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On-column Dimethylation with Capillary Liquid Chromatography-Tandem Mass Spectrometry for Online Determination of Neuropeptides in Rat Brain Microdialysate

Rachael E. Wilson, Andrea Jaquins-Gerstl, and Stephen G. Weber

Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, United States

Abstract

We have developed a method for online collection and quantitation of neuropeptides in rat brain microdialysates using on-column dimethylation with capillary liquid chromatography - tandem mass spectrometry (cLC – MS²). This method addresses a number of the challenges of quantifying neuropeptides with cLC - MS. It is also a completely automated and robust method for the preparation of stable isotope labeled - peptide internal standards to correct for matrix effects and thus ensure accurate quantitation. Originally developed for tissue-derived proteomics samples (Raijmakers, et al., 2008), the efficacy of on-column dimethylation for native peptides in microdialysate has not been demonstrated until now. We have modified the process to make it more amenable to the time scale of microdialysis sampling and to reduce the accumulation of non-volatile contaminants on the column and thus, loss of sensitivity. By decreasing labeling time, we have a temporal resolution of one hour from sample loading to elution and our peptide detection limits are in the low pM range for 5 μ L injections of microdialysate. We have demonstrated the effectiveness of this method by quantifying basal and potassium stimulated concentrations of the neuropeptides leu-enkephalin and met-enkephalin in the rat hippocampus. To our knowledge, this is the first report of quantitation of these peptides in the hippocampus using MS.

Graphical Abstract

SUPPORTING INFORMATION

Dimethylation SI.pdf. Contains a figure illustrating probe placement, additional mass spectra data for the labeling tests, and additional *in vivo* data.

INTRODUCTION

Neuropeptides are heavily involved in cell signaling in the brain, with roles in neurotransmitter modulation^{1–3}, reward behaviors⁴, and cognition^{5,6}. Unsurprisingly, they are implicated in a number of central nervous system disorders including addiction^{7,8} and depression⁹. Despite their importance, the details of the distance- and time- scales over which they function are largely unknown.¹⁰ *In vivo* investigations of extracellular peptides, typically by microdialysis,^{11,12} are challenging due to the neuropeptides' low extracellular concentrations (pM^{13–18}) and the potential for loss of peptides due to sample handling¹⁶, enzymatic hydrolysis,^{19,20} and adsorption^{21,22}.

Ideally, online measurements of peptides in microdialysate by capillary liquid chromatography-mass spectrometry (cLC-MS) should be well-suited for the analysis of extracellular processes involving peptides. Online measurements reduce sample handling and cLC-MS is suited to the small volumes and low concentrations expected^{15,16} of peptides in dialysate²³. As far as we are aware, only Haskins, et al. have used *in vivo* microdialysis with online LC-MS for the analysis of neuropeptides.^{16,24} While one of these works focused on the discovery of novel peptides²⁴, the other involved the quantification of basal and potassium stimulated levels of the neuropeptides met-enkephalin (ME, YGGFM) and leu-enkephalin (LE, YGGFL) in the anesthetized rat globus pallidus.¹⁶ The authors reported higher peptide levels compared to offline studies, which they hypothesized could be due in part to the effectiveness of the online process to minimize sample loss.¹⁶ Thus, online collection and analysis is preferable to offline methods. However, the authors injected dialysate directly on the analytical column, which resulted in rapid column degradation and necessitated daily replacement. In addition, isotopically labeled standards were not included for quantitation. This is essential¹⁹ because ionization efficiencies depend on the analyte as well as the ionic and solvent composition of the medium. Thus, quantitation cannot be based on detector response alone.¹² While some stable isotope-labeled peptide internal standards are available, it would be expensive and impractical to use them in multi-peptide analyses. An online labeling scheme is preferred.

Offline amine labeling has been used in measurements of polar, low-molecular weight neurotransmitters in microdialysate.²³ The benzoyl chloride work of the Kennedy group²⁵ used ¹²C₆-benzoyl, to label analytes in dialysate *offline* while internal standards were labeled with a “heavy” ¹³C₆-benzoyl group. This method improved quantitative analysis of microdialysate for 70 small molecule neurotransmitters and metabolites, including dipeptides.²⁶ An *offline* but *on-column* approach used for quantitative proteomics is the dimethylation of primary amines using formaldehyde and cyanoborohydride.²⁷ Rajmakers, et al. have shown this to be an effective method for protein quantification in tissue-based samples, with higher efficiency and comparable sensitivity as in-solution methods.^{27,28} We theorized that the on-column method could be adapted to the online collection of microdialysate. The potential advantages of this approach are that stable isotope labeled – internal standards (SIL-ISs) for multiple analytes can be generated simultaneously and the labeling reaction rates are compatible with online analysis. Until now, the efficacy of this method has not been demonstrated with native peptides, or with microdialysate²³, or with online *in vivo* sampling.

