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Monitoring of met-enkephalin in vivo with 5-min temporal resolution using microdialysis sampling and capillary liquid chromatography with electrochemical detection

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Abstract

A method based on microdialysis sampling and capillary liquid chromatography with electrochemical detection that allows in vivo monitoring of met-enkephalin with 5-min temporal resolution is described. Sampling was achieved using a concentric microdialysis probe made from polycarbonate membrane material with a 20 kDa cut-off. This probe had an in vitro relative recovery for met-enkephalin of 63% at a dialysis flow-rate of 0.6 μl/min. Separations were performed using 7 cm×25 μm I.D. fused-silica capillary columns packed with 5 μm Alltima C_{18} particles. A carbon fiber microelectrode was used as the detector electrode. The mass detection limit for met-enkephalin with this system was 40 amol. With on-column preconcentration, up to 2 μl of sample could be loaded onto the column resulting in concentration detection limits as low as 20 p*M* for met-enkephalin. Direct injection of dialysate, collected at 5-min intervals, allowed determination of met-enkephalin concentrations in the rat globus pallidus under basal and K^+-induced depolarization conditions.

Keywords: Enkephalins

1. Introduction

Neuropeptides are an important group of neuroactive substances which can act as neurotransmitters, neuromodulators, and neurohormones throughout the central nervous system [1]. Compared with other classes of neurotransmitters, such as monoamines or amino acids, neuropeptides are more difficult to detect and monitor in the extracellular space of the brain because they tend to be found at much lower concentrations. For example, peptides are typically found in the picomolar range, while monoamines are present at nanomolar concentrations and excitatory amino acids are present at micromolar concentrations [2].

By far the most common method for monitoring neuropeptides in the extracellular space of the brain is microdialysis sampling coupled with radioimmunoassay (RIA) [3–5]. An important consideration in using microdialysis for chemical monitoring is the temporal resolution that is possible [6,7]. High temporal resolution is especially desirable in neurochemical applications where rapid extracellular concentration changes of neurotransmitters are often

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associated with neurological events or manipulations [8]. With microdialysis sampling, temporal resolution is usually determined by the amount of time required to remove a detectable quantity of analyte. This time is determined by two factors: (1) absolute recovery, defined as the amount of material removed from the sampling environment per unit time by the dialysis probe and (2) the mass detection limit of the assay used with the sampling probe. High absolute recovery and low mass detection limits allow fractions from the microdialysis sample stream to be collected frequently for high temporal resolution. Absolute recovery tends to be quite low with analytes which are present at low concentrations like neuropeptides.

As suggested above, the low level of neuropeptides in vivo makes it difficult to achieve high temporal resolution when monitoring using microdialysis. The temporal resolution possible with microdialysis–RIA is typically about 20–30 min [3–5]. A good example is the case of monitoring met-enkephalin in the globus pallidus, a region rich in met-enkephalin, where the extracellular concentration is estimated to be 150 p*M* [3]. In this application, the dialysis flow-rate was 2.7 μl/min and the absolute recovery was approximately 36 amol/min. The RIA had a mass detection limit of 0.2 fmol; therefore, it was necessary to collect dialysate at 30-min intervals (81 μl fractions) in order to recover a quantifiable amount of peptide. Thus, the temporal resolution for this application was limited to 30 min. While 30-min sampling intervals have been valuable for many studies of neuropeptides, improved temporal resolution will greatly improve the opportunity to study the function and regulation of these compounds. Further improvements in temporal resolution for microdialysis-based monitoring of neuropeptides will require developing assays that have mass detection limits superior to RIA. With better mass detection limits, dialysate fractions could be collected at shorter intervals for better temporal resolution.

Capillary electrophoresis (CE) and capillary liquid chromatography (LC) are microscale separation techniques that have mass detection limits of a few attomoles or better (for review see [9]), and therefore have the potential to significantly improve temporal resolution for microdialysis- based chemical monitoring. Several recent papers have addressed the issue of using CE, especially with laser-induced fluorescence detection, to improve temporal resolution for microdialysis sampling in the brain [10–13].

