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Identification and Quantification of Neuropeptides in Brain Tissue by Capillary Liquid Chromatography Coupled Off-Line to MALDI-TOF and MALDI-TOF/TOF-MS

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Capillary liquid chromatography (CLC) coupled off-line with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and TOF/TOF-MS were explored for identification and quantification of neuropeptides in microwave-fixed rat brain tissue. Sample was separated by gradient elution on 50-μm-inner diameter reversed-phase columns at 180 nL/min. Effluent was mixed with matrix solution and transferred to a MALDI target plate by pulsed electric field deposition, yielding sample spots with 200−300-μm diameter. Mass detection limits as low as 2 amol, corresponding to 1 pM concentration, were achieved for neuropeptides. CLC−MALDI-TOF-MS analysis of microwave-fixed rat striatum tissue yielded detection of over 400 distinctive peaks. CLC−MALDI-TOF/TOF-MS allowed identification of 10 peptides including 3 novel peptides. Quantification was evaluated using substance P as analyte and $^{15}N_3$-labeled substance P as an internal standard. Quantification of substance P revealed ~6.8-fold higher levels than previously reported in the rat striatum. This increase is attributed to use of microwave fixation, which prevented degradation of the peptide, aggressive extraction procedures, and accounting for oxidation of substance P in the analysis. These results demonstrate that CLC−MALDI-TOF-MS is a versatile tool for neuropeptide analysis in brain tissue by allowing for detection, identification, and quantification.

Neuropeptides constitute a large family of bioactive compounds that participate in transmission and modulation of signals in the central nervous system (CNS). Analysis of neuropeptides is complicated by their large number (hundreds of active neuropeptides are known) and their low concentrations (~5−100 pM) in CNS tissues and fluids. Developing knowledge of peptide functions is limited by a paucity of techniques with sufficient sensitivity and selectivity to identify and quantify neuropeptides in complex CNS samples. In this work, we explore the use of capillary liquid chromatography−matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (CLC−MALDI-TOF-MS) and CLC−MALDI-TOF/TOF-MS for neuropeptide detection, identification, and quantification in mammalian brain tissue.

Peptidomic methods are rapidly becoming an important route to study neuropeptides in CNS. For mammalian tissues, the most common approach is CLC−electrospray ionization (ESI)-tandem mass spectrometry (MS/MS).[1−5] This method has allowed detection and identification of impressive numbers of peptides; however, it is unlikely that all the neuropeptides in tissue are actually measured by this approach because many known peptides are not detected in CNS samples.[5] MALDI is a complementary ionization technique to ESI; therefore, the combination of the two is expected to improve the yield of peptides that may be detected over use of either method alone.[6−9] Indeed, MALDI-MS analysis has proven powerful for studies of inverterbrate systems where specific neurons can be isolated and directly analyzed.[10] Despite its potential, the use of MALDI-MS for neuropeptide analysis in mammalian tissue has been limited to direct profiling of tissue by MALDI or atmospheric pressure-MALDI-MS.[11−14] While pow-

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erful, these methods are more suited for characterization of spatial distributions of peptides rather than complete coverage of the peptidome. It is expected that the use of LC to separate peptide mixtures prior to MS analysis will improve the coverage of the peptidome by reducing ion suppression[15] and resolving isobaric species.[16,17]

Off-line coupling of CLC to MALDI-MS has been applied to the analysis of neuronal tissue homogenates of invertebrates.[18,19] In these approaches, LC fractions were collected and then deposited by dried-droplet deposition[19] or contact capillary transfer.[18] This work demonstrated analysis of femtomole quantities and identification of neuropeptides was achieved using postsource decay (PSD) spectra.[18]

We build upon this prior work by using CLC coupled off-line to MALDI-TOF-MS to analyze mammalian tissue. High sensitivity is critical for analysis of mammalian neuronal tissue because peptides tend to be present in just a fraction of the neurons removed from a sample, resulting in low overall concentration. For this reason, CLC with on-line sample preconcentration was used. In addition, samples were transferred to the MALDI plate by pulsed electric field deposition ("pull-down" deposition),[20] which generates small sample spots and 100-fold improvement in sensitivity over dried-droplet methods.[21] These factors allowed achievement of low-picomolar concentration detection limits corresponding to low-attomole quantities injected onto the column. Tandem MS by TOF/TOF was explored to generate detailed structural information for peptide identification. Quantification of substance P (SP) in tissue by using stable-isotope labeled internal standards[22−24] was also demonstrated. Finally, we present the important role of sample preparation in achieving accurate quantification of neuropeptides in brain tissue. Thus, CLC−MALDI-TOF-MS is demonstrated as a versatile technique for detection, identification, and quantification of neuropeptides in mammalian brain tissue.

EXPERIMENTAL SECTION

Materials. All solvents and chemicals used were of analytical grade. Organic solvents and water were obtained from Burdick & Jackson (Muskegon, MI). α-Cyano-4-hydroxycinnamic acid (CHCA), sodium dodecyl sulfate (SDS), acetic acid, ascorbic acid, trifluroacetic acid (TFA), and peptide standards were purchased from Sigma (St. Louis, MO). Potassium chloride was obtained from ICN Biomedicals Inc (Irvine, CA). [$^{15}N_3$]SP was synthesized by Protein Structure Facility at the University of Michigan (Ann Arbor, MI). Hydrogen peroxide was obtained from Fisher Scientific (Chicago, IL).

Tissue Sample Preparation and Peptide Extraction. For microwave-fixed tissue, male Long Evans rats, 4−8 weeks of age (Charles River Laboratories, Raleigh, NC), were sacrificed by focused microwave irradiation (~3.5 kW applied power for 1.5 s) using a Muromachi Microwave Fixation System model TMW (Stoelting Co., Wood Dale, IL). For nonfixed tissue, rats were sacrificed by decapitation followed by rapid (90 s) removal of the brain from the skull. Striatum samples were removed whole by free-hand dissection (prepared with a Rat Brain Matrix, MyNeurolab.com) and stored at −80 °C until peptide extraction.[25] All animal procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health. The animal facility was accredited by the American Association for Accreditation of Laboratory Animal Care.

