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Capillary liquid chromatography with MS³ for the determination of enkephalins in microdialysis samples from the striatum of anesthetized and freely–moving rats

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In vivo microdialysis sampling was coupled to capillary liquid chromatography (LC)/electrospray ionization quadrupole ion trap mass spectrometry (MS) to monitor [Met]enkephalin and [Leu]enkephalin in the striatum of anesthetized and freely–moving rats. The LC system utilized a high-pressure pump to load 2.5 µl samples and desalt the 25 µm i.d. by 2 cm long column in 12 min. Samples were eluted with a separate pump at ∼100 nl min⁻¹. A rapid gradient effectively separated the endogenous neuropeptides in 4 min. A comparison was made for operating the mass spectrometer in the MS² and MS³ modes for detection of the peptides. In standard solutions, the detection limits were similar at 1–2 pM (2–4 amol injected); however, the reproducibility was improved with MS³ as the relative standard deviation was <5% compared with 20% for MS² for 60 pM samples. For dialysate solutions, reconstructed ion chromatograms and tandem mass spectra had much higher signal-to-noise ratios in the MS³ mode, resulting in more confident detection at *in vivo* concentrations. The method was successfully used to monitor the peptides under basal conditions and with stimulation of peptide secretion by infusion of elevated K⁺ concentration. Copyright © 2005 John Wiley & Sons, Ltd.

INTRODUCTION

Neurons chemically communicate by releasing neurotransmitters and neuromodulators that interact with receptors on neighboring cells. An important route to understanding brain function is to monitor levels of neurotransmitters *in vivo* under different behavioral, physiological or pharmacological states. A powerful method of *in vivo* neurochemical monitoring is microdialysis sampling combined with chemical analysis of collected fractions. *In vivo* microdialysis has been widely used for monitoring some classes of neurotransmitters including amino acids, catecholamines and indoleamines.[1–3] This sampling technique has been used much less frequently for neuropeptides even though many neurotransmitters are peptides (at least 100 peptidergic neurotransmitters are known) and they have been implicated in many important functions.[4] A limiting factor in using microdialysis for neuropeptide monitoring is the extremely low concentration of peptides recovered by microdialysis sampling probes. Dialysate concentrations of endogenous peptides usually range from 1 to 100 pM;[5,6] therefore, even after collecting fractions for 10 min at 1 µl min⁻¹, only attomole quantities may be available in the entire sample. Hence analytical methods used for neuropeptides in dialysate must have both excellent concentration and mass detection limits.

The most common approach to the analysis of neuropeptides is immunoassay, particularly radioimmunoassay (RIA). This method can yield detection limits of 100 amol in microliter fractions, thus achieving adequate sensitivity.[6] While generally considered highly specific, great care must be taken in applying RIA because of the potential for cross-reactivity with peptides that contain similar epitopes.[7,8] Other techniques such as capillary liquid chromatography (LC) with electrochemical detection have been used for peptide analysis of dialysate with some success, although positive identification of compounds at such low levels is problematic with that technique.[9,10]

Advances in capillary LC coupled with tandem mass spectrometry have made this approach highly attractive as a method for determination of peptides *in vivo*. In this method, samples may be concentrated and desalted on a reversed-phase column. Elution at low flow rates provides

efficient electrospray ionization, yielding low attomole detection limits. Successful monitoring of endogenous peptides has been performed using a triple-quadrupole mass spectrometer operated in the single reaction monitoring mode[11,12] and a quadrupole ion trap (QIT) in the MS^2 mode.[13] Methods that allow full-scan MS^n increase confidence in measurement by yielding sequence specific analysis.[14]

In principle, further improvements in sensitivity and selectivity may be achieved by utilizing higher stages of mass spectrometry.[15,16] With each stage of mass spectrometry, both chemical noise and signal decrease; however, chemical noise decreases faster.[17,18] Therefore, the signal-to-noise ratio (S/N) should increase with higher stages of mass spectrometry as long as electronic noise does not become limiting.[19] While this principle is well known, it has rarely been applied to the determination of trace species. MS^3 has been primarily used in structural characterization or to investigate fragmentation pathways;[20–22] however, more recently, MS^3 data were utilized to quantitate structural isomeric[23] and isobaric mixtures[24] and various toxins[25,26] with good reproducibility and improved detection limits. Gas chromatography coupled to MS^3 detection is becoming important in quantitative environmental[27] and drug screening.[28] Improved sensitivity and selectivity make MS^3 an attractive method of detection for complex samples (i.e. biological samples such as microdialysate) that would otherwise be difficult to analyze accurately.