Capillary LC with electrochemical detection (ED) [14] is another attractive technique to couple with microdialysis. Capillary LC–ED would be especially useful for monitoring tyrosine and tryptophan-containing oligopeptides, such as the opioid peptides, since these peptides are electroactive and can be detected directly without derivatization [15–17]. A limitation of capillary LC–ED is that even though its mass detection limits are low enough to possibly allow improved monitoring of peptides by microdialysis, its concentration detection limits are not sufficient to detect basal levels of peptides in dialysates. This problem is caused by the fact that low mass detection limits in capillary LC–ED are usually achieved with minuscule injection volumes, on the order of a few nanoliters, and relatively high concentrations. In one report for example, a detection limit of 36 amol for amino acids was achieved with capillary LC–ED when using a 7-nl injection volume corresponding to a concentration detection limit of 5 n*M* [18]. While the attomole detection limit is impressive, the nanomolar concentration detection limit would not be sufficient for detecting peptides in dialysate. A potential solution to this problem is to preconcentrate dilute samples on-column to take advantage of the low mass detection limit and improve the concentration detection limit [19,20].

In the present work, we utilize preconcentration on-column in conjunction with capillary LC–ED to monitor met-enkephalin in dialysates. Met-enkephalin was chosen for these studies since it should be easily detectable by ED [16,17] and is an important neuropeptide [21–23]. The biomedical significance of met-enkephalin is due to its implications in neurological disorders such as Huntington's [24,25] and Parkinson's disease [26–28], as well as its involvement in regulation of several important physiological functions such as pain transmission [29] and prolactin secretion [30]. The technique described here allows met-enkephalin to be monitored with 5-min sampling intervals, which is a six-fold improvement in temporal resolution over that previously possible using RIA or mass spectrometry (MS) for detection [3,31]. The improved temporal resolution is due primarily to the high mass sensitivity of capillary LC–ED relative to these other techniques. The method should be generally applic-

able to monitoring a variety of tyrosine and tryptophan-containing oligopeptides in the brain. The possibility of further improvement in temporal resolution is also discussed.

2. Experimental

2.1. Reagents and mobile phase

Unless specified otherwise, all chemicals were purchased from Sigma (St. Louis, MO, USA). The aqueous portion of the mobile phase (solvent A) for reversed-phase capillary liquid chromatography was 1 m*M* phosphate buffer in 10 m*M* sodium sulfate adjusted to pH 7.0. The organic phase (solvent B) was prepared by mixing 40% (v/v) of aqueous buffer with 60% acetonitrile. Artificial cerebral spinal fluid (aCSF) used for microdialysis perfusion consisted of 145 m*M* NaCl, 2.68 m*M* KCl, 1.01 m*M* $MgSO_4$, and 1.22 m*M* $CaCl_2$. The high K^+ perfusate solutions for stimulation experiments consisted of 2.62 m*M* NaCl and 145 m*M* KCl with other salts the same as aCSF.

2.2. Preparation of capillary reversed-phase columns

Capillary columns were prepared by previously described techniques [32] using 7–8 cm lengths of 25 μm I.D., 360 μm O.D. fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) as the column blanks. Columns were slurry-packed with 5 μm Alltima C_{18} (Alltech, Deerfield, IL, USA) reversed-phase particles (slurries consisted of 10 mg packing material in 3 ml acetonitrile) at 1.2 to 1.4 MPa using a pneumatic amplifier pump (Cat. No. 1666, Alltech).

2.3. Capillary LC

A schematic of the LC system used in this work is shown in Fig. 1. Mobile phase flow was generated using a SSI 232D HPLC gradient pump (Scientific Systems, State Collage, PA, USA) through a splitter tee. A two-valve injection system was designed to minimize both waste of sample and dead volume between sample and column. The first six-port two-position valve (Valco Instruments, Houston, TX, USA) was used to connect the sample capillary and the pump. The outlet of the sample capillary was connected to a cross (Valco) which was constructed so that 360 μm O.D. capillaries fit snugly inside. One port of the cross was connected to a second six-port switching valve, another was connected to the column, and the last port was normally plugged. The sample capillary and column were positioned inside the cross under a microscope with a small gap between them in order to minimize dead volume. After the capillaries were correctly positioned, the last port of the cross was sealed with a plug. The use