We made a number of modifications to convert the Rajmakers method from tissue-based samples to the online analysis of microdialysate. Here, we demonstrate the effectiveness of this method by measuring basal and potassium-stimulated levels of LE and ME in the rat hippocampus. These endogenous opioids are involved in learning and memory formation in the hippocampus.⁵

Our method is able to achieve peptide detection limits in the low pM range for 5 μ L injections and has a temporal resolution of one hour. While certainly this needs to be decreased, it is faster than the previous on-column dimethylation work with tissue-based samples²⁸ and comparable to the online microdialysis work of Haskins, et al.¹⁶ This method also improves the robustness of online collection, significantly increasing the number of samples that can be analyzed without human intervention. To our knowledge, this is the first report of online collection, labeling, and analysis of neuropeptides in *in vivo* microdialysis samples and the first report of enkephalin quantitation in the hippocampal extracellular space using cLC - MS.

EXPERIMENTAL

Peptide Standards

Stock solutions of LE (American Peptide, Sunnyvale, CA), ME (Sigma-Aldrich, St. Louis, MO), and yaGfl (GL Biochem, Shanghai, China) standards were prepared by dissolving the solid as received from the manufacturer in water, which was obtained from a Millipore Milli-Q Synthesis A10 purification system (Billerica, MA), to a concentration of 1 mM. Serial dilutions were then used to prepare 5 nM standards of each peptide. LE, ME, yaGfl standard mixtures were then further diluted to the desired concentration (usually 100 pM) in either water or a modified Ringer's solution consisting of 148 mM NaCl (EMD-Millipore, Darmstadt, Germany), 1.2 mM CaCl_2 (EMD-Millipore), 2.7 mM KCl (Sigma-Aldrich), and 0.85 mM MgCl_2 (Fisher Scientific, Fair Lawn, NJ) at pH 7.4. All standards were made fresh daily.

Labeling Reagents

The labeling reagents were prepared in the following way. Triethylammonium acetate (TEAA) buffer was prepared by adding 100 mM acetic acid (Fisher Chemical) to 100 mM triethylamine (Sigma) to achieve a pH of 7.40. A volume of 350 μ L of the TEAA buffer was placed into an autosampler vial (Thermo Scientific, Rockwood, TN) followed by 3.50 μ L of 0.6 M sodium cyanoborohydride (Sigma) and 3.50 μ L of 4% formaldehyde (J.T. Baker, Phillipsburg, NJ). Due to the toxicity of formaldehyde vapors, the labeling reagent components should be prepared in a fume hood. The mixture was then vortexed and placed in the refrigerated autosampler at 5 $^{\circ}\text{C}$. The heavy labeling reagent was prepared using the same procedure but with formaldehyde- d_2 (Sigma). TEAA, cyanoborohydride, and formaldehyde solutions were made fresh daily.

HPLC Setup and Conditions

A Dionex Ultimate 3000 HPLC system (NCS-3500RS, Thermo Scientific, Germering, Germany) was used for cLC. The system consisted of an online degasser, a low-flow loading

pump, a nanoflow analytical pump, an integrated column oven housing two 10-port Cheminert high-pressure 1/16" valves (VICI, Valco, Houston, TX), and a WPS-3000 RS autosampler. For the analytical pump, channel A contained 0.5% acetic acid in DI water and channel B contained 0.5% acetic acid in Optima-grade methanol (MeOH, Fisher Scientific). The loading pump mobile phase consisted of 0.5% acetic acid in a 98:2 water: methanol mixture. Peptide standards and labeling reagents were housed in the temperature-controlled autosampler at 5 °C.

