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DETERMINATION OF LEUCINE ENKEPHALIN AND METHIONINE ENKEPHALIN IN EQUINE CEREBROSPINAL FLUID BY MICROBORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY ZONE ELECTROPHORESIS COUPLED TO TANDEM MASS SPECTROMETRY

WOLFGANG M MUCK[a] and JACK D HENION*

Equine Drug Testing and Toxicology Program, New York State College of Veterinary Medicine, Cornell University, 925 Warren Drive, Ithaca, NY 14850 (U S A)

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SUMMARY

The performance of microbore high-performance liquid chromatography and capillary zone electrophoresis, both equipped with on-line tandem mass spectrometric detection capability, was evaluated critically for the determination of endogenous amounts of leucine enkephalin and methionine enkephalin in equine cerebrospinal fluid Using an identical sample clean-up and enrichment procedure, capillary zone electrophoresis–mass spectrometry is limited in its concentration detection capacity owing to its much smaller injection volume Leucine enkephalin was identified in post-mortem equine cerebrospinal fluid at the 1–5 ng/ml level by liquid chromatography–mass spectrometry

INTRODUCTION

Leucine enkephalin (LE=YGGFL) and methionine enkephalin (ME=YGGFM) are pentapeptides with potent opiate agonist activity, first isolated from pig brain by Hughes et al in 1975 [1]. Since then, several research groups have been engaged in the elucidation of various functions of the "endogenous opioid system" with its opiate receptors and the enkephalins and endorphins as its natural ligands. Owing to this increased activity in brain

[a]On leave from the Department of Pharmacy and Food Chemistry, Wurzburg University, 8700 Wurzburg, F R G

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neuropeptide research, substantial progress in our understanding of the structure, distribution and action of the endogenous opioid peptides has been made over the last decade Their complete clinical, pharmacological and therapeutic implications, however, are still not well understood [2]

The correlation of low levels of endogenous substances with physiological effects and/or therapeutic measures can only be accomplished by reliable detection and identification of the trace amounts of target compounds in biological fluids and tissues A variety of different approaches have been described for the determination of enkephalins, based predominantly on radioimmunoassays (RIA) and radioreceptorassays (RRA) [3] High-performance liquid chromatography (HPLC) is currently the method of choice as the major sample pretreatment and sample fractionation step for studies of biological samples [4] Liquid chromatographic determination of opioid peptides with photodiode-array [5], fluorescence [6] and electrochemical detection [7] has been reported in the literature

Mass spectrometry (MS), offering the highest level of molecular specificity compared with that obtained by the above-mentioned analytical techniques [8], has played a crucial role in the first identification of enkephalins [1] These analytes have often served as popular model compounds to establish new biomedically relevant MS technology [9] Therefore, it is not surprising that recent studies in neuropeptide profiling emphasize the key role of mass spectrometry as an essential tool for the unambiguous identification of these analytes [10] The merits of MS in peptide and protein research were discussed recently by Biemann and Martin [11] in an excellent overview Desiderio and co-workers [12–14] have pioneered the extensive use of MS and tandem MS (MS–MS) for the determination of enkephalins and other neuropeptides in biological tissue extracts Usually, deproteinated and desalted biological samples are subjected to gradient reversed-phase HPLC separation and the collected fractions are screened by immunological techniques such as RIA and RRA [15] Unambiguous confirmation of the target peptides is then accomplished by MS using field desorption [16] and more recently fast atom bombardment (FAB) [17] The successful application of this methodology, especially when the MS–MS operation mode is utilized, is well documented [18–21] However, off-line MS analysis of collected HPLC fractions is tedious, labor intensive and includes additional sample handling steps which may produce sources for error and imprecision in the final quantitative result On-line HPLC procedures with comparable MS–MS capabilities [22], therefore, should be even more attractive

Another "high-performance" separation technique, capillary zone electrophoresis (CZE) [23], has received considerable interest owing to its potential as an analytical tool for the separation of complex mixtures containing biopolymers [24] The recent realization of CZE–MS [25,26] will accelerate the

increasing popularity and importance of CZE, especially in the biomedical community.