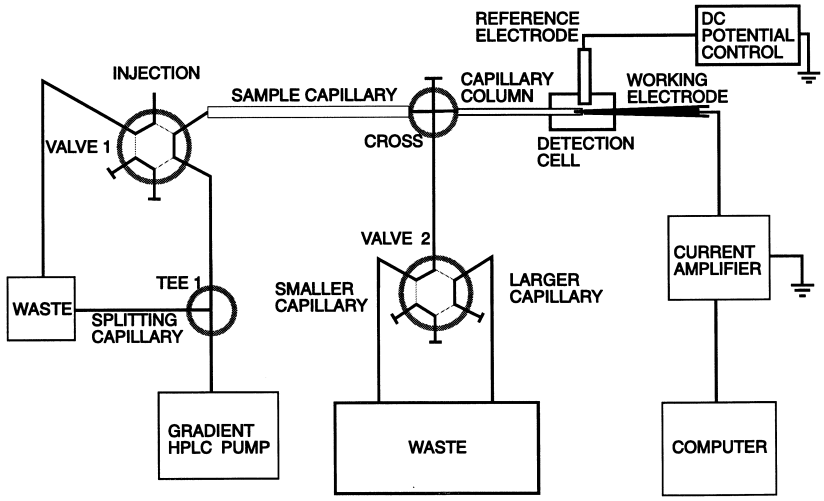

Fig. 1. Block diagram of capillary LC–ED system. See text for detailed description.

of a cross instead of a tee facilitated capillary positioning. The second six-port switching valve allowed the cross to be connected to two different splitter capillaries, the larger one 15 cm×50 μm I.D. and the smaller one 6 cm×25 μm I.D.

Injections were made by switching the sample capillary from the high pressure flow path using the first valve. The second valve was connected to the larger I.D. splitting capillary and a micro syringe used to load sample into the sample capillary. Such loadings left the sample capillary filled and excess sample in the larger splitter capillary with insignificant sample entering the column because of the flow resistance. To transfer sample onto the column, the second valve was closed and the sample capillary reconnected to high pressure by switching the first six-port switching valve. After the sample was loaded onto the column, the second valve was switched to the smaller I.D. splitting capillary. This splitter served to (1) allow faster displacement of mobile phase in the sample capillary to give more prompt response to the gradient and (2) to provide extra pulse dampening to reduce the pumping noise. For all of the separations and injections approximately 1.3 MPa was applied onto the separation column giving a flow-rate of ~2 nl/s and a linear velocity of 0.6 cm/s.

To maintain the best detection limits and minimal drift from large volume injections the aqueous buffer was prepared fresh and filtered every day before the experiment. In addition, after loading the column was rinsed with 5% solvent B–95% solvent A for 5 min before gradient elution started. Gradient elution started at 5% solvent B, linearly changed to 50% solvent B in 5 min, kept at this composition for 2 min, and then stepped back to 5% solvent B.

2.4. Electrochemical detection

A 9-μm diameter, 2-mm long carbon fiber electrode was used as the detector electrode [33]. The detector electrode was inserted into the outlet of the column using a micropositioner. The outlet of the column was mounted in a cell containing 1.0 *M* NaCl as electrolyte and fitted with a Ag/AgCl reference electrode. Unless stated otherwise, the detector electrode potential was set at 1.0 V versus this reference using a battery and voltage divider. The detector current was amplified by a SR570 current preamplifier (Stanford Research Systems, Sunnyvale, CA, USA). Data were collected using a 486 personal computer (Gateway, Sioux City, SD, USA) and data acquisition board (AT-MIO-16F-5, National Instruments, Austin, TX, USA). Software was developed in-house using LABWINDOWS (National Instruments).

2.5. Microdialysis

Microdialysis sampling was performed using CMA/10 probes (CMA/Microdialysis, Acton, MA, USA) made from polycarbonate membrane material with a 20 kDa cut-off. The concentric probes had 0.5-mm diameters and 4-mm tip lengths. Per manufacturer specifications, the probe was rinsed with ethanol and then buffer for 5 min each before use. It was found that an overnight rinse with aCSF buffer further decreased impurity peaks in chromatograms of dialysate. During experiments, the probe was perfused with aCSF using a microliter syringe pump (Harvard Apparatus 553206, South Natwick, MA, USA) at 0.6 μl/min unless stated otherwise. Dialysate samples were collected every 5 min and immediately stored at −4°C. Samples were equilibrated to room temperature before capillary LC–ED analysis.

