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Analysis of substance P in rat brain by means of immunoaffinity capture and matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry

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Abstract

Interest in the analysis of low abundance neuropeptides particularly using matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI–TOF–MS) is increasing because these neuropeptides are essential to the mechanism of transportation and the metabolism. This article describes an immunoprecipitation procedure that is suitable for MALDI–MS analysis of substance P (SP), a neuropeptide, in rat brain tissues. Substance P was precipitated from brain tissue extracts by immunoprecipitation with antibodies directed against SP, and are analyzed by MALDI–TOF–MS. Mass spectrometric analysis showed a singly charged $[M+\mathrm{H}]^+$ ion peak that corresponded to the SP molecular mass and was observed with a detection error of 1.6%. The average mass errors between the observed and theoretical molecular mass were within the 0.11 Da range. Capillary zone electrophoresis analysis was subsequently performed, and the effects of the different separation parameters were examined. Beginning with milligram quantities of brain tissue, picomole quantities of SP could be detected using this method.

Keyword: Substance P

1. Introduction

Substance P (SP) is a peptide neurotransmitter that belongs to the tachykinin family of neuropeptides, and was first discovered by Von Euler and Gaddum (Fig. 1) [1]. It is distributed widely in the peripheral and central nervous system, where it acts as a neuromodulator [2] and is released by various stimuli. High levels of SP are located in the regions of the forebrain such as the hypothalamus, septum and the amygdala. It has been implicated in the perception of painful stimuli throughout the body, nociception, smooth muscle control, allergic responses, inflammation, glandular secretion, vomiting reflexes, defensive behavior, changes in the cardio-vascular tone, vasodilation and is also known to contribute to the hippocampel excitability and status epilepticus [3]. Abnormal levels of SP are associated with disorders such as schizophrenia and Huntington's, Parkinson's and Alzheimer's diseases [3–6]. Due to the presence of SP in a variety of tissues along with its participation in various diseases, the quantitative analysis of SP is important and is expected to yield more information concerning SP in the tissue. High-performance liquid chromatography (HPLC) combined with a radio-immunoassay (RIA) is the most sensitive and widely used method for detecting and quantification SP [7]. However, these methods are hampered by many problems. Although HPLC is relatively selective and accurate, the sensitivity is compound dependent, the retention time is not unique and developing the method can be time consuming. In RIA, there is some difficulty in preparing the specific antibodies that discriminates between related peptides. In addition, there is the risk of antibody cross-reactivity with other molecular species and the method does not possess molecular specificity.

Recently, there has been a rapid increase in the use of mass spectrometry (MS) in biological science [8,9]. Biological mass spectrometry is based on the micro-scale immunoaffinity capture of the target antigen followed by mass-specific identification and quantification with a greater

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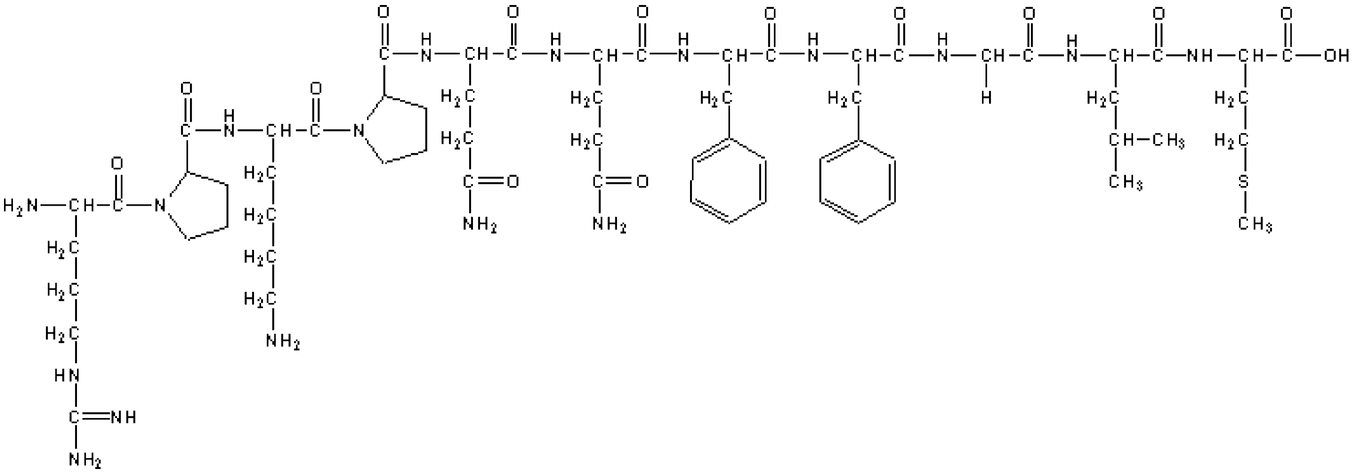