Capillary columns of 50 μ m ID were prepared in-house using polyimide coated fused silica (Polymicro, Phoenix, AZ) that was packed at 6000 psi using the downward slurry method as described previously²⁹. The packing material consisted of Acquity BEH 5 μ m C18 stationary phase (Waters, Milford, MA) suspended in HPLC-grade isopropanol (Fisher Chemical). Methanol was the packing solvent. The outlet was fritted prior to packing by mixing a 25% formamide (Acros, NJ) solution with KASIL® 1 potassium silicate (Kasil, PQ Corporation, Valley Forge, PA), which was then used to moisten a glass microfiber filter (Whatman, UK).³⁰ The capillary was dipped onto the filter paper and placed in an oven at 300 °C for 12 hours. After packing, columns were trimmed to 4.5 cm and connected to a pulled silica tip using a PTFE sleeve. Pulled tips were also prepared in-house using a laser puller (P-2000, Sutter Instruments, Novato, CA) and 25 μ m ID capillaries, which were trimmed to a final tip ID of approximately 10 μ m and length of 2 cm. The pre-column consisted of an Acclaim PepMap 100 C₁₈ column (Thermo Scientific) packed with 3 μ m particles and dimensions of 75 μ m ID x 2 cm length.

Peptides were eluted at 0.3 μ L/min using the following gradient: a step of 2–20% B (nominally in 0.1 minutes) then 20–40% B in 5 minutes with a hold at 40% B for 2 minutes. A ramp of 40–95% B over 1 minute was then used to remove any highly-retained analytes present in the dialysate. The mobile phase was returned to 2% B (nominally in 0.1 minutes) and re-equilibrated for 7 minutes. A 3-minute column wash with 95% B was then performed to prevent carryover. The total analysis time was one hour.

Mass Spectrometry

The cLC was coupled to a Thermo Scientific LTQ XL linear ion trap MS using nanospray in positive ion mode. A locally constructed stage housed a 6-port 1/16" VICI high-pressure Cheminert valve (Valco) connected to a liquid junction tee (Idex, Oak Harbor, WA) by a 25 μ m ID x 10 cm long fused silica capillary. Voltage was applied to the tee at the head of the column. The selected precursor ions used for MS² fragmentation and daughter ions used for generating extracted ion chromatograms (EIC) and determining peak area can be found in Table S1. The precursor and daughter ions were chosen for each peptide based on information from direct infusions of 5 μ M standards into the MS at 0.1 μ L/min. Peaks were identified based on MS² fragmentation patterns and retention times of the standard peptides as well as comparisons to expected fragmentations based on sequence (prospector.ucsf.edu/prospector/cgi-bin/mssearch). The LC was operated using Chromeleon Xpress with DCMS link to Xcalibur for MS operation and chromatograms were analyzed using Xcalibur Qual Browser.

On-Column Labeling Procedure

There are three stages in the online method (Fig. 1): pre-concentration of peptides in the sample (Fig. 1A), reaction of peptides adsorbed to the pre-column with light reagent followed by introduction of unlabeled peptide as internal standards and then reaction of peptides adsorbed to the pre-column with heavy reagent (Fig. 1B), and finally injection of the isotopically labeled analytes and standards onto the analytical column. The total cycle time is one hour. In more detail, Fig. 1A shows perfusate from the probe continuously filling a 5 μ L sample loop on Valve 2. Once per hour Valve 2 is switched allowing peptides to adsorb at the head of the pre-column. We have given this step four minutes at 5 μ L/min to ensure complete emptying of the sample loop and sufficient de-salting. Valve 2 is reset to the “fill” position. The second stage has three steps (Fig. 1B) that use the autosampler. The autosampler has five vials as follows: vials 1 and 2 contain light labeling reagent and 5% formic acid, respectively. Vial 3 contains the sought-for peptides (100 pM) that will, when labeled, act as isotopically labeled internal standards. Vials 4 and 5 contain the heavy labeling reagent and 5% formic acid, respectively. The sequence represented in Fig. 1B is thus, first, labeling of the adsorbed analyte peptides with light reagent followed by quenching, injection of the sought-for peptides, then labeling them with heavy reagent following by quenching. Each of the five solutions was loaded into a 5 μ L loop and then introduced to the pre-column at 5 μ L/min for three minutes. The result is on-column reaction of adsorbed peptides with the injected reagents. The third stage is simpler (Fig. 1C). Valve 3 was switched so that the pre-column is in line with the analytical pump and the analytical column for separation.