2.6. Surgical preparation and procedures

Male Sprague–Dawley rats weighing 300–425 g were anesthetized with subcutaneous injections of 100 mg/ml of chloral hydrate. The initial injection was 4.0 ml/kg. Booster injections of 2.0 ml/kg were given every 30 min until the animal no longer exhibited limb reflex. After surgery, the rat was kept unconscious with subcutaneous administration of 1.0 ml/kg chloral hydrate as needed. Once the rat was secured in the stereotaxic apparatus, the microdialysis probe was placed in the globus pallidus ventral pallidum to the coordinates −1.0 mm anterior–posterior, −3.0 mm medial–lateral, +9.0 mm dorsal–ventral from bregma [34]. Basal level chromatograms were taken until they stabilized which was typically 2 h after insertion of the dialysis probe.

2.7. Data processing

To correct for drift in the chromatographic traces, some chromatograms were processed using a variant of median filtering described elsewhere [35]. For typical chromatograms where the data acquisition rate was 5 Hz and the peak width was 15 s, a window size of about twice the peak width was used to calculate the medians of raw data. By subtracting the corresponding medians from raw data, the baseline drift could be largely eliminated. If more than one peak was inside the filter window, and the total peak width (sum of each peak width) was more than 50% of the selected window size, a larger median filter window was chosen.

3. Results and discussion

3.1. Electrochemical detection of met-enkephalin

For detection of met-enkephalin, the best signal-to-noise ratio was obtained with +1.0 V applied to the electrode. The conversion efficiency was calculated to be 100% at this voltage and flow-rate. For example, for injection of 2.0 fmol, the peak area was 191±13 pC. Assuming a 1 electron transfer, we would expect an area of 193 pC at 100% conversion. Under these conditions, the root mean square (RMS) noise level was typically 300 fA and the detection limit, calculated as the amount of analyte injected that would yield a peak three times the RMS noise, was 40 amol or 2 n*M* for a 20-nl injection. Detection limits for capillary LC–ED have been reported as low as 1–10 amol [14] and for CE–ED as low as 0.5 amol [36,37] for catechols. The higher detection limit for met-enkephalin was due to several reasons. First, the detection of tyrosine-containing peptides required higher voltages than catechols which in turn increased the background noise. The noise increase is exacerbated by the fact that EDTA, a commonly used mobile phase additive to reduce background noise, actually caused an increase in background noise when the detector electrode was higher than +0.8 V (data not shown). A second reason for the higher detection limit was that the gradient used during the separation increased the background drift and noise relative to the other cases where isocratic separations were performed. Considering these factors, it is expected that the detection limit in this case would be comparable to that achieved for derivatized amino acids (36 amol) in a similar chromatographic system [18].

In order to maintain the lowest detection limits and stable responses, it was necessary to avoid electrode deterioration caused by the peptide. Electrode fouling caused a 20% decrease in response after injection of 1 fmol of met-enkephalin. Fouling of electrodes has been seen before with electrochemical detection of tyrosine-containing peptides and can be attributed to adsorption of oxidation products [38]. The fouling effect was eliminated by applying a triangular waveform between −0.90 V and +1.10 V at 300 V s−1 for 20 s at the beginning of a series of chromatograms. After this pretreatment, the electrode stability was improved so that six consecutive injections of 1 fmol met-enkephalin gave a relative standard deviation of 7.4%. This electrode waveform has previously been reported by Edmonds and Ji [39] for activating carbon electrodes.

3.2. Use of large injection volumes

The mass detection limit of 40 amol for met-enkephalin by capillary LC–ED is superior to that obtained by RIA thus suggesting its possible application to assay of microdialysis samples; however, the relatively high concentration detection limit of 2 n*M* with a 20-nl injection would make capillary LC–ED unsuitable for that application. Therefore, we explored on-column preconcentration to further improve the concentration detection limit.