Fig. 1. Structure of substance P.

selectivity, accuracy and efficiency. A mass spectrometric immunoassay (MSIA), which was first developed by Nelson et al., relies on affinity capture (antibody–antigen recognition) of the analytes from the biological matrix prior to MS analysis [10]. Hutchens and Yip introduced affinity based purification techniques combined with matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI–TOF–MS) [11]. The main advantage of this technique is its high specificity of detection of the intrinsic molecular masses of the antigens or fragment ions, as well as it being a rapid and sensitive technique. By detecting the antigen at its specific molecular mass, artifacts due to antibody cross-reactivity in RIA can be overcome.

Matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry has become a valuable and important analytical tool in biological research owing to its ability to analyze low concentrations of peptides and proteins in a complex biological mixture [12,13]. Among electron spray ionization and MALDI–TOF–MS techniques, MALDI is generally applied to the direct analysis of peptides in complex biological samples such as tissues [14] due to its soft ionization source, which prevents decomposition of fragile biomolecules, high sensitivity, easily interpretable mass spectra and relatively low susceptibility to salts and detergents. Muddiman et al. reported the detection and quantify cyclosporin A in the blood using MALDI–TOF–MS [15]. Several reports have described the use of MALDI–TOF–MS for the analysis of neuropeptides [9,16–18].

This paper focuses on a quick method for detecting and determining the SP level in rat brain tissue using MALDI–TOF–MS. The method is based on analyzing the SP level in the rat brain by immunoprecipitation, in which the SP polyclonal antibodies are raised, followed by solid phase extraction. The level of SP is then determined by measuring its molecular mass using MALDI–TOF–MS. The ion signal intensities were used to estimate the concentration of SP using the working curves constructed using external standards. The Influence of the laser on the signal intensities and sample preparation techniques including flight time deviation for accurate mass measurements are discussed. In combination with MALDI–TOF–MS, capillary zone electrophoretic (CZE) analyses were also performed and the results were compared.

2. Experimental

2.1. Reagents

Substance P, α-cyano-4-hydroxycinnamic acid (HCCA) was obtained from Sigma (St. Louis, MO, USA). The mass spectrometry calibration kit was obtained from Sigma–Aldrich Chemic Gmbh (Steinheim, Germany). The substance P goat polyclonal IgG was obtained from Santa Cruz Biotec (Santa Cruz, CA, USA). The protein G Sepharose was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The rat brains were stored at −80 °C before preparing the extracts. All the chemical reagents, the solvents used for sample preparation, buffer preparation and analysis were of analytical reagent grade. The samples were prepared with deionized water obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA)

2.2. Tissue extraction and peptide rich fractions

The frozen brain tissues were quickly weighed (∼423 mg) and suspended in 1 M acetic acid (4 ml) and were boiled for 15 min. After cooling to room temperature the sample was homogenized and centrifuged at 20,000 × *g* for 20 min at 4 °C. The resulting supernatants were saved, while the pellet was again washed with 1 M acetic acid and centrifuged. The pellet was discarded and the two supernatants were combined. The supernatants were lyophilized and redissolved in PBS (pH 8.0). The insoluble materials were removed by centrifugation at 20,000 × *g* for 10 min at 4 °C, and aliquots were stored at −20 °C for further analysis. The total protein concentration in the brain tissue extract was determined using a micro bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL, USA). The peptide rich fraction was prepared from the brain tissue extract using a Sep-Pack C18 cartridge (Waters, Division of Millipore). The cartridge was activated with acetonitrile (ACN) followed by 1% trifluoroacetic acid (TFA). Equal portions of 1% TFA was added to the samples and applied to the cartridge. The cartridge was washed with 1% TFA, and 3 ml ACN:1% TFA (60:40) was used to elute the peptides. The solvent was evaporated, lyophilized to dryness and reconstructed in PBS.