Striatum tissue (20−40 mg) was placed into cold (4 °C) homogenization solution (0.25 mg/μL) containing 145 mM KCl, 8 mM SDS, and 0.25% acetic acid. The tissue/homogenization solution was set on ice for 1 h and sonicated every 10 min for 5 s using a sonic dismembrator (Fisher Scientific) on power setting 3 followed by 30 s of vortexing. The homogenate was centrifuged at 20000*g* for 30 min at 4 °C. The supernatant containing both proteins and neuropeptides was removed from the resulting tissue pellet and filtered using a Microcon YM-10 centrifugal filter (Millipore) fitted with a 30-kDa membrane to separate proteins from peptides [3]. The filter device was then placed into a centrifuge and spun at 14000*g* for 45 min. The peptide-rich filtrate was frozen in 15-μL aliquots by liquid N_2 and stored at −80°C until analysis. A portion of the homogenization solution without the added tissue was retained and used as control sample for spotting onto the MALDI plate.

Methods. *Solutions for Sample Preparation.* CHCA was recrystallized in ethanol/water (70:30, v/v) and dissolved in acetonitrile/0.1% TFA (50:50, v/v) as a saturated solution. The peptides were made up as 1 mM stock solutions in water, frozen in aliquots, and further diluted in 0.1% TFA before use.

Sample Preparation Using Ziptip. A C_{18} Ziptip (Millipore, Billerica, MA) was initially wetted by aspirating with acetonitrile/water (50:50, v/v) twice followed by rinsing with 0.1% TFA solution twice (10 μL each rinse) as instructed by the manufacturer. The 15-μL brain tissue samples were aspirated into the tip and dispensed out 7−10 times to bind the peptides to the stationary phase in the Ziptip. The stationary phase was washed by aspirating and dispensing to waste 10 μL of 0.1% TFA three times. Peptides were eluted by aspirating and dispensing 5 μL of acetonitrile/0.1% TFA (50:50, v/v) through several cycles into a sample tube and then mixed 1:1 (v/v) with CHCA solution in acetonitrile/0.1% TFA (50:50, v/v) before deposition onto a MALDI plate.

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Quantification of SP from Striatum Tissue. The 15-μL striatum sample was spiked with 2 μL of 500 nM synthetic [$^{15}N_3$]SP and then 5 μL of hydrogen peroxide to oxidize the SP. CLC separation was performed using a 1-h gradient, and collected spots were analyzed by MALDI-TOF-MS as described in the Instrumentation section. Spectra acquisition and processing were performed using Masslynx software (Micromass, Milford, MA). Over 200 shots were summed to generate spectra for quantification. The spectra were smoothed and baseline subtracted. The relative intensity of oxidized SP (SP (O)) to [$^{15}N_3$]SP (O) was measured as the summed peak area of the first three monoisotopic peaks of SP (O) divided by the summed peak area of the first three monoisotopic peaks of [$^{15}N_3$]SP (O). With this ratio, the SP concentration in the sample was calculated from a standard curve. The standard curve was generated using 5 μL of stock solutions of 1 mM commercial, pure SP, and 1 mM synthesized [$^{15}N_3$]SP oxidized by adding equal volumes of hydrogen peroxide. Standard solutions were prepared from the two stock solutions that contained 2.5 μM [$^{15}N_3$]SP and 0.15−5 μM SP. Each solution was mixed with an equal volume of CHCA and deposited via the dried-droplet method. The spots were analyzed by MALDI-TOF-MS as described above. A standard curve was constructed by plotting the relative intensity of SP (O) to [$^{15}N_3$]SP (O) as a function of the relative concentration of SP to [$^{15}N_3$]SP.

Instrumentation. *CLC.* CLC columns were packed in-house according to a previously described method.[26] Briefly, 13-cm lengths of fused-silica capillary (Polymicro Technologies, Phoenix, AZ) with 50-μm i.d. were slurry-packed with 5-μm, 100-Å C_{18} stationary phase (Alltech, Deerfield, IL) at pressure of 3500 psi. Only 8 cm of the capillary was packed. Each packed column was then flushed and conditioned using acetonitrile/0.1% TFA (50:50, v/v) with pressure above 1500 psi for 30 min.

Mobile-phase flow was controlled using a MicroPro HPLC pump (Eldex, Napa, CA), and the mixing of the two mobile phases was performed with a Y-connector (Upchurch Scientific, Oak Harbor, WA) (Figure 1a). The flow was split (20-μm i.d., 360-μm o.d., 150 cm long) via a stainless steel T connector (Upchurch Scientific) before entering the six-port electrically actuated injection valve (Valco Instruments Co. Inc., Houston, TX). Mobile phase A consisted of water with 0.1% TFA (v/v) while mobile phase B of acetonitrile with 0.1% TFA (v/v). Sample injection volume was controlled by injection time. In the preconcentration experiment, peptide standards were injected for 3 s and 5 or 30 min, and a gradient from 5 to 50% B in 10 min was used for separation. For tissue samples, each sample was injected for 30 min, desalted with 5% B for 20 min, and then separated with a 1-h gradient: from 5 to 35% B in 20 min, fixed at 35% B for 10 min, from 35 to 50% B in 5 min, and then fixed at 50% for 10 min. Control sample was injected for 30 min, desalted for 20 min, and then separated with the same gradient as used for peptide standards. The flow rate over the CLC column after splitting was measured from injection of ascorbic acid during isocratic elution and detected by a UV detector (Spectra 100, Spectra-Physics, Mountain View, CA) at 214 nm.

CLC−MALDI Interface. Matrix was delivered by a syringe pump (Harvard Apparatus, So. Natick, MA) at 25 nL/min and mixed postcolumn with CLC effluent via a micro Y connector (Valco Instrument, Houston, TX) that was mounted on an XZ translation stage (Newport, Irvine, CA) (Figure 1). A capillary (50-μm i.d., 180-μm o.d., 1.5 cm long) in the center port of the Y connector served as an outlet. The end of the outlet capillary was 2−3 mm above a MALDI target plate that was mounted on a motorized XY stage. The motorized stage for automatic fractionation has been described elsewhere.[21] Pulsed electric field deposition was used to form sample spots.[20] In brief, a negative potential was applied to the capillary, polarizing the droplet and pulling it down to the target plate (Figure 1b). Syringe needle and CLC column were grounded; −2 kV was applied for 300 ms every 5 s to the target plate through a high-voltage power supply (Spellman

