitors for angiotensin I-converting enzyme from thermolysin digest of dried bonito. *Biosci. Biotechnol. Biochem.* **1992**, *56*, 1541−1545.

(13) Fujita, H.; Yokoyama, K.; Yoshikawa, M. Antihypertensive effect of thermolysin digest of dried bonito in spontaneously hypertensive rat. *Clin. Exp. Pharmacol. Physiol.* **1995**, *Suppl. 1*, S304−305.

(14) Fujita, H.; Yoshikawa, M. LKPNM: a prodrug-type ACE-inhibitory peptide derived from fish protein. *Immunopharmacology* **1999**, *44*, 123−127.

(15) Wyatt, P. J. Light scattering and the absolute characterization of macromolecules. *Anal. Chim. Acta* **1993**, *272*, 1−40.

(16) Folta-Stogniew, E.; Williams, K. R. Determination of molecular masses of proteins in solution: implementation of an HPLC size exclusion chromatography and laser light scattering service in a core laboratory. *J. Biomol. Tech.* **1999**, *10*, 10−22.

(17) Biemann, K. Contributions of mass spectrometry to peptide and protein structure. *Biomed. Environ. Mass Spectrom.* **1988**, *16*, 99−111.

(18) Roepstorff, P.; Fohlman, J. Proposal for a common nomenclature for sequence ions in mass spectra of peptides. *Biomed. Mass Spectrom.* **1984**, *11*, 601.

(19) Bruins, A. P. Mechanistic aspects of electrospray ionization. *J. Chromatogr.* **1998**, *794*, 345−357.

(20) Hopfgartner, G.; Bean, K.; Henion, J.; Henry, R. Ion spray mass-spectrometric detection for liquid chromatography: a concentration or a mass-flow-sensitive device? *J. Chromatogr.* **1993**, *647*, 51−61.