2.3. Immunoprecipitation

A 400 μl portion of the SP sample was incubated with 5 μl of the SP antibody diluted 1/400 (v/v) in PBS overnight at 4 °C. A 20 μl Sepharose protein G beads (washed with buffer H: β-glycerolphosphate 50 mM, 1.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin A, pH 7.3) suspension was added and the sample was incubated for 2 h. The sample was then centrifuged for 10 min at 15,000 × *g*. All the liquids were removed using a flat pipette tip and replaced with 400 μl PBS. The samples were washed three times. After the third wash, the SP bound to the antisera was eluted with 20 μl of 0.5% TFA (v/v), centrifuged for 15 min at 15,000 × *g* and the supernatant was stored in a fresh vial. This elution step was repeated once again using 20 μl of 0.1% TFA (v/v). The supernatant were pooled and stored at −20 °C.

2.4. MALDI–TOF–MS

The mass spectrometry measurements were performed on a HP G2025A system (Hewlett-Packard, Palo Alto, CA, USA) linear type time-of-flight mass spectrometer. The ionization was initiated with a pulsed N_2 laser (3 ns duration) emitting at 337 nm. The pressure in the ion chamber was maintained at between 1×10^{-7} and 4×10^{-7} Torr (1 Torr = 133.322 Pa). The ions generated by the laser pulses were accelerated typically to 28 KeV ($1\,\text{eV} = 1.602 \times 10^{-19}$ J) energy and were ejected into the field free region of the TOF mass spectrometer. The ions were detected by a dual microchannel plate, which was biased to 1900 U. For data acquisition, the detector signal was pre-amplified and digitized using a digitizing oscilloscope (1G samples/s) at a rate of 500 MHz bandwidth (9350AM, LeCory, Chestnut, Ridge, NY, USA). All mass spectra were recorded in a positive linear mode. Unless otherwise stated, every spectrum obtained for each sample is a mean of 40 laser shots with a laser power setting to a high threshold (result and discussion section). No smoothing was performed on the spectra. The mass spectra were processed using the data acquisition package (HP G2025A, TOF ware A 03.00 version 2.1, Pittsburgh, PA, USA). The external calibration was performed as a two-point calibration using two standard peptides [Angiotensin II (Ang II) and ACTH fragment 18-39 (ACTH)] bracketing the desired mass range. The mass measurement accuracy was ±0.05 Da. All the samples were analyzed under constant experimental conditions and a minimum of ten spectra were accumulated on each sample in order to determine the reproducibility of spectra.

The matrix solution was freshly prepared each day by making a 20 mg/ml of HCCA in 50% ACN/0.1% TFA (v/v), which was recrystallized from ethanol, and stored in the dark. A stock solution containing 1 mg/ml of standard SP and the external standards Ang II, ACTH (1 pmol/μl) were prepared in 50% ACN/0.1% TFA (v/v). In order to make an analyte/matrix deposit ($1{:}10^5$), the samples were prepared using the dried-droplet method. One microliter of either the standard SP or the immunoprecipitated sample and a 1 μl mixture of the external standard solution was added to 1 μl of a HCCA solution in an Eppendroff tube. The tube was shaken vigorously on a vortex mixer, and then centrifuged with a microcentrifuge for 1 min. The resulted mixture was immediately deposited into a 10-position gold plated sample probe. The samples were air dried at the ambient temperature using a cover-box for the sample slide in order to avoid differences in the crystallization process, which was followed by acetone redeposition. The sample probe was then loaded into an ion source.