One-column Approach versus Two-column Approach

Labeling was performed using a one-column setup and a two-column setup. In the one-column setup, pre-concentration and separation occurred on a single column. For the two-column setup, pre-concentration occurred on a pre-column while the separation occurred on a separate analytical column. Figure 1 illustrates the two-column setup. The valve configuration for the one-column setup was identical except the column was positioned between the loading pump and the analytical pump on Valve 3, taking the place of the pre-column. The sequence of events was also the same for the one- and two-column approach, except that for the one-column approach, all samples and reagents were injected directly onto the analytical column, with the eluate going into the inlet of the MS rather than to waste.

Labeling Tests

For *in vitro* labeling tests, the Ringer's standard consisted of either 50, 100, or 200 pM mixtures of LE, ME, and yaGfl in Ringer's solution, which was injected via syringe pump in the exact manner used for an animal experiment. The aqueous standard consisted of either 50, 100, or 200 pM mixtures of LE, ME, and yaGfl in water, which were injected using the autosampler in the same way described for the animal experiments. The concentrations and labeling conditions were randomized and repeated for $n = 3$ replicates. Samples were randomized to prevent bias due to carryover effects at higher concentrations and to

demonstrate that the method is reproducible when going from high to low concentrations and vice versa. Table S2 shows the ion transitions used.

Microdialysis and Surgery

All procedures involving animals were carried out with the approval of the Institutional Animal Care and Use Committee of the University of Pittsburgh. Male Sprague-Dawley rats (250–375g, Charles River, Wilmington, MA) were intubated and anesthetized with isoflurane (S-3 Baxter Healthcare, Deerfield, IL) at 0.5 % by volume and wrapped in a homoeothermic blanket (EKEG Electronics; Vancouver, BC, Canada), (n=4). Body temperature was maintained at 37.5 °C using a heating pad. The rats were placed in a stereotaxic frame (David Kopf Instruments; Tujunga, CA). Microdialysis probes (216 µm o.d., 2 mm in length) were constructed with hollow fiber dialysis membrane (Spectra-Por RC Hollow Fiber; MWCO: 13,000 Da, 200 µm i.d., Spectrum Laboratories, Inc.; Rancho Dominguez, CA) and fused silica outlet lines (150 µm o.d., 75 µm i.d., Polymicro Technologies; Phoenix, AZ) as described elsewhere.³¹ The scalp was shaved and the skull was exposed. A craniotomy was performed and the dura was carefully removed away to expose the brain. The incisor bar was adjusted so that the dorsal-ventral measurements at lambda and bregma were no more than 0.01 mm apart (flat skull). Microdialysis probes were lowered into the hippocampus to a final position of 5.6 mm anterior to bregma, 4.6 mm lateral from midline and 5.0 mm below dura³² over a 30-minute period. The probes were perfused throughout all implants with Ringer's at 0.500 µL/min using a syringe pump (Harvard Apparatus, Holliston, MA). Potassium stimulation was conducted using a Ringer's solution containing 100 mM KCl. The concentration of NaCl was adjusted to maintain ionic strength. The high potassium Ringer's was administered via a VICI Valco 10-port valve housed in the LC column oven. After 10 minutes of high potassium stimulation the valve was switched to resume perfusion with the standard Ringer's solution. On completion of testing, the rats underwent intracardial perfusion and the brains were removed for verification of the probe location (Fig. S1).

Microdialysis Probe Recovery

The microdialysis probe recovery was tested *in vitro* by placing a probe in a solution of 100 pM LE and ME in Ringer's after removal of the probe from the rat brain. 100 pM yaGfl in Ringer's was perfused through the probe at 0.5 µL/min and the outlet of the probe was connected directly to the cLC sample loop as shown in Figure 1. At least three replicates were obtained for each measurement.

Data Analysis

Peak integration was automated using the Xcalibur Qual Browser. Each peak was manually inspected prior to area calculation. Relative areas were obtained by dividing the peak area of the light peptide by that of its heavy analog. An analysis of variance (ANOVA) was used to compare basal relative areas for LE for each of the four rats with the null hypothesis being that the mean relative area is the same for each rat (with 95% confidence). A p-value of 0.0004 indicated that the null hypothesis should be rejected and a *post hoc* Tukey test revealed that the measured relative areas for Rat 1 were significantly different from those of Rats 2, 3, and 4. For this reason, the measured values for each rat are presented individually.

The ANOVA was repeated in the same way for ME. Although it indicated that there was no significant difference in measurements for each rat ($p = 0.18$), in most cases we were only able to measure ME once in each rat. Thus, we have chosen to report ME values for each rat individually. All statistics were performed using StatPlus in Excel. Values are stated as the mean $\pm$ the standard error of the mean (SEM).