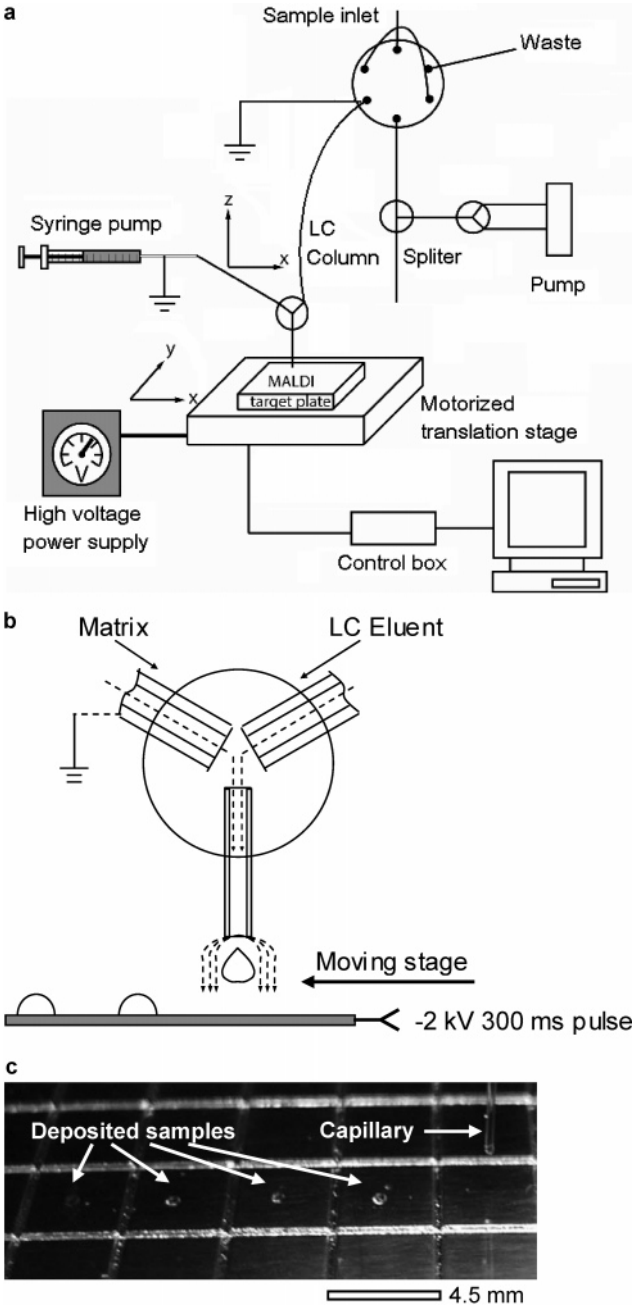

Figure 1. Diagram of the CLC−MALDI system (a); diagram of postcolumn matrix addition and pulsed electric field deposition (b); small sample spots (200−300 μm) obtained on a MALDI target plate by pulsed electric field deposition (c). Operation of components is described in the text.

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CZE 1000R, Hauppauge, NY) to achieve multiple deposition every 5 s. The dwell time for each spot was 15 s for peptide standards, 40 s for tissue samples analyzed by MALDI-TOF-MS analysis, and 2 min for MALDI-TOF/TOF-MS analysis.

Mass Spectrometry. MALDI-TOF mass spectra were acquired with a MALDI TOF Spec 2E mass spectrometer (Micromass) equipped with a delayed extraction source and 337-nm pulsed (4 ns) N_2 laser operated in reflectron mode. All mass spectra were obtained in positive ion mode with 20-kV source voltage, 2.2-kV extraction pulse voltage, 19.98-kV extraction voltage, 16-kV focus voltage, and suppression mass of 500 m/z with scan extended to 5000 m/z. The size of the laser spot was 150 × 250 μm. Mass spectral data were generated by summing 10−50 scans (each scan corresponds to 5 laser shots) into a single spectrum in Masslynx software (Micromass). External calibration was performed on spectra of tissue samples using five peptide standards, angiotensin II, neurotensin, adrenocorticotropic hormone fragment 18−39 (ACTH 18−39), insulin chain B oxidized, and ACTH 1−39. An in-house Labview program was used to convert the data into a matrix, subsequently rendered with MATLAB (The MathWorks, Inc., Natick, MA) into contour and three-dimensional (3D) surface mass chromatograms. Peak lists were generated from each spectrum by Masslynx and combined with an in-house-made PEAL program.

Tandem mass spectra were acquired on an Applied Biosystems 4700 Proteomics analyzer (TOF/TOF) (Foster City, CA). MS spectra were acquired in reflector positive ion mode with the source voltage set at 20 kV and the grid voltage at 13.8 kV. Peptide masses were acquired for the range from 800 to 4000 m/z, with a focus mass of 1800 m/z. MS spectra were summed from 1200 laser shots from an Nd:YAG laser operating at 355 nm and 200 Hz. An automated plate calibration was performed using five peptide standards (masses 900−2400 m/z) in six calibration wells at the corners and (vertical) middle left and right sides of the plate. This plate calibration was used to update the instrument default mass calibration, which was applied to all MS and MS/MS spectra. MS/MS spectra were acquired in MS/MS 1 kV positive mode, with the source voltage set at 8.0 kV and the grid voltage set at 6.8 kV. Spectra were manually acquired for 8000 laser shots or fewer if the peptide fragment signal-to-noise ratio (S/N) was no longer increasing. Fragmentation of the peptides was induced by the use of atmosphere as a collision gas with a pressure of $\sim 6 \times 10^{-7}$ Torr and a collision energy of 1 kV.

Peptide Sequencing and Protein Precursor Identification. All MS/MS spectra were analyzed as SEQUEST DTA files using the on-line version of Mascot (Matrix Science, http://www.matrixscience.com) with the Swiss Prot protein database for *Rattus norvegicus* and the MS/MS Ion Search algorithm with the following parameters: peptide tolerance ±2.0, MS/MS tolerance ±1.0. Sequences were confirmed by Mascot if the score indicated significant homology.

RESULTS AND DISCUSSION

Characterization of CLC Columns and Preconcentration. The CLC system was initially characterized using a standard mixture of Arg-vasopressin, bradykinin, angiotensin II, neurotensin, and SP separated with a 10-min gradient elution at 180 nL/min and UV detection. Reproducibility of the retention times for the five peptides was 0.3−1.3% ($n = 3$) using the same column. Among different columns, the reproducibility of retention times was 0.4−1.3% ($n = 3$). A simple approach to achieving good concentration limit of detection (LOD) is to preconcentrate analytes by injecting samples dissolved in a weak mobile phase. To determine the maximum volume that could be injected, 20 nL to 10 μL of aqueous sample containing the peptides at 100 nM was injected and the peak areas measured. A linear relationship between the peak area and injection volume up to 5.4 μL (30-min injection) was obtained, indicating a 5.4-μL sample (86 column volumes) loading without sample loss. The mass loading range of the column was measured by injecting a fixed volume (360 nL) while increasing the analyte concentration and recording peak areas. A linear increase in peak area was obtained for up to 3.6 pmol, indicating the maximal mass that could be loaded onto such a column.