2.5. Capillary electrophoresis

Capillary electrophoresis separations were performed on a Waters (Milford, MA, USA) Quanta 4000 capillary electrophoresis system with a UV 185 nm detector in an untreated fused-silica capillary column (35 cm × 75 μm i.d.) (Polymicro Technologies, Phoenix, AZ, USA). The capillary electrophoretic separation buffer consisted of 100 mM phosphate pH 2.5. All the buffers and sample solutions were filtered through a 0.45 μm filter and then degassed. The system was operated at 6 kV. The electropherograms were recorded on a Waters 746 data module. The capillary was conditioned prior to their first use by rinsing with 0.1 N NaOH for 30 min and then with water for 20 min. In order to obtain reproducible results, at the start of each run, the capillary was washed with 50% ACN, water, 0.1 N NaOH followed by a separation buffer. The hydrodynamic injections were performed by raising the sample reservoir 10 cm higher than the collection reservoir.

3. Results and discussion

3.1. Immunoaffinity

The main purpose of this study was to develop a MALDI–TOF–MS method based on an immunoprecipitation procedure for analyzing biomolecules in complex biological mixtures. Due to the complex biological fluid mixtures, it was necessary to isolate low concentrations of the analyte of interest from the biological fluid prior to the MALDI–MS analysis. Otherwise, large amounts of other analytes might suppress the analyte ion signals of interest. Immunoaffinity capture of the analyte from the biological mixture is a method that pre-concentrates the analyte and provides the most selective form of analyte isolation [10]. Mass spectrometric detection of the immunoaffinity captured analyte is specific because the analyte signals are observed at characteristics *m*/*z* values. In this study, the SP in the rat brain tissue samples were isolated by an immunoprecipitation method in which the samples were incubated with the SP polyclonal antibodies immobilized on Protein G beads. High affinity polycolonal SP antibodies were used to specifically purify and enrich the SP. During incubation, the SP was captured on the antibody, and after a series of washes the immunoprecipitated samples were subjected to a MALDI sample preparation step. Mass spectrometric analysis of the immunoprecipitated sample confirmed the presence of SP at their specific *m*/*z* 1348.8. Therefore, this technique offers the unique possibility of quantifying SP. Affinity purification of the antibodies directed toward small peptides was hampered by reduced antibody affinity for a small antigen bound to a solid support.

3.2. Optimization of MALDI–TOF–MS parameters

The presence of basic residues in the peptides greatly enhanced the efficiency of protonation, and their absence resulted in moderate ion signals [19]. Substance P has an arginine at the N-terminal and has better ionization efficiencies with a higher signal intensity than peptides without basic residues, and can suppress the signals from another analytes. In this study, the positive ion mass spectra were recorded because the negative ion signals were typically less intense for the same laser fluence and spot. α-Cyano-4-hydroxycinnamic acid (HCCA) is a commonly used matrix for peptide and protein analysis. As HCCA and the biomolecule combination yield a high ion abundances, even for contaminated samples, HCCA was chosen as the matrix for this study. In addition, HCCA gives good signal intensity for a broad range of analyte molecules.

The laser power was found to play a more important role, affecting the peak intensity in UV MALDI. The ionization efficiencies of the peptides are independent of the levels of the laser power. In this study, the laser energy was the first step taken to investigate its effect. In order to determine the laser power (high threshold) at which small amounts of SP ions can be detected, MS analyses were performed with different laser power ranging from the minimum intensity necessary to evoke a signal to the maximum intensity, which was very close to the detector saturation. Through a series of experiments using SP concentrations ranging from 50 to 250 fmol/μl, the laser intensity was set to an initial minimum energy, which was increased until an acceptable data signal was acquired. Among the different levels of laser power tested, the laser energy used, which showed an acceptable SP ion signal was found to be 5.6 μJ (data not shown). The sensitivity was tested and is expressed in terms of detection limit by carrying the experiments with a standard SP at the above mentioned concentrations. The spectra recorded from the samples with a SP concentration >100 fmol/μl showed SP ion signals. At the high concentrations tested, the detector did not become saturated. At low concentrations (50 fmol/μl), the signal intensity was too low and it was more difficult to identify a signal.