RESULTS AND DISCUSSION

On-column Dimethylation

While the efficacy of the on-column dimethylation procedure has been shown elsewhere,²⁷ this technique was originally developed for proteomics applications, which have a few significant differences compared to online microdialysis applications. First, proteomics samples typically consist of tryptic digests derived from tissue homogenates. Samples go through several purification and pre-concentration steps, which also enable higher ion counts. The microdialysis probe typically excludes large proteins, resulting in relatively clean samples, but dialysate still contains a high concentration of salts which must be separated from analytes to achieve efficient ionization and detection. Thus, a de-salting step was added following sample loading. Low endogenous concentrations (pM-nM) in the extracellular space combined with limited microdialysis recoveries result in significantly less analyte mass on-column than tryptic digests, so while the previous work utilized larger volumes of labeling reagents (40 μ L), we have reduced these volumes to 5 μ L, which also reduces labeling time. We note that each labeling step only required three minutes. This reduction in time was necessary to achieve a practical temporal resolution for online microdialysis sample collection and near real-time measurements. Previous work utilized a sodium phosphate buffer for the labeling reagents. We have chosen to use triethylammonium acetate instead to reduce the accumulation of non-volatile salts on the column, which result in decreased sensitivity and column lifetime, particularly for the one-column setup.

Labeling Tests

It is imperative to establish the completeness of the labeling procedure and to confirm the absence of cross-labeling and mixed-labeling which would occur if the first of the two labeling reactions was not effectively complete. Recall that the autosampler contains five vials: light reagent and quench solution (vials 1 and 2), aqueous peptide standards (vial 3), and heavy reagent and quench solution (vials 4 and 5). We determined completeness using two tests: 1) The Ringer's standard (a surrogate for the sample) was injected followed by the sequence of labeling steps (Fig. 1B) *except* the introduction of the aqueous standards, *i.e.*, vials 1, 2, 4, 5 were injected in series; and 2) The Ringer's standard was *not* injected. All steps in Fig. 1B, including the introduction of the aqueous standard, were injected in sequence. In the first case, the peptide is exposed to both light and heavy reagents but should only be light labeled. The presence of the heavy labeled peptides would indicate incomplete reaction. In the second case, only heavy labeled peptides should be present. The presence of light labeled peptide would indicate carryover of the light labeling reagent. In addition, if we observed differences in the amount of light and heavy label between the two tests, we would infer an effect of incomplete removal of salt on the reactions.

We did not detect unlabeled peptide, cross-labeling, or mixed products over a set of concentrations of peptides (Figs. S2 to S4). Ions were present in the expected ratios based on relative concentration (1:1). Thus, any amount of unlabeled peptide is below our detection limit and thus the labeling is apparently complete at these concentrations. Given that the ratios between light and heavy peptides were as expected, we conclude that the presence of salts in the Ringer's solution did not affect the labeling efficiency or ionization efficiency compared to the aqueous standard. Thus, we conclude that this is an effective method for labeling. Previous reports^{27,33,27} have investigated the labeling efficiency of the dimethylation reaction at varying concentrations and for various applications and have had similar findings.

One-column Setup

Initially, we investigated the use of online labeling using a single column. With this approach, the loading pump directs all sample and reagents onto the analytical column and waste is directed into the MS source. This method can be easily adapted to existing LC-MS setups and does not necessarily require additional equipment, such as valves. Fig. 2 depicts a calibration curve of LE generated using the one-column setup, where the relative area was determined by dividing the peak area of the light peptide (prepared in Ringer's) by the peak area of the heavy peptide (100 pM LE in water). The plot should be linear with an intercept equal to approximately zero, yet by eye the points do not describe a good straight line (slope ($\pm$ SEM) is 0.01130 ± 0.00092) and the intercept ($\pm$ SEM) is 0.671 ± 0.047 . Reproducibility was also a challenge, as indicated by large SEMs for replicate measurements. A significant contribution to this poor calibration is the decrease in MS sensitivity after several hours of repeat injections. This was likely due to the accumulation of non-volatile salts from the Ringer's solution at the MS inlet. This problem could be prevented if one has a method for preventing eluate from entering the MS source, such as diverting the liquid stream from the glass emitter tip using N₂.¹⁶ Another issue was rapid column degradation, which has been reported by others in similar applications^{16,35,16}. Interestingly, this was not an issue with aqueous standards, only those prepared in Ringer's. Larger-bore columns may minimize this effect¹⁸ but trade-offs in sensitivity must be considered. We found the one-column method useful for method development, especially since it requires minimal changes to existing setups and procedures. It could also be utilized for short-term studies occurring over a few hours but longer studies require a more robust setup. Thus, we pursued a two-column approach.