In this paper, we describe the first application of capillary LC–MS^3 to quantify peptides in microdialysate. The method is demonstrated to have improved selectivity and S/N for complex samples compared with LC–MS^2. Utilization of the method for measurement of [Met]enkephalin and [Leu]enkephalin reveals information on the regulation of the release of these pentapeptides into the extracellular space. As these peptides are agonists for opioid receptors, information on their regulation is important for studies of pain inhibition[29] and addiction among other processes.[30]

EXPERIMENTAL

Chemicals and reagents

Benzoin methyl ether, glycidyl methacrylate, toluene, trimethylolpropane trimethacrylate and 2,2,4-trimethylpentane were purchased from Sigma-Aldrich (St. Louis, MO, USA) and high-purity water, methanol and acetone from Burdick & Jackson (Muskegon, MI, USA). Liquid chromatography and spectrophotometry grade anhydrous alcohol was purchased from J. T. Baker (Phillipsburg, NJ, USA), glacial acetic acid from Fisher Scientific (Fairlawn, NJ, USA), hydrofluoric acid (HF) and sodium chloride from Acros Organics (Morris Plains, NJ, USA) and calcium chloride, magnesium sulfate and potassium chloride from ICN Biomedicals Inc., (Aurora, OH, USA). Peptide standards of [Met]enkephalin (YGGFM), [Leu]enkephalin (YGGFL) and neurotensin 1–11 (pELYENKPRRPY) were purchased from Sigma. Fused-silica capillary was purchased from Polymicro Technologies (Phoenix, AZ, USA).

Animals

Adult male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing between 295 and 399 g were used for all experiments. A total of 12 animals were used, eight for anesthetized and four for freely–moving experiments. The rats were kept in a temperature and humidity controlled room with 12 h light/dark cycles with food and water available *ad libitum*. Each rat was used only once and sacrificed on conclusion of the experiment. All animals were treated as approved by the University of Michigan Unit for Laboratory Animal Medicine (ULAM) and in accordance with the National Institute of Health (NIH) *Guidelines for the Care and Use of Laboratory Animals*.

Surgery

For experiments on freely–moving animals, aseptic surgical implantation of guide cannulae was performed approximately 1 week prior to recording experiments. Animals were anesthetized with initial intraperitoneal (i.p.) injections of 90 mg kg^{-1} ketamine (Fort Dodge Animal Health, Fort Dodge, IA, USA) and 15 mg kg^{-1} xylazine (Lloyd Laboratories, Shenandoah, Iowa, USA) with booster injections of 30 mg kg^{-1} ketamine given to maintain anesthesia. Guide cannulae (CMA Microdialysis, North Chelmsford, MA, USA) were positioned and implanted using a stereotaxic instrument (David Kopf Instruments, Tujunga, CA, USA) into the striatum (1 mm anterior and 2.8 mm lateral of bregma to a depth of 8 mm from dura)[31] and secured using cranioplastic cement (Plastics One Inc., Roanoke, VA, USA) and skull screws. Microdialysis probes (CMA Microdialysis) were implanted on the day of experimentation.

For experiments performed on anesthetized rats, animals were initially given an i.p. injection of 400 mg kg^{-1} chloral hydrate (Sigma) prepared in an isotonic salt solution with 50 mg kg^{-1} and booster injections given as needed to maintain the surgical plane of anesthesia. Probes were positioned as above and implanted in the striatum at the same coordinates. The probes were perfused for 1 h after implantation prior to data collection.

Microdialysis

Microdialysis probes with 4 mm active length and 20 kDa molecular weight cut-off (CMA/12, CMA Microdialysis) were used for all experiments. The microdialysis probe was connected to the capillary LC system using an ~50 cm length of 75 µm i.d. × 360 µm o.d fused-silica capillary tubing. The capillary used in freely–moving experiments was lengthened to ~90 cm to accommodate animal movement and covered with poly vinyl chloride (PVC) tubing (0.010 in i.d. × 0.030 in o.d; Small Parts Inc., Miami Lakes, FL, USA) to prevent breakage. Initial experiments with freely–moving rats utilized a swivel to prevent tangling of the microdialysate capillary, but proved unsuccessful as particulates generated inside the swivel obstructed column flow. This problem was resolved by using a swivel-less system (Raturn, Bioanalytical Systems, West Lafayette, IN, USA).