In order to maximize the signal quality and reproducibility, the laser intensity, the sample preparation methods, the crystallization process and the *m*/*z* ranges were carefully adjusted. When the samples are analyzed by MALDI–MS, ionization is a very important step and in order to reduce variability from run-to-run/spot-to-spot, it is necessary to make homogeneous crystals on a sample probe. Different sample preparation methods were attempted in order to achieve this, and the best result was obtained by a dried-droplet technique. Even though it showed lower signal intensities than other methods (such as the thin layer method), it showed a better reproducibility of the peak intensity from different shots. In addition, the acetone redeposition technique was used to improve the sample homogeneity and buffer tolerance. One drop of acetone was deposited on to the dry sample. As the acetone evaporates, the sample recrystallized to form a more homogenous spot. In order to improve the shot-to-shot reproducibility and minimize the effects of variations in sample preparation, 10 samples were prepared for each HCCA–analyte combination. Every mass spectrum was recorded as the sum of 40 consecutive spectra collected from a selected spot on the probe tip, which gives rise to identical results. In each sample, the average peak intensity values were taken for analysis. Some of the experiments showed adduct peaks and removal of which was unnecessary for the analysis.

3.3. Calibration curves

Using the above procedure, the calibration plots were generated from a high quality spectrum to determine the amount of SP in the brain tissues. Since the sample preparation and laser parameters were kept unchanged between each run and all samples were analyzed under constant experimental conditions, it is reasonable to assume that the generated species were similar for the different homologues. Only the amplitudes (peak intensities) of the ion signals for each species ($[SP + H]^+$, $[Ang\ II + H]^+$ and $[ACTH + H]^+$) were repotted to generate the calibration plot and to determine the amount of SP. The signal detected by MALDI–TOF–MS corresponds to the amounts of the peptides that were excited by the laser.

A mass spectrometry system was calibrated using Ang II and ACTH as the calibration standards. In this study, Ang

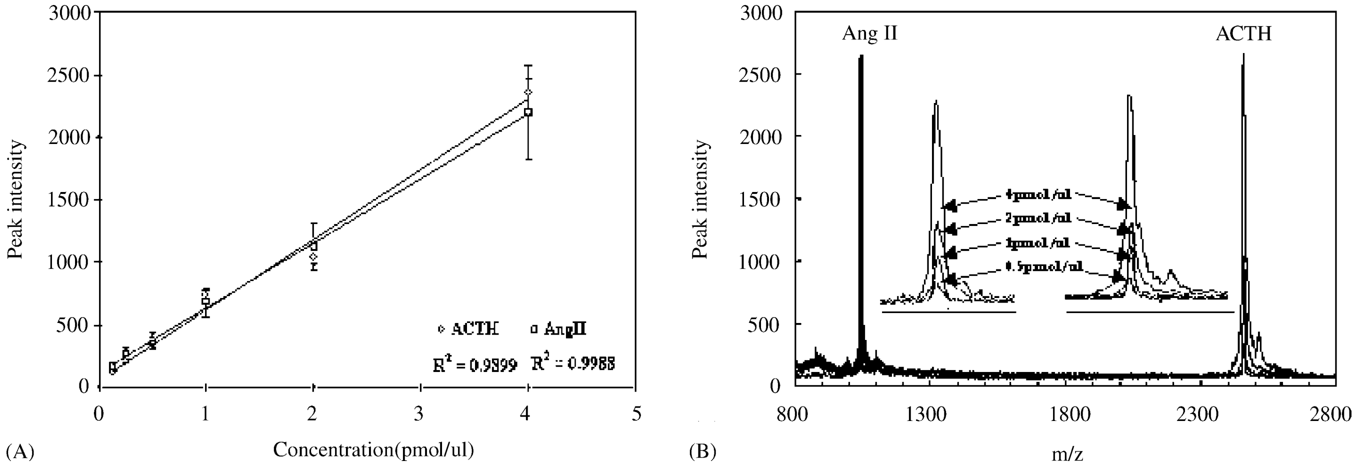

Fig. 2. (A) External standard (Angiotensin II, ACTH 18-39) working curve. Angiotensin II and ACTH intensity vs. its concentration. Each point represents plot of the ion peak intensity for a function of solution concentration. (B) Representative spectra of data used to generate working curve. The inset contains an expansions of peaks corresponds to the $[M + \mathrm{H}]^+$ ion region (only four data concentrations were shown).