Two-column Setup

It is well-known that internal standards are necessary for MS² quantitation to correct for matrix effects.¹⁹ This is particularly true for dialysate samples (Fig. 3). Fig. 3A depicts the peak areas of Ringer's standards that were labeled using the light reagent. In comparison, Fig. 3B shows the same data, but expressed as the ratio of the peak area of the light labeled peptide to that of the heavy labeled peptide (100 pM in water). The addition of the isotopic standard resulted in improved reproducibility and linearity (Table 1). These results further demonstrate the efficacy of this method, as ratios of light to heavy are as expected based on relative concentrations. In Fig. 3, yaGfl was present at a concentration of 100 pM in both the Ringer's and the aqueous standards. Though both are normally distributed, the relative

standard deviation of the distribution of yaGfl relative areas is considerably smaller than that for the peak area of yaGfl alone (Table 2). Thus, inclusion of the heavy peptide improves reproducibility of dialysate measurements.

In Vivo Microdialysis

To demonstrate the utility of this method for studying dynamic changes in endogenous neuropeptides *in vivo*, we monitored LE and ME concentrations in the rat hippocampus. Fig. 4 depicts recorded LE and ME levels throughout the sampling time for a single rat. This was a typical profile obtained for each of the rats (Fig. S5). Interestingly, with each rat we saw an initial high peptide level immediately after probe implantation. This phenomenon has been observed in other neuropeptide microdialysis studies.^{14,37} The calibration curves in Fig. 3B were used to convert the relative areas, such as those shown in Fig. 4, to concentrations for each rat. Estimated dialysate concentrations of LE and ME can be found in Table 3. ANOVA revealed that basal concentrations of Rat 1 were statistically different from the others (see Experimental). This could be because the probe for Rat 1 was slightly outside the targeted area where the other probes were located (Fig. S1).

Potassium stimulation is a commonly employed method used to verify the presence of a neuropeptide.^{38–40} Fig. 5 shows the typical EIC and MS spectra for peptides following stimulation with potassium. Each peptide was easily identifiable under these conditions. Thus, we are observing endogenous neuropeptide release. Peptide response decreased with repeated potassium stimulation. This has been observed elsewhere.¹⁷ We observed a statistically significant 2.8-fold increase in LE during potassium stimulation compared to basal levels (Fig. S7). We did not detect a significant increase (defined as $p < 0.05$) in ME during potassium stimulation (Table S3). This was because basal levels were often below the detection limit.

At the end of the sampling time, the microdialysis probe was removed to test for *in vitro* recovery. The recoveries $\pm$ SEM were $14.9 \pm 1.1\%$ for LE and $13.3 \pm 2.4\%$ for ME ($n = 3$). Probe recovery was determined after *in vivo* use to prevent carryover of standards prior to rat experiments and to show that the probe was properly functioning throughout the course of the experiment. Thus, we conclude that this method is useful for observing dynamic changes in neuropeptide concentrations in the extracellular space.

Since this is the first report of online labeling and MS quantitation of neuropeptides in rat brain microdialysates and the first study quantifying enkephalin levels in the hippocampal extracellular space using MS, there is a lack of literature with which to compare our results. The closest work consists of two reports, both conducted by Rocha, et al., in which microdialysis followed by radioimmunoassay (RIA) was used to compare total opioid peptide (defined as peptides with YGGFM... or YGGFL... N-terminal sequences) levels in the hippocampus of control rats to those with induced seizures. They reported total opioid concentrations in control animals of 11.2 ± 2.5 pM⁴¹ and 24.5 ± 2 pM,⁴² without correction for probe recovery. These values are similar to ours. They also observed approximately 1.5–41 and 4-fold⁴² increases in total opioid levels when stimulating with 100 mM potassium for 10 minutes. These values are similar to the relative changes in LE levels that we observed under the same stimulation conditions.