Probes were conditioned for at least 15 min with 70% ethanol at 3.0 µl min^{-1} prior to use. Artificial cerebral

spinal fluid (aCSF) containing 145 mM NaCl, 2.68 mM KCl, 1.01 mM $MgSO_4 \cdot 7H_2O$ and 1.22 mM $CaCl_2 \cdot 2H_2O$ was perfused at 0.6 µl min^{-1} through the probe using a Harvard syringe pump embedded in the mass spectrometer throughout the experiments. For experiments requiring stimulation for neuronal depolarization, the aCSF solution perfusing the probe was switched by means of valve to a high K$^+$ aCSF solution (30 or 70 mM for 15 min at the same flow rate) with NaCl reduced by a comparable amount to maintain ionic strength. All aCSF solutions were syringe filtered (0.02 µm, Anotop 10, Whatman, Maidstone, UK) immediately preceding use.

Microdialysis probe recovery was determined *in vitro* using solutions containing 580 pM [Met]enkephalin and 600 pM [Leu]enkephalin in aCSF at 37 °C. Recoveries averaged $20 \pm 10\%$ and $40 \pm 10\%$ ($n = 8$) for [Met]enkephalin and [Leu]enkephalin, respectively.

Following a recording session, the animal was sacrificed and the brain immediately removed and frozen at −80 °C until histology was performed. Microdialysis probe placement was verified by visual examination of 30 µm sections taken via cryostat (CM1850, Leika, Bannockburn, IL, USA).

Capillary liquid chromatography−mass spectrometry

In order to prepare an integrated chromatography column−electrospray emitter tip,[32] ∼500 µm long macroporous photopolymer frits were formed within 20 cm lengths of 25 µm i.d. × 360 o.d. fused-silica capillary *in situ* 5 cm from the terminus. The polyimide coating on the capillary was removed by rotating the capillary between two metal electrodes through which an electrical arc (7640 V, 60 Hz) was passed until the polyimide was eliminated. An additional 1 cm region of polyimide located ∼4 cm from the ending was removed for laser pulling by burning over a flame. Remaining debris was wiped away using a methanol-moistened Kimwipe (Kimberly-Clark Corporation, Roswell, GA, USA). Capillaries were filled with a polymer solution as described elsewhere.[13,33] The region to be pulled was concealed from UV light with a 330 µm i.d. PEEK tubing sleeve (Upchurch Scientific, Oak Harbor, WA, USA). The remaining region was exposed to UV radiation (254 nm, Spectroline, Westbury, NY, USA) for 30 min. Capillaries were flushed with acetone then dried with helium gas for 2 min each using an in-house built pressure bomb operated at 500 psi. The capillary was pulled to a fine point using a P-2000 CO$_2$ laser puller (Sutter Instrument Co., Novato, CA, USA). A two-line program was cycled once (line 1: heat = 300, velocity = 30, delay = 128, pull = 0; line 2: heat = 300, velocity = 30, delay = 128, pull = 125) with the micrometer set at 786.0 for glass pulling. The resulting tip was etched in 50% HF for 40 s then dipped in water to create a ∼3 µm electrospray emitter. A 2 cm length of the capillary was packed using an acetone slurry of reversed-phase particles (5 µm, Alltima C18, Associates Inc., Deerfield, IL, USA) at 200 psi as described elsewhere.[34] Although macroporous photopolymer frits could be batch produced and stored, we found it necessary to pack columns daily in order to alleviate tip breakage and prevent column drying. It was additionally more facile to pack columns daily rather than maintain them
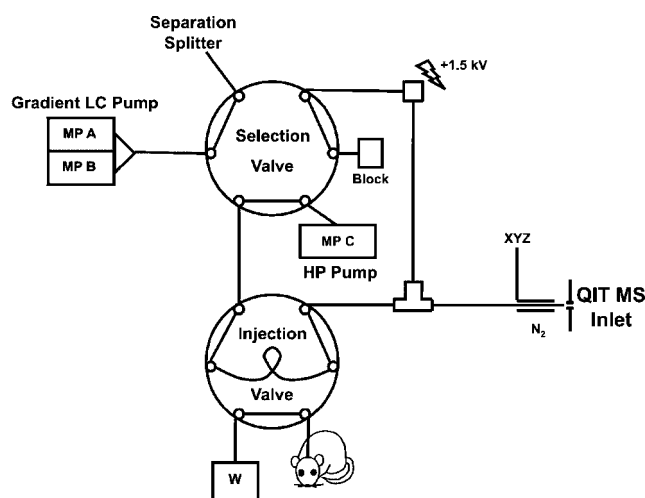

Figure 1. Diagram of the capillary LC−MS system.

overnight as particulates or aCSF crystals could clog the tip after extended use with microdialysate samples.