The ease of preconcentration is a key advantage of capillary LC over CE for detection of trace-level analytes in biological samples such as microdialysates and is the main reason that we chose capillary LC instead of CE for this application [19]. In on-column preconcentration, the sample is injected in a weak mobile phase so that it is retained at the head of the column and concentrated. Analytes are eluted with a stronger mobile phase after the less retained solutes are washed out. The greater the retention factor (k) for analyte in the sample solution, the greater the preconcentration that can be achieved. When the k value in the injecting solution is too low, the peaks can begin to migrate down the column

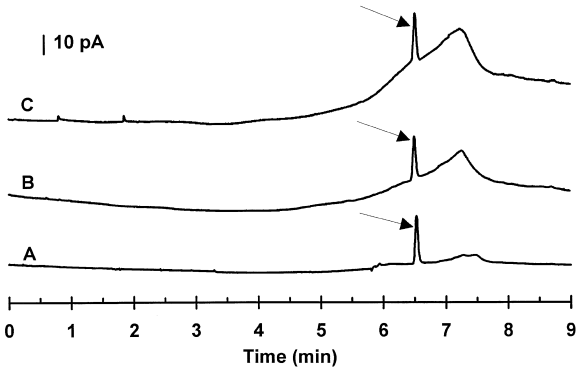

Fig. 2. Comparison of chromatograms for different volume injections. Met-enkephalin injections were (A) 10 nl of 80 n*M*, (B) 100 nl of 8.0 n*M* and (C) 1000 nl of 0.80 n*M*. Separation conditions are given in Section 2.

during a large volume injection resulting in broadening or breakthrough giving rise to variable retention times and peak areas or excessive peak widths.

We found that the Alltima C_{18} stationary phase allowed excellent retention of met-enkephalin when injected in dialysate solutions as illustrated by the data in Fig. 2 and Table 1. In this experiment, the total amount of analyte was kept constant while the injection volume was increased. As shown by the figure and quantified in the table, a constant peak area was obtained indicating no loss of the analyte during preconcentration. The retention time was also unaffected by the injection volume indicating that the met-enkephalin did not elute during the sample loading. Finally, the variances of the analyte peak were also unchanged indicating no broadening of the zone during preconcentration. These results show that even with a sample volume of 2 μl, which is equivalent to at least 59 column volumes (capillary volume is estimated to be 34 nl), the met-enkephalin did not significantly migrate through the column. These results indicate that the concentration detection limit can be improved by over 100-fold when compared with an injection volume of 20 nl on the column (see Table 1).

For all subsequent work we utilized 1-μl injection volumes. Larger injection volumes could be used for better concentration detection limits; however, this would result in a diminishing return in concentration detection limit for injection time. For example, increasing injection volume from 0.2 μl to 1 μl improves the detection limit five-fold to 40 p*M* for an increase in injection time of 6.6 min. (At 2 nl/s, the injection time was 1.6 min for 0.2 μl and 8.3 min for 1 μl). A further two-fold improvement in detection limit to 20 p*M* would require another 8.3 min for injection for a total of 16.6-min injection time. This time is significant relative to the total analysis time.

Quantitative aspects of the system were explored using 1-μl injection volumes. To achieve complete loading of 1 μl onto the column using a 1-μl sample capillary, it was found 3 μl of sample had to be injected into valve 1 (see Fig. 1). Presumably, this overfilling requirement was due to dead volume and inefficient rinsing of the tubing used in the system and a more efficient design could reduce the amount of sample needed to make 1-μl injections. Using 1-μl injections, the system had linear dynamic range for met-enkephalin from 100 p*M* to at least 10 n*M*. A typical calibration curve covering this concentration range was given by the following equation:

Table 1
Effect of large injection volume on characteristics of met-enkephalin peak

Injection volume[a] (nl)	Concentration of sample (n*M*)	Retention time (s)	Height of peak (pA)	Limit of detection[b] (p*M*)	Variance of peak (s^2)
10	80.0	382	23.1	4200	0.13
30	26.7	383	24.2	1300	0.14
100	8.00	387	20.4	470	0.14
500	1.60	386	21.5	89	0.13
1000	0.80	388	23.3	41	0.14
2000	0.40	384	22.1	22	0.13

[a] For each injection, the mass injected was the same.
[b] Calculated as the concentration that would give a signal three times the RMS noise.

peak area (pC)=88.4 pC/n*M*×concentration (n*M*)+ 14.4 pC with a correlation coefficient of 0.998.