II and ACTH were used as external standards to normalize the $[\mathrm{SP} + \mathrm{H}]^+$ ion peak intensities. For this, a working curve was first generated for these external standards. Angiotensin II and ACTH were premixed with HCCA and crystallized using a dried-droplet method. The laser power was regulated so that the peak intensity of the spectrum would never exceed a fixed counts. Singly charged Ang II and ACTH ion peak intensities were used to generate the curve. Fig. 2A and B shows the working curve consisting of samples containing 0.125–20 pmol/μl each of Ang II and ACTH and the representative spectra of the data (only four concentration points were showed) used to for the working curve, respectively. Regression analysis resulted in the following linear equations $y = 516.4x + 121.73$ (Ang II), $y = 560.17x + 79.32$ (ACTH) with correlation coefficients of 0.9988 (Ang II), 0.9899 (ACTH), respectively, where x is the Ang II and ACTH concentrations and y the analyte peak intensity. The standard error of mean bars was used to report replicate measurements of the MALDI mass spectra.

The SP working curves were constructed for a standard SP concentration series by measuring the peak height mass spectral response as a function of the SP concentration. Angiotensin II, ACTH (1 pmol/μl each) and different SP concentrations were premixed with HCCA. The crystallized mixture was deposited onto the sample probe and the mass spectra were recorded using constant conditions. The mass spectral peak intensity of the $[\mathrm{SP} + \mathrm{H}]^+$ ion for the SP concentrations ranging from 0.5 to 20 pmol/μl was normalized to the ($[\mathrm{Ang\ II} + \mathrm{H}]^+ + [\mathrm{ACTH} + \mathrm{H}]^+$) peaks, and the normalized response was plotted as a function of the SP concentration in Fig. 3A. The Ang II and ACTH ion peaks were adjusted to a fixed spectral peak intensity at a laser energy of 5.6 μJ (same laser energy used to acquire the external

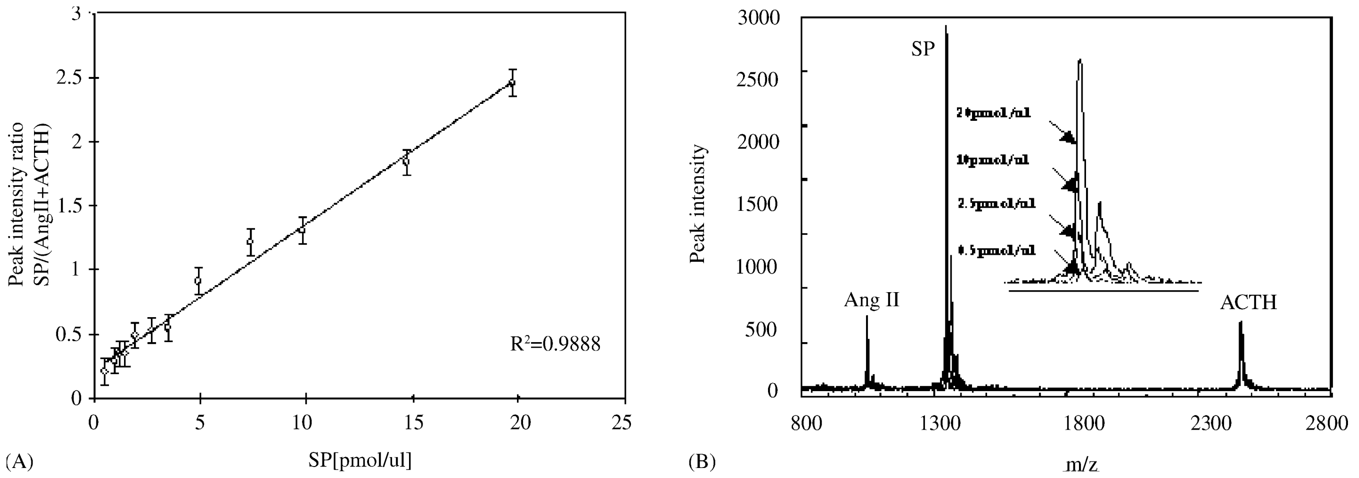

Fig. 3. (A) SP working curve. $[\mathrm{SP} + \mathrm{H}]^+$ ion peak intensity (normalized to Angiotensin II and ACTH ion peak intensities) vs. SP concentration. Each point represents plot of the $[\mathrm{SP} + \mathrm{H}]^+$ peak intensity for a function of solution concentration. (B) Representative spectra of data used to generate working curve. The inset contains an expansion of peaks corresponds to the $[\mathrm{SP} + \mathrm{H}]^+$ ion region (only four data concentrations were shown).