The capillary LC system was comprised of a dual pumping system utilizing an elevated pressure (3400 psi) pump (Haskel Inc., Burbank, CA, USA) for sample loading (7 min) while washing (5 min) and a lower pressure (∼600 psi) HPLC pump (MicroPro, Eldex Laboratories, Napa, CA, USA) for separation and elution (4 min) of the peptides. The different pressures allowed for high flow rates for sample loading and column washing while keeping low flow rates (∼100 nl min^{-1}) for a more efficient chromatographic separation and increased electrospray ionization efficiency. Two six-port valves (C2, Cheminert, Valco Instruments, Houston, TX, USA) connected the system as shown in Fig. 1 to allow for pump selection and sample injection. Dialysate was loaded online into a stainless-steel sample loop (5 µl, Valco Instruments) for injections. All connections were constructed using 75 µm i.d. × 360 µm o.d. capillary, except for the link between the injection valve and the splitting-tee immediately preceding the column, which was 50 µm i.d. × 360 µm o.d.

A rapid gradient was utilized for separation. Mobile phase A was composed of 1% acetic acid, 5% methanol in water and mobile phase B consisted of 1% acetic acid in methanol. Sample loading and washing were performed with 1% acetic acid in water. The mobile phase program consisted of an isocratic step (40% B, 1 min), a linear increase to 75% B over 30 s, followed by an isocratic step at 75% B for 1 min. All solutions were filtered weekly with an aluminum oxide filter (0.02 µm, Anodisc 47, Whatman) prior to use.

The chromatography system was coupled to the mass spectrometer (LCQ Deca, ThermoFinnigan, San Jose, CA, USA) using electrospray ionization (ESI). A +1.5 kV potential was applied to a liquid junction downstream of the splitting-tee (see Fig. 1). The emitter tip was positioned ∼0.5 mm from the heated capillary of the mass spectrometer using a micromanipulator operated in the *x*, *y* and *z* directions as described previously.[13]

The QIT was operated with the following parameters: positive mode, automatic gain control (AGC) on, maximum AGC time = 300 ms, $q = 0.25$, isolation width = 3 m/z, activation time = 30 ms, normalized collision energy = 40 and

33% for [Met]enkephalin, 42 and 33% for [Leu]enkephalin, mass range m/z 105.0–800.0, and the default number of microscans and target count values. Optimization of the ion optics was achieved by tuning during constant infusion of 180 nM neurotensin 1–11 in 50% methanol/1% acetic acid at 100 nl min^{-1} using MS² with collision energy = 35%. [Met]enkephalin and [Leu]enkephalin ions were isolated and fragmented using the MS³ pathways of m/z 574 → 397 → 278 + 323 + 380 and 556 → 397 → 278 + 323 + 380, respectively. Collision energies were optimized using constant infusion of enkephalins in 50% methanol/1% acetic acid with a flow rate of 100 nl min^{-1}. The identity of the granddaughter peaks used for quantification was based on a previous study.[35]

RESULTS AND DISCUSSION

Comparison of MS² and MS³

Initial experiments were performed to compare analytical figures of merit for the capillary LC–MS² and LC–MS³ determination of [Met]enkephalin and [Leu]enkephalin in standard solutions. Calibrations were performed by injecting standards dissolved in aCSF over the concentration range 60–1800 pM (Fig. 2). Each standard was analyzed in triplicate by both MS² and MS³ (the MS mode was alternated for each injection) before advancing to a higher concentration standard. Periodic injection of blank solutions between standards revealed that carry-over did not occur from previous injections when following the protocol described in the Experimental section. (Carry-over was observed if samples were dissolved in water, but was not noticeable if samples were dissolved in aCSF.) For all measurements, peak areas from the reconstructed ion chromatograms (RIC) were used for quantification as this proved more reproducible than peak heights. For both MS² and MS³ detection, linear responses were obtained over the concentration range tested with $R^2 > 0.99$ (see Fig. 2).

Detection limits, calculated as the concentration that would generate S/N = 3 in the RIC, were similar for both
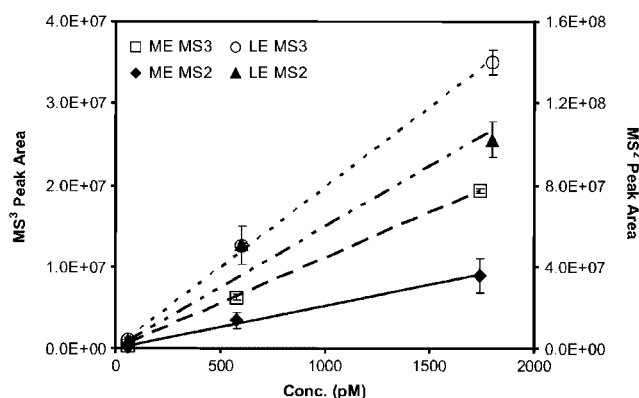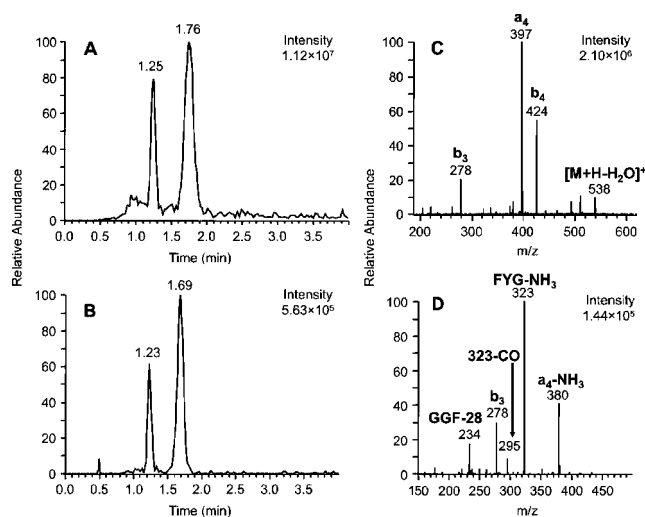