The large preconcentration used with these capillary columns resulted in several practical problems. The first was that the preconcentration step concentrated impurities in the water and buffers resulting in baseline drift and extra background peaks. For example, in Fig. 2 a large broad peak, presumably due to detection of preconcentrated impurities, increased with injection volume. It was found that many of these interferences could be decreased by rinsing the column with 5% solvent B for 5 min as described in Section 2.5. A comparison of injections with and without the rinsing step illustrating the decrease in background is shown in Fig. 3. Even with rinsing, a significant background peak remained as shown in Fig. 3. Furthermore, this peak was somewhat variable as seen by comparing the chromatograms of Figs. 2 and 3 which were obtained on different days. This variability was presumably due to different levels of impurity caused by differences in filtering and water quality. Another problem encountered with large volume injections was that the electrode sensitivity decreased by up to 50% after loading a 1-μl sample dissolved in high salt solutions such as aCSF. The reason for this deactivation is unclear; however, it was avoided by setting the potential of the detector electrode at 0.0 V during the loading of large samples. Despite these problems, the method allowed reliable detection of trace-level of met-enkephalin with a simple large-volume injection technique.

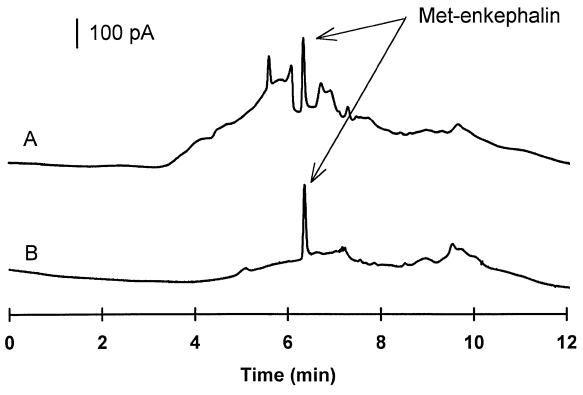

Fig. 3. Effect of column rinsing on chromatograms (A) 5 n*M* met-enkephalin was injected without rinsing the column with 5% solvent B (B) the same met-enkephalin concentration was injected and the column was rinsed for 5 min with 5% solvent B after injection as described in the experimental section. Injection volumes and sample solvent were the same for both chromatograms. Separation conditions are given in Section 2.

3.3. Characterization of microdialysis probe

Temporal resolution in microdialysis sampling is typically limited by the mass sensitivity of the analytical method and the absolute recovery of the probe. Absolute and relative recoveries for met-enkephalin at 37°C by the probe utilized in this work are shown in Fig. 4. In order to maximize signal and temporal resolution, we used a flow-rate of 0.6 μl/min since at this flow-rate the absolute recovery begins to level off. At this flow-rate the relative recovery was 63±3%. Below this flow-rate, less analyte were removed per unit time and temporal resolution was compromised. Above this flow-rate, there was more dilution and larger samples had to be injected. Thus, temporal resolution may be enhanced at a higher flow-rate, but this is achieved at a higher flow-rate.

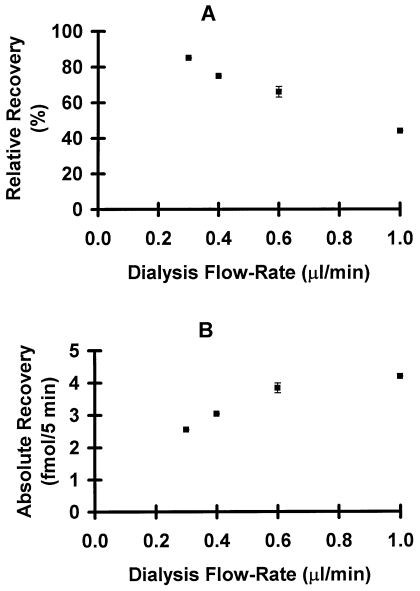

Fig. 4. Relative recovery and absolute recovery of the microdialysis probe at different flow-rates at 37°C. The met-enkephalin concentration was 2 n*M*.