Characterization of Deposition Device. As shown in Figure 1b, matrix was mixed postcolumn with CLC effluent through a low dead-volume Y-connector and then deposited onto the MALDI target plate. The use of a small o.d. outlet capillary (180 μm) and application of voltage pulses every 5 s minimized sample adherence to the end of the outlet capillary, thus preventing cross-contamination between adjacent spots. The low postcolumn volume after mixing (~150 nL) helped retain separation quality. The peak width for each peptide (from UV detection) before mixing with matrix was ~10 s. When the same mixture was analyzed by CLC−MALDI-TOF-MS with a 15-s dwell time for each spot, the peptides were detected in one or two spots, indicating inconsequential band broadening for this system. (Even with no postcolumn band broadening, a 10-s-wide peak may be detected in two 15-s spots due to peak splitting into adjacent spots.) In principle, spots could be taken more frequently to avoid broadening associated with the fraction collection; however, this would split the sample into small fractions thereby decreasing sensitivity. In addition, it would require many more spots to be analyzed per chromatogram.

With pulsed electric field deposition at 5-s intervals and 205 nL/min flow rate (matrix and CLC effluent mixture), sample spots were ~200−300 μm (Figure 1c); which is much smaller than the 2-mm spots obtained by dried-droplet deposition. Due to the small laser spot size (150 × 250 μm) used in the MALDI-TOF mass spectrometer, a greater portion of small sample spots can undergo ionization than larger samples, thus achieving consistently high sensitivity. This strategy has been used in several ways to achieve high-sensitivity MALDI analysis.[21,27,28]

Detection Limits. The good sensitivity of the method is illustrated by the mass chromatograms for a mixture of five peptides at 100 pM each shown in Figure 2. For this sample, 0.9 μL was injected corresponding to 90 amol of each peptide loaded onto the column. All peptides were baseline separated, eluted in no more than two spots, and detected with S/N > 10. LODs were determined by calculating the concentration or mass injected onto the column that would give S/N of 3 by assuming a linear relationship between S/N and concentration. To minimize error from this assumption, the concentration of sample used was

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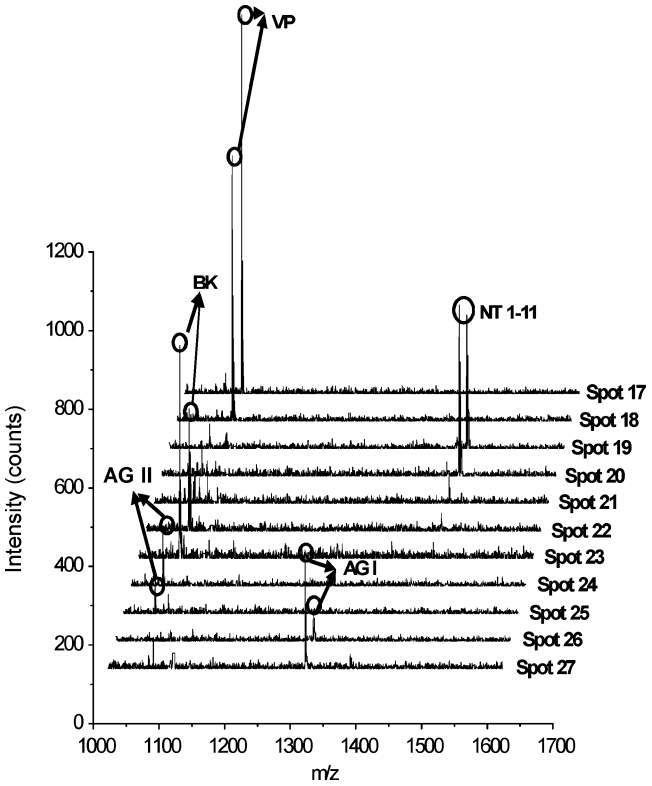

Figure 2. Mass spectra acquired at different times in CLC−MALDI-TOF-MS for separation of 100 pM each of vasopressin (VP), bradykinin (BK), angiotensin II (AG II), AG I, and neurotensin 1−11 (NT 1−11). The 900 nL corresponding to 90 amol of each peptide was loaded onto the CLC column. Separation used a linear 10-min gradient. Each spot was acquired for 10 scans. Spots shown cover 4.3−7.3 min of the chromatogram.

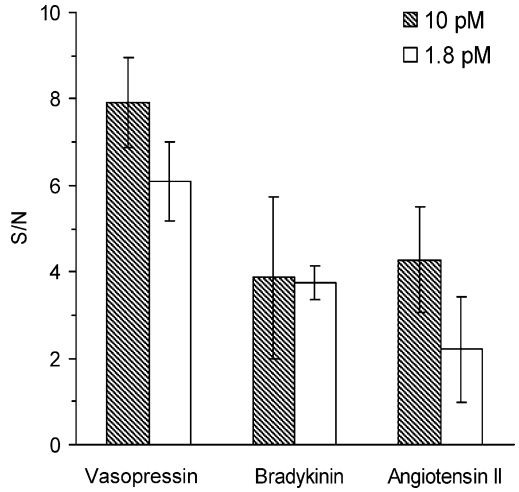

Figure 3. S/N of peptides in a standard mixture detected by CLC−MALDI-TOF-MS. The 0.9 μL of 10 pM sample and 5.4 μL of 1.8 pM sample were loaded so that in both cases 9 amol of each peptide was injected onto the column. Error bars indicate ±1 standard deviation ($n = 3$).

adjusted to obtain S/Ns in the range of 3−10. Noise was measured as the average peak-to-peak noise at 50 m/z adjacent to the peak. Low-attomole mass LOD (2−20 amol) and low-picomolar concentration LOD (2−23 pM) were obtained for peptide standards ($n = 5$). These results compare favorably with other LC-MALDI methods that achieved attomole-level detection,[20,29] where 300 μm i.d. × 15 cm long reversed-phase columns were used and deposition was achieved using either a piezoelectric flow-through microdispenser with matrix precoated on the target plate[29] or a pulsed electric field onto a prestructured target plate to confine sample spots.[20] In our system, the smaller i.d. column (50 μm), which limits sample dilution,[30] combined with multistep pulsed electric field deposition resulted in small sample spots even on an unmodified stainless steel target plate. This system therefore allows low LOD with simpler (and less expensive) target plates.