Figure 3. Mass chromatograms from separation of 60 pM enkephalin standards with corresponding [Leu]enkephalin spectra using MS² and MS³ for detection. (A) MS² TIC; (B) MS³ TIC; (C) MS² spectrum of [Leu]enkephalin (m/z 556 → 397 + 425); (D) MS³ spectrum of [Leu]enkephalin (m/z 556 → 397 → 278 + 323 + 380). [Met]enkephalin elutes first followed by [Leu]enkephalin. The values above the chromatographic peaks indicate the retention times in minutes. Spectral peaks that correlate with fragmentation are labeled.

methods at 2 pM (4 amol injected) for [Met]enkephalin and 1 pM (2 amol injected) for [Leu]enkephalin (compare the S/N for the methods in Fig. 3). Although the detection limits were similar, the MS³ method proved to be significantly more reproducible as the relative standard deviation (RSD) was 5% at 60 pM by MS³ whereas the same standard gave an RSD of 20% by MS². This difference was maintained at higher concentrations as the RSD was 1% in MS³ and 5% in MS² at 1800 pM ($n = 3$). Although for standard solutions the detection limits were similar, greater background discrimination was observed when analyzing actual dialysate samples (compare the chromatograms of standards in Fig. 3 with dialysate in Fig. 4). For dialysate samples obtained under basal conditions, [Met]enkephalin and [Leu]enkephalin peaks were not visible in the MS² total ion current (TIC) chromatogram but were obvious in MS³ mode (compare Fig. 4(A) and (C)). Similarly, in the RIC, much more baseline fluctuation, presumably from chemical noise, is observed for MS² than for MS³ (compare Fig. 4(B) and (D)). Although the signal present in MS³ was an order of magnitude lower than in MS², the chemical noise had diminished to a greater extent, allowing for increased S/N and improved sensitivity. *In vivo* microdialysate levels as low as 3 pM were detected by MS³; however, the spectral quality degrades at lower concentrations as incomplete fragmentation is observed as compared with higher concentration samples. Peaks are visible at lower concentrations, although unambiguous identification is hampered by partial fragmentation.

MS³ fragmentation spectra were similarly improved over MS² spectra for dialysate samples (Fig. 5). MS² provided two characteristic daughter ions (a₄ = m/z 397, b₄ = m/z 425) along with loss of water from the parent ion (m/z 556 and 538 from [Met]enkephalin and [Leu]enkephalin, respectively),