3.4. In vivo measurements

The requirement of 3 μl of sample for injection and a dialysis flow-rate of 0.6 μl/min suggested that samples could be collected as often as every 5 min if the system had sufficient detection limits to allow determination of the peptide of interest. Based on our detection limit of 40 p*M* for 1-μl injections, the high relative recovery of the probe at 0.6 μl/min, and previous observations by RIA [3], it seemed likely that we could use 5-min sampling times for in vivo measurements of met-enkephalin. Fig. 5 shows chro-

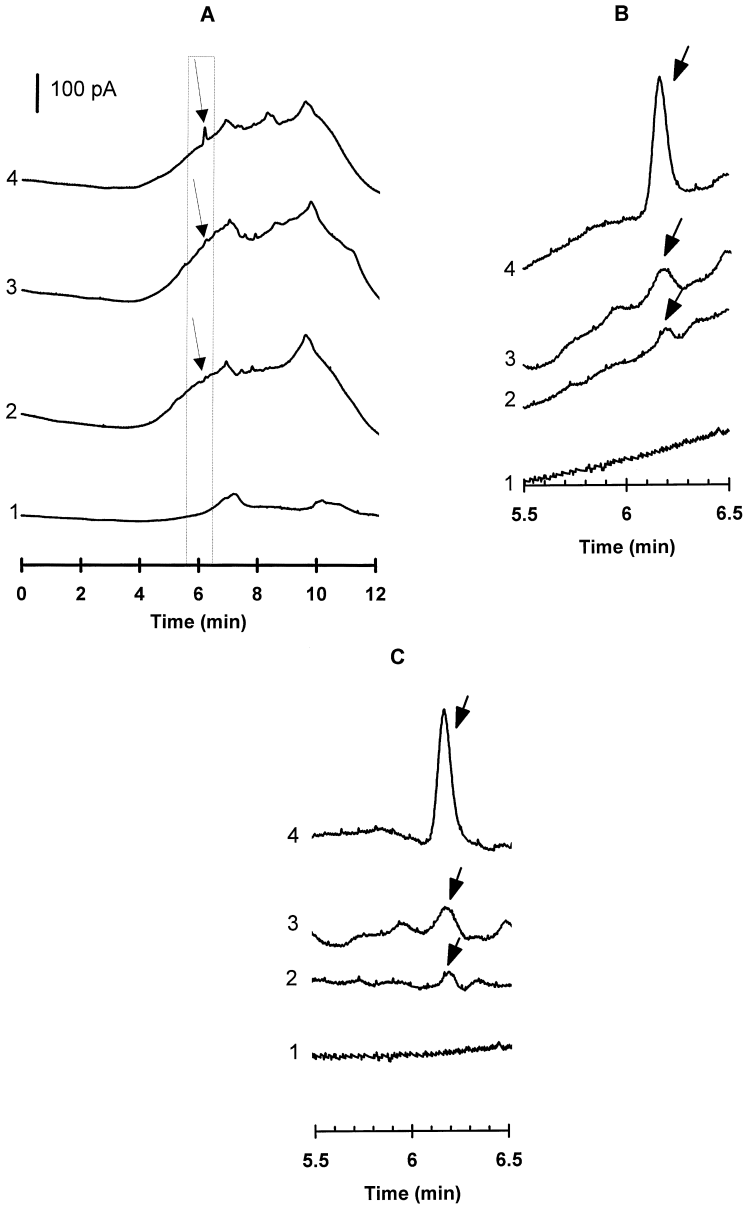

Fig. 5. Chromatograms obtained from (1) 1-μl injection of aCSF, (2) dialysate under basal conditions, (3) dialysate during K^+ depolarization and (4) 5 n*M* met-enkephalin standard. For each injection, the figure shows the complete, unfiltered chromatograms (A), the section immediately around the met-enkephalin peak as outlined by the dashed line (B), and the same section after a median filter has been applied as described in the text (C). The arrow indicates the met-enkephalin peak in each trace. Separation conditions are given in Section 2.

matograms obtained from in vivo experiments utilizing 5-min sampling times. This figure illustrates chromatograms obtained for injection of dialysate under basal conditions, dialysate during perfusion with high K^+, a standard sample, and a blank solution. The large drift in the baseline makes it difficult to discern the peaks unless the median filter is used over the section of interest as seen in Fig. 5C. As shown, the chromatogram from dialysate at basal conditions has a peak that matches the retention time of met-enkephalin at a level just above the detection limit. Furthermore, this peak increases with increasing K^+ in the dialysis probe as expected for a neurotransmitter under depolarizing conditions. Based on the matching retention time, the response to K^+, and the results of experiments in which authentic met-enkephalin was spiked into the dialysate samples (not shown), this peak has been attributed to the detection of met-enkephalin. Using this technique, the dialysate concentration of met-enkephalin was determined to be 111 ± 35 p*M* ($n = 13$) under basal conditions.