standard, standard SP and immunoprecipitated samples) which corresponded to its concentration (1 pmol/μl) and was determined by its working curve (Fig. 2A). The method with constant condition to acquire the entire spectrum makes the quantification. The SP working curve response (Fig. 3A) followed a linear relationship ($y = 0.1144x + 0.2145$) over the concentration range used with a correlation coefficient of 0.9888 and a 0.74% R.S.D., where x is the SP concentrations and y the analyte peak intensity. The standard error of mean was used to report 10 replicate measurements of the MALDI mass spectra. The difference in the system sensitivity for the SP and external standard confirmed that the external standard was correct. Fig. 3B shows representative spectra of the data used to make the calibration curve (only four different SP concentrations were showed). For all the spectra, singly charged $[\mathrm{SP} + \mathrm{H}]^+$, $[\mathrm{Ang\ II} + \mathrm{H}]^+$ and $[\mathrm{ACTH} + \mathrm{H}]^+$ ion peaks were examined. In order to determine the precision of the method whilst constructing the working curves, each experiment was replicated 10 times and the mass spectral data were acquired using identical experimental conditions. The problem of suppressing the less concentrated SP signal in the brain tissue extracts by the more concentrated components (external standard), which was the main problem in quantification, was overcome by selecting low concentrations of the external standards [20].

3.4. *Analysis of rat brain tissue samples*

The immunoprecipitated samples from the two brain tissues (A and B) were subjected to MS analysis, and as mentioned earlier, the mass spectrum was acquired at fixed MALDI conditions. Fig. 4 shows the MALDI–TOF mass spectrum obtained from the brain samples of two individuals after immunoprecipitation. A singly charged $[M + \mathrm{H}]^+$ ion peak corresponding to the SP molecular mass was observed. The SP concentrations were determined in the brain tissue extracts from two individuals and are summarized in Table 1. In order to determine the reproducibility of the method, the immunoprecipitation reactions were performed twice and subjected to MS analysis. The error in reproducibility was <5%. The accuracy and precision were represented as the error (%) and R.S.D. (%), respectively. Intra-day run accuracy and precision were determined to be 7% error and 2.04% R.S.D. Inter-day run accuracy and precision were 11.5% error and 1.05% R.S.D. From 10 consecutive mass spectra, the average mass of two samples A and B were 1348.87 and 1348.91, respectively. The mass errors between that observed and the theoretical molecular mass were in the 51–81 ppm range.

Table 1
MALDI–TOF–MS and CZE quantification of SP in the rat brain

Immunoprecipitated samples	Substance P (pmol/μg protein)	
	MALDI–TOF–MS ($n = 10$)	CZE ($n = 3$)
Sample A	0.12 ± 0.06	0.23 ± 0.04
Sample B	0.36 ± 0.05	0.38 ± 0.02

Protein concentration (μg/μl): Sample A = 2.495; Sample B = 2.201.