The concentration LOD of the method can be further improved by more extensive preconcentration. This was demonstrated by loading 9 amol of peptide using either a 0.9-μL injection of 10 pM sample or a 5.4-μL injection of 1.8 pM sample and comparing the S/N obtained. As shown in Figure 3, there is no significant difference between the S/N achieved for the two injected volumes ($p < 0.05$). The concentration LODs for the three peptide standards with 30-min preconcentration were 1−2 pM. This trace-level detection capability combined with sample purification suggested that the method could be used for determination of neuropeptides in complex media, such as brain tissue extracts.

The peptides used for LOD study all contained at least one basic residue (Lys or Arg). It has been reported that MALDI ionization efficiency is higher for those peptides containing basic residues;[31,32] therefore, we may expect that other peptides will have higher LODs. This also suggests that in complex mixtures the analysis will be biased toward basic peptides; however, this is true of any MALDI method. Given that many neuropeptides contain such residues,[33,34] this is not a significant disadvantage.

Detection of Peptides in Microwave-Fixed Striatum Tissue. We then evaluated this method for peptidomic analysis of tissue extracts from striatum of rats sacrificed by microwave irradiation. A typical mass chromatogram displayed as contour and surface plot is shown in Figure 4a and b for a 60-min gradient separation with 90 spots collected. About 200 distinct MALDI-TOF-MS peaks (800−5000 m/z) were detected in each sample, each with clear isotopic pattern unless unresolved due to the high m/z (>3000). As shown in the figure, most peaks eluted in one or two spots. When the same samples were analyzed using dried-droplet deposition following solid-phase extraction (Ziptip) to desalt and preconcentrate, typically only 20−40 distinct peaks were detected each time ($n = 5$). This large difference in ions detected is attributed to the better sensitivity and separation of the CLC method.

The reproducibility of peptide detection by CLC−MALDI-TOF-MS was studied by analyzing the same extract five times and recording how frequently each peak was detected (see summary

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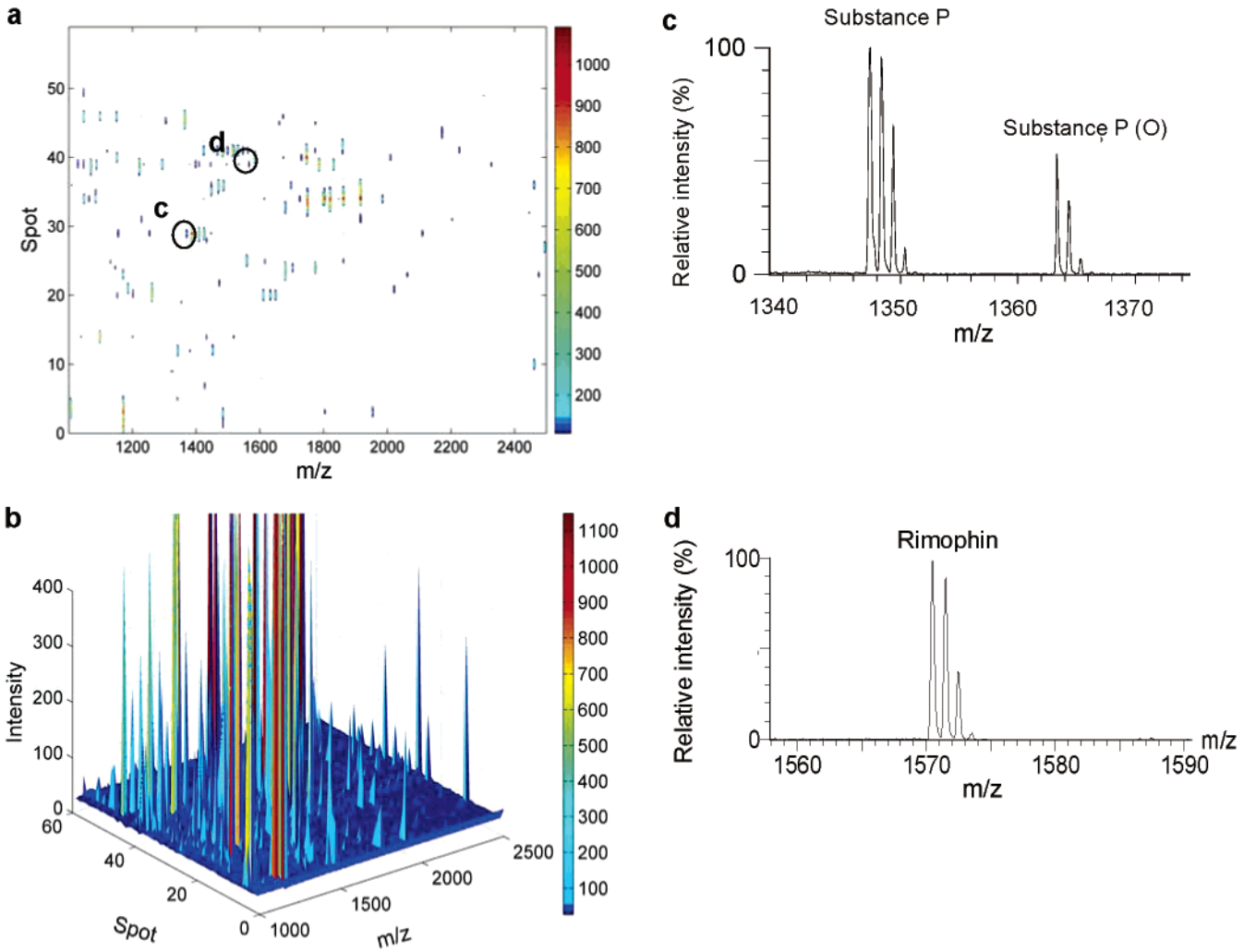

Figure 4. Contour plot (a) and 3D surface plot (b) of the mass chromatogram obtained from CLC−MALDI-TOF-MS analysis of peptides extracted from striatum of rats sacrificed by microwave irradiation. The 5.4 μL of sample was loaded onto the column and separated with a 1-h gradient of 5−50% B. MALDI spots were deposited at 5-s intervals. Spots 0−60 correspond to time 0−41.3 min of the gradient. Plots show the region in which most abundant peptide ions were detected. Mass spectra of spot 29 (c) and spot 41 (d) show SP with SP (O) and rimorphin detected, respectively.