Extracellular neuropeptide concentrations are region-dependent.¹¹ Our measured LE and ME concentrations were near and at times below our detection limits. Three of our probe locations were primarily in the CA1 region of the hippocampus, which has lower enkephalin levels than the nearby dentate gyrus and the CA3.^{49,50} More broadly, RIA and immunohistochemistry measurements have shown that LE and ME levels vary considerably among regions of the brain and are higher in other brain regions, such as the striatum, compared to the hippocampus.^{43–48} Although immunoreactivity is not directly related to extracellular concentrations of the pentapeptides, we speculate that the detection limits of this technique will allow for measurements in many other brain regions.

Use of yaGfl as a Quality Control Indicator

Given the complexity of the experimental setup, we found it useful to include the D-amino acid-containing peptide yaGfl in the perfusate to aid in troubleshooting and as a semi-quantitative measure of the data quality. This synthetic amino acid was chosen because it is similar in length and composition to our analytes of interest and is not hydrolyzed to any detectable extent in the extracellular space.⁴⁹ If a peptide that is hydrolyzed in the extracellular space were used, it would not be a suitable control because it would not be known if decreases in signal observed at the detector were due to instrumental issues or enzymatic hydrolysis. The yaGfl present in the perfusate was light labeled along with the other peptides in the microdialysate. In order to generate a SIL-IS, yaGfl was also contained in the aqueous standard. By pooling data from all four rats, we found that the relative area of yaGfl (ratio of light to heavy peak area) was normally distributed, with a mean of 0.989 and a standard deviation of 0.067 ($n = 23$, Table 4). Using this value, we can quickly assess the quality of an injection. If we obtain a relative area outside the range of 0.85 to 1.13, we suspect (with 95% confidence based on 22 degrees of freedom) that there is some problematic issue, such as a leak. It is especially important to have a control such as this when doing online analysis, which requires rapid troubleshooting to avoid sample loss. Additionally, by comparing the SEM of yaGfl measurements to that for a peptide, we can determine if variations in peptide levels are due to physiological variations or procedural errors. Thus, yaGfl is a useful internal standard to assess point-to-point and rat-to-rat reproducibility.

Retrodialysis of yaGfl is also useful for determining microdialysis probe extraction fraction, which is important for accurate quantitation. This is typically determined *in vitro* but it is helpful to have an *in vivo* method since the environment of the brain is much different than that of a beaker. By comparing the mean relative area $\pm$ SEM of yaGfl in the absence of the microdialysis membrane (Table 2, 1.180 ± 0.022) to the mean in Table 4, the extraction fraction is $16.19 \pm 0.03\%$ ($1 - (0.989/1.180)$ expressed as a percent). Assuming there is no degradation of yaGfl in the extracellular space, the extraction fraction is equal to the probe recovery. This value is within reasonable agreement with the LE and ME recoveries and indicates that yaGfl is useful for determining probe recovery *in vivo*.

CONCLUSION

We have developed an online, automated method for labeling endogenous neuropeptides in microdialysate and corresponding isotopic standards. This allows quantitation of dynamic processes occurring in the extracellular space. We have demonstrated this by quantifying LE and ME in the anesthetized rat hippocampus under basal and potassium-stimulated conditions. To our knowledge this is the first report of online collection and labeling for microdialysis applications and the first quantitative study of endogenous LE and ME in the hippocampus using MS². One drawback of this technique is the time required for labeling. In this study we did not optimize the labeling procedure for maximum time efficiency. We anticipate improving this for future work, both by optimizing labeling steps to achieve complete labeling in the least amount of time and by increasing loading flow rates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic for two-column labeling setup. Valve 1 is housed on the autosampler, Valve 2 is housed in the LC column oven, and Valve 3 is an external valve housed on the MS stage. Valve 2 is a 10-port valve but is depicted with 6-ports for clarity. White indicates a valve that is switching. Labeling is performed in the following way: (A) A 5- μ L sample loop on Valve 2 is filled with microdialysate (MD) and upon switching, the sample is directed onto the pre-column (PC) by the loading pump (LP); (B) A series of five autosampler (AS) injections delivers reagents from vials (In sequential order: light labeling reagent, formic acid, 100 pM peptide aqueous standards, heavy labeling reagent, and formic acid) onto the PC, which react with peptides that have adsorbed onto the PC; and (C) Valve 3 switches, directing flow from the analytical pump (AP) through the PC, eluting peptides off the PC and onto the analytical column-mass spectrometer (LC-MS) by gradient elution.