Fig. 6A illustrates the results of monitoring met-enkephalin concentration in vivo in a single rat with 5-min temporal resolution during an infusion of K^+ in the probe. Fig. 6B shows similar data averaged from five different rats. During infusion of K^+ the met-enkephalin level was immediately elevated to an average of 3.2 ± 1.8 times the basal level. After the K^+ stimulant was removed, the met-enkephalin level lowered, but it did not always stabilize at the basal level.

The met-enkephalin levels obtained in this work are in good agreement with previous results obtained using microdialysis sampling with RIA [3] and MS [31] if the differences in relative recovery are taken in to account. In addition, the change in met-enkephalin is comparable to that previously reported during K^+-stimulation [3,31]. The LC–ED system, however, allowed samples to be collected with six-fold higher temporal resolution than either the RIA or the MS method. This difference can be attributed to a mass detection limit that was five-fold lower and a smaller sample volume requirement. The higher temporal resolution allowed several novel observations to be made. For instance, these results show that neuropeptide concentration increased within the first 5 min of K^+-stimulation. Furthermore, our results show that within the 30-min stimulation

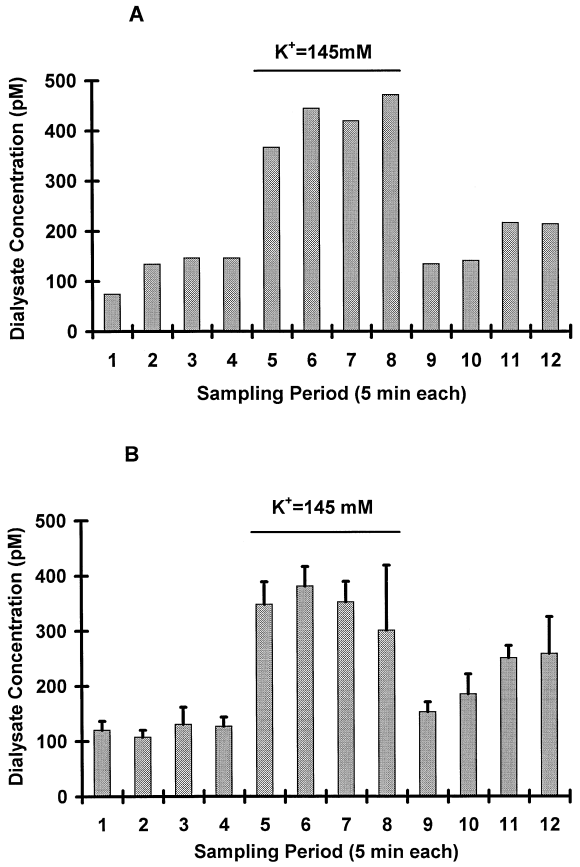

Fig. 6. Monitoring of met-enkephalin level in rat brain with 5-min temporal resolution using capillary LC–ED. The bar indicates application of high K^+ aCSF (corrected for dead volume). (A) is from an individual rat and (B) is the average with one standard deviation from five rats.

period, the release of met-enkephalin is sustained at a constant level. With only 30-min sampling intervals, such observations would not have been possible. In combination with behavioral studies or pharmacological manipulations, the improved temporal resolution should be more valuable.

4. Conclusions

The work presented here demonstrates that aqueous samples can be preconcentrated by over 100-fold on-column to give concentration detection limits as low as 20 p*M* for met-enkephalin. This sensitivity and sample requirement allow capillary LC–ED

combined with microdialysis sampling to be used for monitoring met-enkephalin in vivo with 5-min temporal resolution. The prospects for improving temporal resolution to less than 1 min seem good. First, the sample injector wasted 66% of the 3-μl samples, therefore a three-fold gain could be made by utilizing all of the sample for injection. A system compatible with higher pressures could allow larger volumes to be injected more quickly which, in turn, would allow practical use of larger volume samples. Thus, higher dialysis flow-rates, with subsequent higher absolute recovery could be used, resulting in better temporal resolution. Finally, the possibility of derivatizing peptides using the biuret reaction for electrochemical detection offers the possibility of extending this approach to non-electroactive peptides [40,41].

Acknowledgements

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