in Figure 5a). Most peaks (64%) were detected twice, but only 12% were detected in all repetitions. This effect is not due to inconsistent detection of low-abundance peptides because the detection reproducibility did not significantly improve when only counting the most intense peaks; i.e., 67% were detected twice and 14% were detected in all repetitions (see Figure 5a). Several reasons for the inconsistent coverage were revealed by further analysis of the data. In some cases, the ^{13}C monoisotopic peak was chosen as the recorded mass by the MassLynx software instead of the ^{12}C. This effect was more prevalent for the higher m/z peaks. For example, in one analysis, a peak with mass 2480.75 was reported as 2481.75 because the ^{12}C monoisotopic peak was too small, but in others, it was properly assigned as 2480.75. As a result, this mass would not be considered detected in both analyses. Another factor related to the limited resolution of the MALDI-TOF instrument (up to 10 000). Peaks over 3000 m/z did not have a clear monoisopotic pattern and therefore sometimes were not recognized by the software. In other cases, ions with close m/z partially overlapped and only one peak was selected by the software. The peak selected depended on the relative intensity, which was variable. Finally, because the mass spectrometer used required manual alignment of the laser with the sample spot, it was possible to misalign the laser and obtain low signals for peptides.

The above results suggest that, to achieve higher coverage of the peptidome, repeated analysis is required. As shown in Figure 5b, analysis of seven extracts from two different rats results in a progressively higher total number of peaks observed. By the fifth repetition, however, little improvement is seen. With these repetitions, a total of 400 peaks distinct from control homogenization solution were detected in the striatum. These peaks are likely peptide peaks according to their isotopic pattern and CLC elution time.

Peptide Identification. We explored the possibility that peptides separated by CLC could be identified by MALDI-TOF/TOF-MS followed by database searching using Mascot. For these analyses, chromatograms were collected in 12 spots (2 min each). Figure 6 shows a sample MS/MS spectrum from these analyses obtained for a peptide ion with m/z 1386.70 where the b and y ion series were clearly detected. The peptide was found to have sequence of SPQLEDEAKELQ from proenkephalin A precursor with Mascot score of 99. Internal fragments such as PQ and PQLE

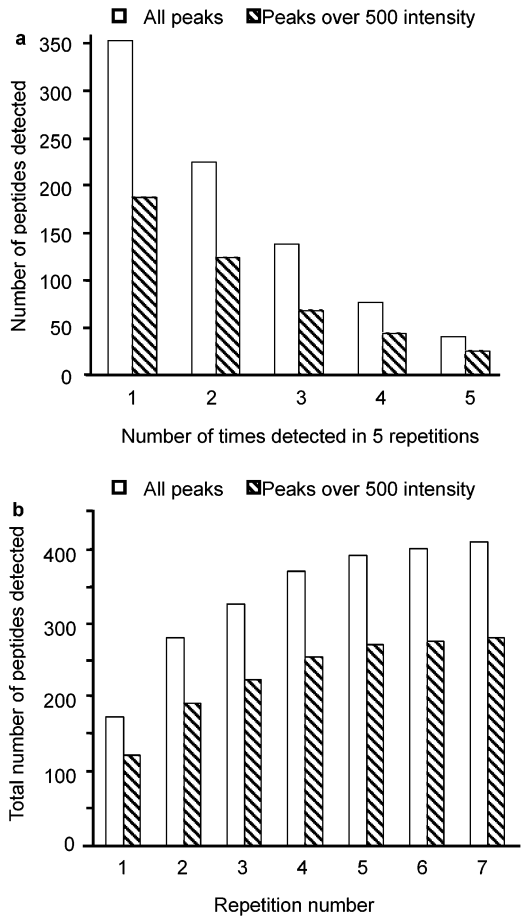

Figure 5. Reproducibility of peak detection and effect of repetitive analysis on peptidome coverage. (a) The reproducibility of peptide detection was assessed by analyzing a sample of microwave-fixed striatum from one rat 5 times by CLC−MALDI-TOF-MS. Less than 50 of the peptides were detected in all 5 repetitions. (b) The effect of repetitive analysis on the peptidome coverage was evaluated by analyzing samples from two rats a total of 7 times. The total number of detected peptides increased asymptotically as the number of repetitions increased, reaching a stable value after the fourth or fifth repetition depending on the intensity of the peaks used.

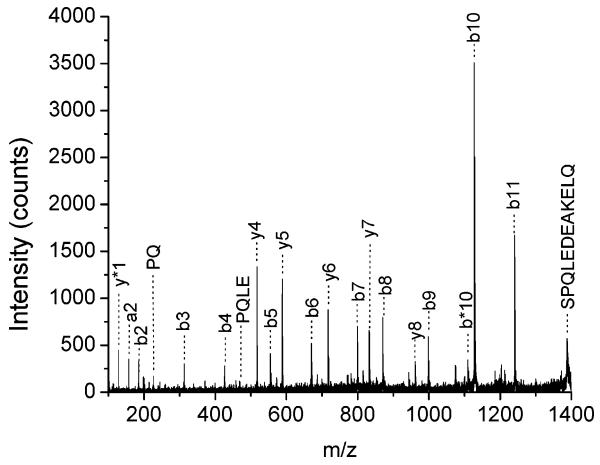

Figure 6. Example of MS/MS spectrum acquired by CLC−MALDI-TOF/TOF-MS for peak at *m*/*z* 1386.73 from a sample of microwave-fixed rat striatum. The peptide was identified as SPQLEDEAKELQ by Mascot. Characteristic ions are labeled.

were detected, illustrating the increased structural information available from high energy collision induced dissociation that is not present using PSD.[18,35] A total of 10 peptides (including oxidized and unoxidized SP) were successfully identified with significant homology as summarized in Table 1. Sample TOF spectra for SP and rimorphin are shown in Figure 4c and d, illustrating the quality S/N for identified peaks in the CLC−MALDI-TOF-MS analysis. Of the peptides identified, eight are either neuropeptides or portions of precursors to known signaling molecules. The other two peptides appear to be degradation products of other abundant proteins in the brain. Three out of 10 peptides were not previously identified as indicated in the table. The results suggest that TOF/TOF analysis may be useful for peptide identification from brain tissue samples separated by CLC.