Figure 2.

Calibration curve for LE using the one-column setup. The relative area is defined as the area of the light peptide (in Ringer's) divided by the area of the heavy peptide (100 pM LE in water). Error bars represent the SEM for $n = 2$ replicates.

Figure 3.

Quantitation of LE (blue), ME (black), and yaGfl (100 pM, red) (A) based on the peak area of the light labeled peptide and (B) based on the relative area, in which the peak area of the light peptide (in Ringer's) is divided by the peak area of the heavy peptide (100 pM in water). Black squares with blue centers indicate overlapping data points. Error bars represent the SEM of n = 2 replicates

Figure 4.

Profile of LE (blue) and ME (black) levels during sampling of a single rat. The first point (t=0 hours) was collected 15 minutes after probe implantation. Shaded areas represent points collected during 10-minute potassium stimulation. The relative area is defined as the peak area of the light peptide divided by the peak area of the heavy peptide.

Figure 5. Peptide detection during potassium stimulation. A) Chromatograms and corresponding mass spectra at the point of elution of B) light LE, C) heavy LE, D) light ME, and E) heavy ME during 10-minute stimulation with 100 mM KCl Ringer's. Light and heavy yaGfl spectra can be found in Figure S6.

Table 1

Slope and Intercept of the Calibration Curve Obtained using the Peak Area of the Light Peptide (Fig. 3A) and the Relative Peak Area^a (Fig. 3B)

Peptide	Peak Area of Light Peptide		Relative Peak Area	
	Intercept ^b ($\pm$ SEM ^c)	Slope ^d ($\pm$ SEM)	Intercept ^e ($\pm$ SEM) $\times 10^4$	Slope ^f ($\pm$ SEM) $\times 10^4$
LE	399 $\pm$ 337	76 $\pm$ 3	373 $\pm$ 115	91.7 $\pm$ 1.1
ME	-23 $\pm$ 194	48 $\pm$ 2	-26 $\pm$ 96	96.47 $\pm$ 0.98

^aDefined as the peak area of light peptide divided by the peak area of its heavy analog

^bUnits of Peak Area

^cSEM = standard error of the mean

^dUnits of (Peak Area)(Conc.)⁻¹

^eUnits of Relative Area

^fUnits of (Relative Area)(Conc.)⁻¹

Table 2

Mean and Standard Deviation^a of Light yaGfl Peak Areas (Fig. 3A) and Relative yaGfl Peak Areas^b (Fig. 3B)

Parameter	Peak Area of Light yaGfl ^c	Relative Peak Area ^d
Mean	16700	1.18
Standard Deviation	2200	0.078
Relative SD	0.13	0.066
Median	16400	1.21

^aStatistics are based on n = 12 measurements.

^bDefined as the peak area of light yaGfl divided by the peak area of heavy yaGfl

^cUnits of Peak Area

^dUnitless

Table 3:Estimated Concentrations^{a,b,c} of LE and ME in Dialysate

Rat	LE		ME	
	Basal Conc. (pM) ^c	K ⁺ Stimulated ^d Conc. (pM)	Basal Conc. (pM) ^c	K ⁺ Stimulated ^d Conc. (pM)
1	67 ± 12 (n = 3)	126 ^f	49 ± 8 (n = 3)	84 ^f
2	5.6 ± 1.2 (n = 2)	Not Performed ^e	3.7 ^f	Not Performed ^e
3	4.4 ± 1.2 (n = 4)	8.9 ^f	2.8 ^f	11 ^f
4	2.0 ^f	7.5 ^f	9.0 ^f	14 ± 2 (n = 2)

^aValues are not corrected for probe recovery.^bLevels were quantified using the regression statistics in Table 3^cConcentrations are expressed as the mean ± SEM, where the value in parenthesis indicates the number of measurements in which the peptide was detected^dBased on the first potassium stimulation^ePotassium stimulation was not performed on Rat 2^fMeasurements that do not have SEM values were only measured once (n = 1)

Table 4:Descriptive Statistics^a for the Relative Area^b of yaGfl

Parameter	Value
Mean	0.989
Standard Deviation	0.067
Standard Error	0.014
Median	0.982

^aStatistics are based on n = 23 measurements.^bThe relative area was calculated by dividing the peak area of light yaGfl (from the perfusate) by the peak area of heavy yaGfl (100 pM aqueous standard).