Figure 2. Calibration curves for [Met]enkephalin and [Leu]enkephalin standards prepared in aCSF using MS² and MS³ for detection. Signals are the mean peak areas from reconstructed ion chromatograms following injection of 2.5 μl of standards dissolved in aCSF. Error bars are ±1 standard deviation ($n = 3$ for each concentration). Different y-axis scales are used for MS² and MS³ as indicated.
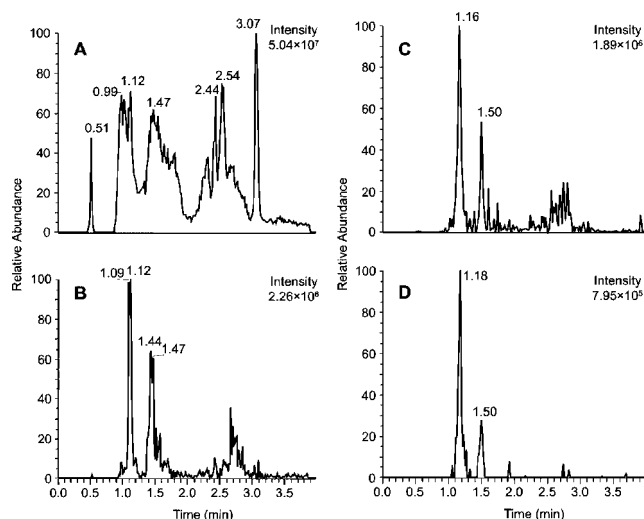
Figure 4. Comparison of LC–MS^2 and LC–MS^3 for samples collected from the striatum of an anesthetized rat under basal conditions (i.e. with aCSF as perfusion fluid). (A) MS^2 TIC; (B) MS^2 RIC (constructed using the base peak of m/z 397 and 425); (C) MS^3 TIC; (D) MS^3 RIC (constructed using the base peak of m/z 278, 323 and 380). [Met]enkephalin elutes first followed by [Leu]enkephalin. The values above the peaks indicate the retention times in minutes.
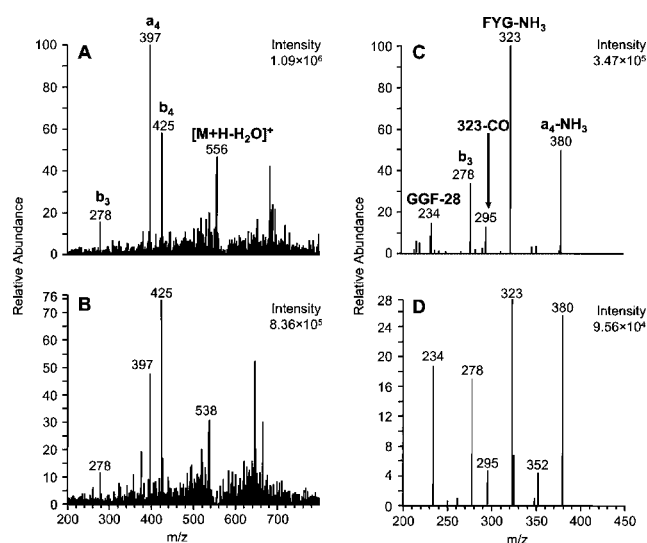

Figure 5. Comparison of MS^2 and MS^3 spectra for enkephalins acquired from dialysate samples collected from the striatum of an anesthetized rat under basal (non-stimulated) conditions. (A) MS^2 of [Met]enkephalin (parent ion m/z 574); (B) MS^2 of [Leu]enkephalin (parent ion m/z 556); (C) MS^3 of [Met]enkephalin (parent ions m/z 574 → 397); (D) MS^3 of [Leu]enkephalin (parent ions m/z 556 → 397). Peaks that correlate with fragmentation are labeled in the [Met]enkephalin spectra (omitted from the [Leu]enkephalin spectra for clarity).

MS^3 spectra can be assigned to the peptides, whereas the MS^2 spectra contain an abundance of extraneous ''noise'' peaks that are not correlated with the peptides. These peaks are due to interferences that have not been eliminated through the first level of ion isolation and fragmentation. Thus, for trace level analytes in complex samples, the additional selectivity of MS^3 improves the reproducibility, spectral quality and S/N. The cleaner spectra provided by MS^3 may also be useful for proteomic applications since it is expected that better peptide identification could be obtained by database searching programs such as SEQUEST or Mascot when spectra used for analysis are free of chemical noise.

In vivo monitoring of enkephalins by microdialysis coupled with capillary LC–MS^3

In order to demonstrate the suitability of capillary LC–MS^3 for *in vivo* monitoring, the method was used to analyze microdialysis samples collected online at 16.5 min intervals under basal conditions and during infusion of 70 mM K$^+$ through the probe in both awake, i.e. freely–moving ($n = 4$ animals), and anesthetized animals ($n = 4$) (Fig. 6). K$^+$ infusion stimulation evoked a substantial increase in peptide levels (see Fig. 6 and Table 1), due, presumably, to depolarization of neurons and subsequent stimulation of exocytosis. Stimulation in awake animals did not cause overt behavioral changes although quantitative analysis of movements was not performed. A second stimulation
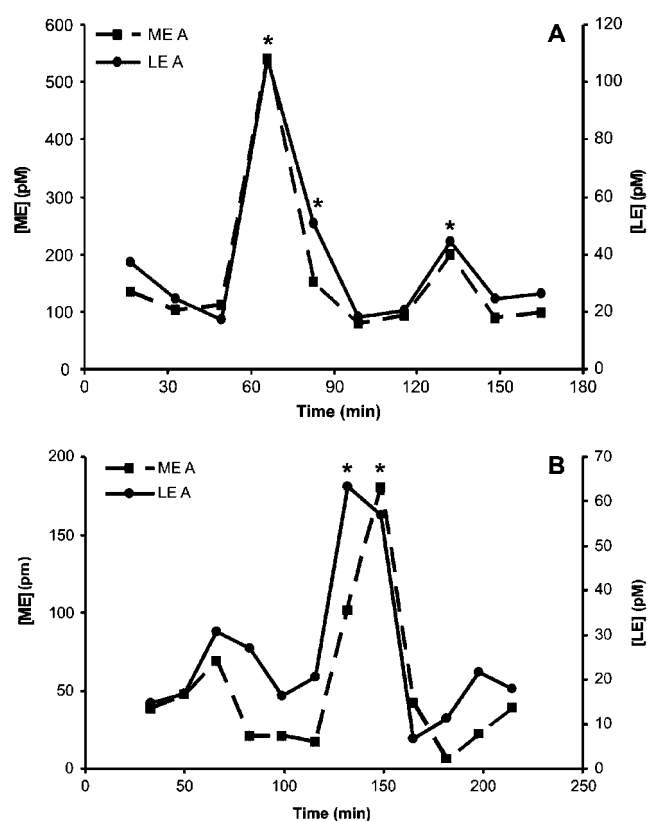