The 10 peptides identified are notably fewer than the 80−100 peptides identified in tissue samples by CLC−ESI-MS/MS methods;[3,5,36] however, these initial experiments, which were performed to determine the feasibility of neuropeptide identification by the MALDI-TOF/TOF approach, were not optimized. Only ~50% of the chromatogram was collected and analyzed. Furthermore, the volume of fractions collected was much larger than what was used for the TOF analysis. These steps were taken to minimize the number of spots to be analyzed because of limited availability and high cost of instrument time; however, this procedure may also adversely affect sensitivity by not fully resolving the mixture and allowing ion suppression. Furthermore, conditions such as matrix concentration and sample deposition were optimized for the TOF instrument and not the TOF/TOF. Because the latter generally requires more sample, it may be expected that different ratios of sample and matrix may be useful. Optimization of conditions for the TOF/TOF instrument may therefore be expected to yield identification of more peptides and make this approach more competitive with the CLC-ESI methods.

With the limited identification possible by the conditions used for TOF/TOF analysis, the identity and source of the other 390 detected peaks is unclear. Evidence from other peptidomic studies of rat CNS tissue indicates that many of the other peaks detected are also peptides, and possibly neuropeptides, present in vivo. In a parallel study in our laboratory, the same samples were analyzed using CLC−ESI-quadrupole ion trap (QIT) MS operated in data-dependent mode.[5] This method, which allowed acquisition of MS/MS spectra and peptide identification using the database searching algorithms SEQUEST and Mascot, yielded identification of 108 peptides. In addition, previous studies have analyzed similar samples by CLC−ESI-QTOF-MS and reported 89 identified peptides.[3,4] Comparison of the peaks detected in our samples to those identified in these previous studies revealed that 81 of the 390 unidentified peaks had masses that matched identified peptides within the 100 ppm mass accuracy of the instrument used. These 81 peptides included several bioactive compounds such as neurotensin, thymosin β-10, thymosin β-4, somatostatin, big PEN, little SAAS, cerebellin, and neuropeptide E-I. We selected three of the matching peptides (neurotensin, neuropeptide E-I, thymosin β-10) and compared the retention times of the peptide standard to the

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Table 1. Summary of Peptides Identified by CLC−MALDI-TOF/TOF-MS in Striatum of Rats in Microwave-Fixed Tissue[a]

mass (detected)	mass (calcd)	sequence	peptide	protein precursor
1048.49[b]	1048.57	PSISQAYLGI		neuropeptide Y receptor type 4
1098.77	1098.60	YGGFLRKYP		β-neoendorphin-dynorphin precursor
1170.62[b]	1170.63	NAPPEPVPPPR		neurosecretory protein VGF precursor (VGF8a protein)
1228.74	1228.72	YGGFLRKYPK	α-neo-endorphin	β-neoendorphin-dynorphin precursor
1347.82	1347.74	RPKPQQFFGLM	substance P	protachykinin 1 precursor
1363.81	1363.73	RPKPQQFFGLM (O)	substance P	protachykinin 1 precursor
1386.73	1386.68	SPQLEDEAKELQ		proenkephalin A precursor
1466.75	1466.65	VGRPEWWMDYQ		proenkephalin A precursor
1570.97	1570.89	YGGFLRRQFKVVT	rimorphin	β-neoendorphin-dynorphin precursor
1858.97[b]	1858.92	GSDGDSVPVSPQPAPPSPPA		homeobox protein engrailed-1 (Mo-En-1)

[a] Mass indicates $(M + H)^+$. M (O) indicates methionine sulfoxide. [b] Indicates peptides that have not been identified by ESI-MS/MS methods.

endogenous compound that was detected. All three had matching retention times providing further evidence for the identity of the peptides. The observation of matching masses and retention times provides good evidence that some of the 390 unidentified peaks detected by the CLC-MALDI method are endogenous peptides of interest. It will be fruitful therefore to pursue unambiguous identification of the peaks using either higher mass accuracy instruments or more sensitive and optimized MS/MS.

It is possible that some of the unidentified masses are peptides or other compounds that are detected are not present in vivo but are artifacts produced during the sample preparation procedure. Some protein degradation is unavoidable during sample processing, and the observation that 2 of the 10 identified peptides are protein degradation products illustrates this problem. (Although without further study we cannot rule out the possibility that these peptides were not present in the tissue.) Similarly, the detection of oxidized SP, which is not expected in vivo, indicates that some chemical alterations may occur during preparation. However, the sample preparation methods used here, in particular microwave fixation,[3], have previously been used to eliminate much of the protein degradation from sample preparation. Indeed, as discussed below in the quantification section, we find higher levels of endogenous neuropeptide than previous quantitative studies, suggesting less degradation of peptides of interest using these procedures.

Quantification of Endogenous SP from Rat Striatum Tissue. While detection of many peptides may be of value as a screen for peptide content or peptide processing, it is also important to quantify peptides and compare levels under different conditions. We determined the possibility of quantifying peptides using SP as a model analyte. The SP ion (1347.73 m/z) was detected in every run in samples from different rats by CLC−MALDI-TOF-MS and also was successfully identified by CLC−MALDI-TOF/TOF-MS, suggesting that it could be reliably detected and quantified. Quantification was based on use of $[^{15}N_3]$SP as an isotope-labeled internal standard. The ratio of the intensity of analyte and internal standard in a given spot was compared to a calibration curve to achieve quantification. Over 200 shot spectra were summed for each sample spot to generate stable relative intensities.[23]

A complication of determining SP is the tendency of its methionine residue to be oxidized, either in solution or on the target plate, forming a sulfoxide bond.[37,38] We typically observed two peaks for SP, 1347.73 (SP) and 1363.68 (oxidized SP or SP (O)) (Figure 4c) that were partially resolved by chromatography. This complicated quantification because the ratio of reduced form and oxidized form of SP is variable, unknown, and could differ for the internal standard and analyte depending on when oxidation occurs. Initial attempts to block all oxidation were not successful; therefore, to circumvent this problem, we quantitatively oxidized SP by treating the sample with H_2O_2 after internal standards were added.

The oxidized $[^{15}N_3]$SP and endogenous SP eluted together (Figure 7a and b); although, in some cases the peptides were split into two adjacent spots on MALDI target plate. When the peak was split in two spots, the relative intensities of the analyte and internal standards were always consistent with each other and the average ratio was used for quantification. Using this approach yielded reproducible ratios (RSD < 8%) for all concentrations tested and linear calibration curves ($R^2 = 0.99$; see Figure 7c) with a slope RSD of 1.3% ($n = 6$).