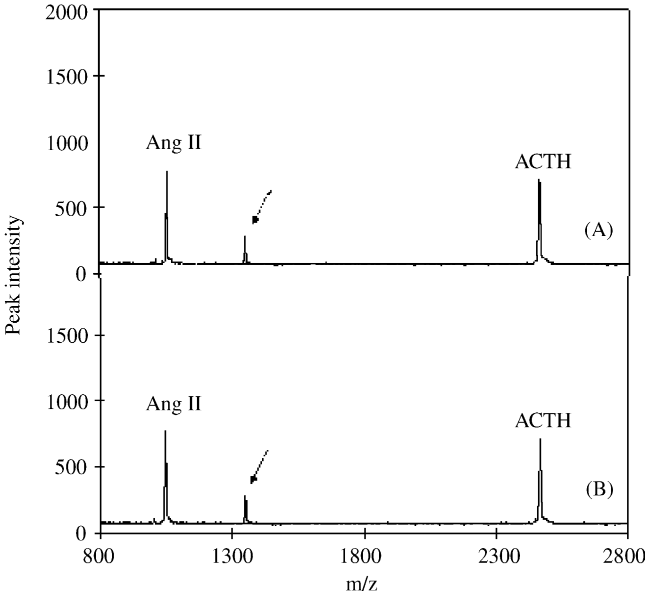

Fig. 4. MALDI–TOF mass spectra of SP immunoprecipitated samples from two individuals (A and B). The signal marked with arrow corresponds to SP molecular mass.

The ability to accurately measure the flight time of the ions is a requirement for accurate TOF mass measurements. These effects were minimized using proper and careful sample preparation techniques. Whilst accumulating the mass spectrum of the immunoprecipitated samples, the flight times were measured and the deviations in the flight time were calculated. Fig. 5A and B shows the flight time deviation from the average flight time for two individual samples (A and B). The average deviation in time was approximately 300 ppm.

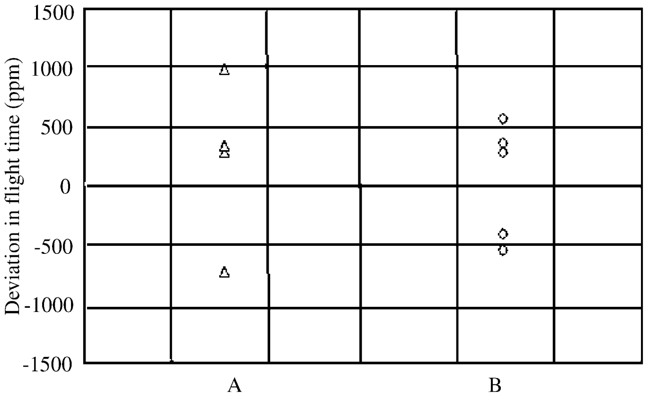

Fig. 5. Plot of variation in flight time (ppm) of $[\mathrm{SP} + \mathrm{H}]^+$ ions from two individual brain samples (A and B).

3.5. Capillary electrophoresis

In addition to MALDI–TOF–MS analysis, a CZE method coupled with UV detection was been developed [21]. The detailed method is discussed in [21], which involves investigating the optimum conditions to separate and analyze SP. Briefly, the CZE separation parameters such as the buffer type, pH, the concentration and applied voltages were the steps used to determine the optimum electrophoretic conditions. The optimal conditions are as follows: 100 mM phosphate buffer at pH 2.5, 35 cm × 75 μm i.d. untreated fused-silica capillary column, 6 kV applied voltage and 185 nm wavelength UV detection. The immunoprecipitated samples were spiked with a known amount of standard SP and subjected to CZE at the optimum conditions. Quantitative analysis was performed using a calibration curve (normalized peak area versus concentration), and a good linear relationship was obtained over the whole concentration range. The correlation coefficients were >0.96. The quantified levels of SP are summarized in Table 1. The data shows the comparative results for the SP concentrations in the in brain tissue extracts obtained from MADLI–TOF–MS and CZE. Comparative quantitative analysis showed good agreement with the results obtained using MALDI–MS and CZE.

4. Conclusions

This study showed that a combination of immunoprecipitation and mass spectrometry provides a specific approach for measuring the SP. pmol range sensitivities were observed using the fixed MALDI–MS parameters, and no special sample preparation methods or sample deposition techniques were required. Because of the high dependence of the absolute ion signal yield on the laser irradiance and the sample preparation, SP was prepared with an external reference standard, and the signals of which were used to normalize the SP signal. The results show a linear relationship between the molecular ion signals and the SP concentrations in solution using MALDI–TOF–MS. Mass spectrometric analysis of the immunoprecipitated sample showed a singly charged ion peak that corresponded to the SP molecular mass. In addition, the component of the immunoprecipitates were detected and analyzed by CZE. Both techniques could measure picomole amounts of SP beginning with milligram amounts of brain tissue. The method is highly sensitive and can be applied to other bioactive peptides in a complex biological mixture.

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