Figure 6. *In vivo* [Met]enkephalin and [Leu]enkephalin microdialysate concentrations over time from an individual (A) anesthetized and (B) freely–moving rat. 70 mM K$^+$ was infused for 15 min in order to stimulate a response. Fractions affected by the stimulation are marked with asterisks.

as seen in Fig. 5. MS^3 yielded five granddaughter ions (GGF − 28 = m/z 234, b$_3$ = m/z 278, FYG − NH$_3$ − CO = m/z 295, FYG − NH$_3$ = m/z 323 and a$_4$ − NH$_3$ = m/z 380). (This fragmentation pattern is similar to that reported previously for these peptides.[35]) Nearly every peak in the

Table 1. Summary of microdialysate levels of [Met]enkephalin (ME) and [Leu]enkephalin (LE) from striatum of anesthetized ($n = 8$ for basal and $n = 4$ for stimulated) and freely moving rats ($n = 4$)[a]

Rats		[Met]Enkephalin concentration ± SEM[b] (pM)	[Leu]Enkephalin concentration ± SEM[b] (pM)	ME : LE	ME : LE (corrected)
Anesthetized	Basal	110 ± 20*	36 ± 6	3.1 : 1	8.2 : 1
	Stimulated	670 ± 90**	180 ± 30*	3.7 : 1	10.1 : 1
Freely moving	Basal	50 ± 10	23 ± 3	2.2 : 1	2.0 : 1
	Stimulated	190 ± 30	60 ± 10	3.2 : 1	7.6 : 1

[a] Stimulated results were obtained by perfusing 70 mM K$^+$ aCSF solution for 15 min. Results from anesthetized rats were significantly higher than those from freely–moving animals as determined by a t-test (*$P < 0.025$, **$P < 0.005$) except for basal [Leu]enkephalin.
[b] SEM, standard error of the mean.

performed ∼60 min after the first in anesthetized animals also evoked significant release over basal levels but was reduced compared with the first (see Figs 6(A) and 7). The diminished response may reflect the inability of neurons to replenish peptide stores in the short time allowed between stimulation.

We observed that a lower concentration of K$^+$ (30 mM) infused for comparable times did not evoke any change ($n = 4$ animals) in peptide levels (see Fig. 7). This concentration of K$^+$ would generally be considered sufficient to depolarize neurons and cause release of neurotransmitter; however, it is often found *in vitro* that peptides require more vigorous stimulation (i.e. neuronal depolarization) to be secreted.[36,37] Therefore, our observations of a higher threshold for stimulated release are consistent with this model of peptide regulation.

These results also allow us to evaluate the effect of chloral hydrate anesthesia on the enkephalins. Anesthesia resulted in an increase in both basal and stimulated enkephalin levels except for basal [Leu]enkephalin levels (see Table 1). The increase in extracellular enkephalins observed here may participate in the anesthetic properties of chloral hydrate. Our results are seemingly in contrast to previous reports that

Figure 7. [Met]enkephalin and [Leu]enkephalin levels resulting from perfusion of aCSF with increasing concentrations of K$^+$ for 15 min into an anesthetized rat. Values are reported as a percentage of basal level. Basal levels were determined as the average of 2–5 assays collected prior to stimulation in each animal. Error bars represent ±1 standard error of the mean (SEM).

suggested a decrease in [Met]enkephalin basal release under chloral hydrate anesthesia *in vivo* based on measurements of tissue [Met]enkephalin and metabolites.[38] This difference may be related to the measurement techniques used. Micro-dialysis provides an index of extracellular concentration, which is a balance of release and degradation, whereas the tissue measurement is an estimate of release with several assumptions including no alterations in degradation rates. Based on these differences, several possible explanations for the discrepancy may be considered including effects of anesthesia on peptide degradation. Further studies would be required to understand better the effect of anesthesia on the enkephalins.