The quantification method was used to compare the SP level in striatum from microwave-fixed and nonfixed (i.e., animals sacrificed by decapitation) rat brain. For fixed striatum tissue samples, the average relative intensity of SP (O) and $[^{15}N_3]$SP (O) was 1.5 ± 0.1 ($n = 4$), which corresponded to a concentration 56 ± 4 nM based on the calibration curve. This concentration in the sample corresponded to 6.8 ± 0.5 pmol in the striatum or 350 ± 26 pg/mg of tissue (RSD = 7%). For nonfixed striatum tissue, the average relative intensity of SP (O) and $[^{15}N_3]$SP (O) was 0.27 ± 0.02 ($n = 3$), corresponding to 83 ± 6 pg/mg of striatum tissue (RSD = 7%). These results demonstrate the good precision of using an isotope-labeled internal standard method for quantification of endogenous neuropeptides.

The oxidation step used here would not always be required; however, where it is necessary, side effects are expected to be minimal. In principle, other peptides with oxidizable residues may be treated in a similar fashion for quantification. Since isotope-labeled standards would be available for such a treatment, both the standard and endogenous peptides will be treated in the same

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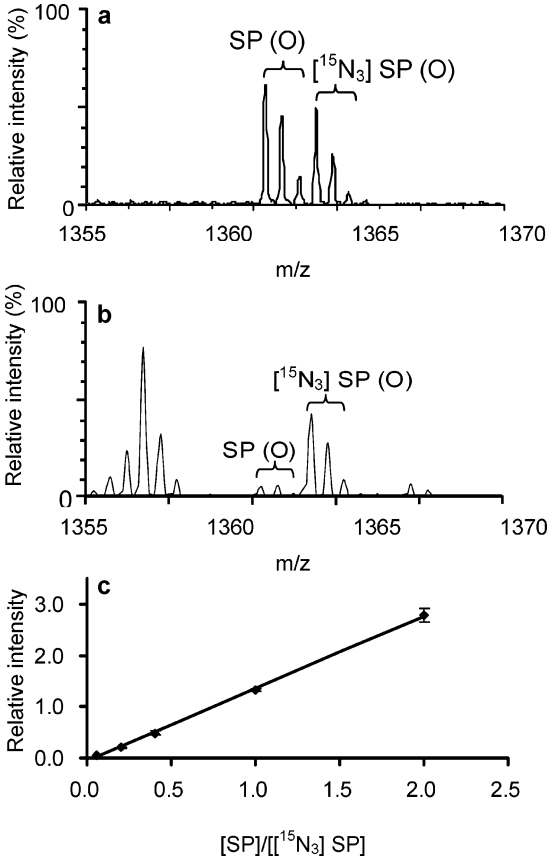

Figure 7. Data used in quantification of SP. (a) A typical MALDI-TOF mass spectrum of coeluted SP (O) and [$^{15}N_3$]SP (O) (internal standard) obtained for analysis of microwave-fixed striatum tissue. Internal standard was added to sample and the mixture treated with hydrogen peroxide to quantitatively oxidize the peptide for quantification. (b) Similar to (a) except for a sample from nonfixed striatum tissue. The lower ratio of SP (O) to [$^{15}N_3$]SP (O) is indicative of lower amounts of SP in the sample compared to the microwave-fixed sample in (a). (c) Calibration curve for SP quantification. Error bars indicate ± 1 standard deviation ($n = 6$). For each sample, 2.5 μM [$^{15}N_3$]SP was added. The relative standard deviation of each concentration on the curve was less than 8%. The relative standard deviation of the slope was 1.3%.

way (as done for substance P in this case) to determine effects of oxidation on retention and molecular weight.

The SP concentration in fixed striatum tissue was 4.2-fold greater than that in nonfixed tissue. This result is in reasonable agreement with a previous study that used radioimmunoassay (RIA) and found a 2.4-fold greater SP concentration in microwave-fixed than nonfixed whole brain tissue.[39] This effect has been attributed microwave fixation to preventing enzymatic degradation by rapidly raising the brain temperature to 80 °C in <1 s and denaturing proteolytic enzymes.[3,39−41] The greater improvement with microwave fixation seen here may be due to differences in the tissue used (we used striatum while the other used whole brain). It may also be due to improvements in microwave irradiators, which presently have higher power than those previously available, that decreased the fixation time sufficiently to result in more effective preservation of the peptide.[42]

The CLC method used here found significantly higher levels of SP than those previously found using RIA.[37,39,43,44] Typical levels were ~1 pmol/striatum[37] compared to 6.8 pmol detected in this work. Certainly one reason for the higher levels is that we have observed higher levels is the use of microwave fixation; however, the nearly 7-fold difference is unlikely to be explained only by in situ tissue fixation. Previously used RIA-based methods did not account for oxidation of SP; therefore, it is possible that these methods underestimated the amount of SP in tissue if oxidation affects antibody binding. Another significant difference is that we used an aggressive extraction procedure that combined mechanical and chemical lysis to improve the yield of neuropeptides isolated from tissue.[5] Because of the high selectivity of the LC−MS method, it is unlikely that the higher levels are due to an interfering substance. Thus, we conclude that this new method has revealed that SP levels in striatum are substantially higher than previously believed. These results demonstrate the importance of sample preparation and high chemical selectivity for accurate measurements of neuropeptide content. It will be of interest to use this method on other peptides that may have similarly been underestimated.

The CLC−MALDI-TOF-MS method appears to be promising for peptide quantification in tissue. Compared to RIA, this method achieves better mass LOD (2−21 amol compared to 100 amol) and comparable concentration LOD (both are of low picomolar).[23] Furthermore, the MS method avoids ambiguities associated with antibody cross-reactivity by allowing sequence-specific detection and detection of modifications, such as the oxidation of methionine. Finally, the CLC method, in principle, would allow multiple peptides to be quantified in one sample. At present the CLC method would be lower throughput than RIA, which can be performed in batch mode; however, separation speed can be greatly increased and the possibility of parallel separations has been reported.[20]

CONCLUSION

CLC−MALDI-TOF-MS is shown to be a versatile and sensitive platform for neuropeptide analysis by allowing detection, identification, and quantification of neuropeptides. Compared with other reported LC−MALDI-MS methods for peptides, the method has equal or better mass LOD for neuropeptide standards (low attomole) and improved concentration LOD (low picomolar). Use of MALDI-TOF/TOF-MS allowed identification of peptides, but more optimization will be required for this to be highly effective for the samples used here. Peptide-specific quantification is possible when using isotope-labeled internal standards. The sample preparation and analysis procedures used resulted in significant improvements in quantification over previous reports. The most intriguing potential application of the method is quantification of groups of identified peptides in a single analysis. The method could therefore be useful for monitoring the expression level changes of identified neuropeptides under different physiological conditions.

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