The enkephalins are produced by proteolysis of proenke-phalin A, prodynorphin and proopiomelanocortin within neurons. Earlier work showed that the [Met]enkephalin: [Leu]enkephalin ratio in striatal tissue was 2.3 : 1.[39–41] Our microdialysis measurements, which detect only extracel-lular levels, reveal a ratio of 2.2 : 1 to 3.7 : 1 depending on the conditions (see Table 1); however, if dialysate lev-els are corrected for differences in recovery by probes for the peptides (see Experimental) then the ratios are 2.0 : 1 to 10.1 : 1. The discrepancy between the tissue and dialysate [Met]enkephalin:[Leu]enkephalin ratios are likely due to extracellular processing of peptides after secre-tion. It is not clear from these results if the higher [Met]enkephalin:[Leu]enkephalin ratio results from more rapid removal of [Leu]enkephalin or production of more [Met]enkephalin in extracellular space. The extracellular [Met]enkephalin:[Leu]enkephalin ratio tends to increase with overall peptide concentration, i.e. when release rates are higher. This correlation suggests that interaction of extra-cellular processing and release rates can alter the mixture of peptides that appear in the extracellular space and hence the message sent by neurons.

Reproducibility and temporal resolution of method for monitoring

Variability for *in vivo* samples was considerably higher than that observed for similar concentrations of standards. The RSDs for the basal concentration within individual rats (at least three measurements for each rat) were from 6 to 57% for [Met]enkephalin and from 7 to 38% for [Leu]enkephalin with an average of 22 and 29%, respectively. Mean basal levels in different rats had RSDs of ∼35% (see Table 1). In contrast, the RSDs of standards at 60 pM were 5%. The intra-rat variability

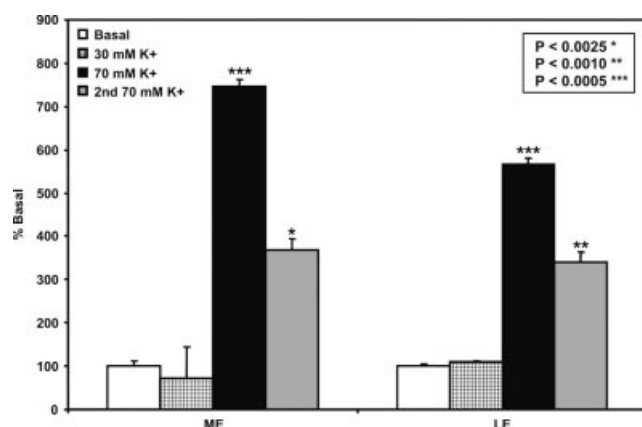may indicate natural fluctuations in peptide levels on the time-scale of these measurements whereas the variability between rats may indicate fluctuations due to differences in rats, probe placement and anesthesia (for anesthetized animals). From these results, it is concluded that the method has sufficient reproducibility to detect significant changes in levels of the peptides *in vivo*.

In previous work we performed monitoring experiments on [Met]enkephalin and [Leu]enkephalin by capillary LC–MS^2 but with 30 min separation times, resulting in a total sampling interval of 41 min.[13] In this work, the separation time was reduced from 30 to 4 min by using a steeper gradient, slightly faster flow rates and decreased column re-equilibration time between separations, thus allowing the sampling rate to be improved 2.5-fold. In principle, the sampling rate could be improved to 4 min because the sensitivity was sufficient to detect enkephalins in 2.5 µl of sample while sampling at 0.6 µl min^{-1}. In the configuration used, most of the sample is shunted to waste because samples were collected online and the complete analysis (loading, rinsing, separation, column equilibration) requires more time than that required to collect sample. Further improvements would require either faster analysis or sample handling that allowed samples to be collected off-line. In preliminary experiments, we have observed that freezing of samples after collection results in substantial loss of sample, therefore off-line analysis will require identification of suitable sample storage conditions.

CONCLUSION

The capillary LC–MS^3 method described here provides detection limits in the low attomole range and is less susceptible to interferences from complex samples than capillary LC–MS^2. Reproducible MS^3 spectra devoid of extraneous peaks were obtained even at low picomolar concentrations in complex mixtures. The improved background rejection may be of use in proteomic applications and is useful for improving quantification at low levels. The sensitivity achieved for enkephalins is sufficient to allow routine monitoring in dialysates collected *in vivo*.

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