As preliminary work for investigations related to pain and pain-relief phenomena in the horse and the possible abuse of enkephalins at race tracks, a method for the determination of leucine enkephalin and methionine enkephalin in equine cerebrospinal fluid (CSF) has been developed The detection of picogram levels of the two pentapeptides was possible by microbore HPLC with on-line MS detection via an ion spray LC–MS interface [27] and a commercially available atmospheric pressure ionization (API) triple quadrupole mass spectrometer In addition, the availability of our recently developed CZE–MS interface [28] allowed a facile change to another separation technique under the same on-line MS conditions, and provided comparative studies to obtain improved evidence for the feasibility and capability of CZE–MS confronted by this real-life bioanalytical problem

The performance of both state-of-the-art coupling techniques for the sensitive and specific determination of endogenous profiles of leucine enkephalin and methionine enkephalin in CSF are presented and discussed with respect to recovery, limit of detection (analyte amount vs concentration), linearity and precision Leucine enkephalin levels at the low ng/ml (ppb) range, obtained for the first time from equine CSF, will serve as a useful database for future pain-related investigations in the horse

EXPERIMENTAL

Chemicals

Leucine enkephalin and methionine enkephalin, the internal standard (D-Ala^2)-leucine enkephalin and the dynorphins 1–3 to 1–7 were purchased from Sigma (St. Louis, MO, U S A) and were used without further purification HPLC-grade ammonium acetate, water, acetonitrile and methanol were obtained from Fisher Scientific (Rochester, NY, U S A) HPLC/Spectrograde trifluoroacetic acid (TFA) with a purity of higher than 99 5% was obtained from Pierce (Rockford, IL, U S A) The 3-ml disposable extraction columns (J T Baker, Phillipsburg, NJ, U S A) packed with 500 mg of C_{18}-bonded silica (Model 7020-03, 40 μm, 60 Å) were used with a vacuum manifold equipped to hold twelve cartridges (Supelco, Bellefonte, PA, U S A)

CSF samples

Cerebrospinal fluid (CSF) was collected post-mortem from the horse (premedicated with 400 mg of xylazine and given a fatal dose of pentobarbital) by puncture of the cerebromedullary cistern (atlanto-occipital joint, 18G/3 5-in spinal needle) Collected samples were frozen immediately (within 1 min of collection) in liquid nitrogen and stored at $-60\,^{\circ}$C until the time of analysis This was done to prevent possible enzymatic degradation of the target peptides

Addition of internal standard and quantification standard

CSF (4 75 ml) was defrosted and 250 μl of a 200 ng/ml solution of the internal standard (IS), (D-Ala^2)-leucine enkephalin, were added, resulting in a final concentration of 10 ng/ml of IS Half of the sample (2 ml) was spiked with 20 ng each of LE and ME, as indicated in the overall sample analysis scheme in Fig 1, and served as the reference sample in this standard addition approach

Protein precipitation

CSF (2 ml) was mixed thoroughly with 2 ml of acetonitrile (Fig 1) for 5 min and the mixture was centrifuged at 3000 rpm (ca 500 *g*) for 10 min (IEC HN-S Centrifuge, Damon, Needham, MA, U S A) The supernatant was evaporated under a gentle stream of nitrogen at 30°C until 1 ml of residue remained A 2-ml volume of 5 m*M* ammonium acetate solution was then added prior to the clean-up step by solid-phase extraction

Solid-phase extraction

The solid-phase extraction columns were conditioned by elution with 5 ml of methanol and 5 ml of 5 m*M* ammonium acetate solution The sample was loaded onto the conditioned column, washed with 5 ml of water and the peptide fraction recovered with 2 ml of acetonitrile–water (80 20, v/v) The eluate was evaporated under a gentle flow of nitrogen at 30°C and stored at −18°C until the time of analysis The samples were reconstituted with vortex mixing in 100 μl of mobile phase for LC analysis or in 100 μl of water for the CZE experiments

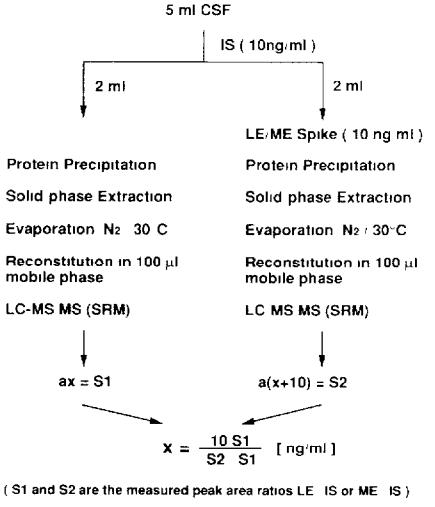

Fig 1 Overview of sample analysis and quantification scheme

Microbore HPLC

The LC system consisted of a Brownlee Labs (Santa Clara, CA, U S A) micropump and a Model 9125 injection valve equipped with a 5-μl sample loop (Rheodyne, Cotati, CA, U S A) Enkephalins were separated with a 100 mm $\times$ 1 mm I D analytical column packed with Spherisorb ODS II (3-μm) particles (Keystone Scientific, State College, PA, U S A) The mobile phase was maintained at a flow-rate of 40 μl/min and consisted of acetonitrile–water (50 50, v/v) with a final concentration of 0 05% TFA The eluent was degassed by purging with helium before use The exit of the chromatographic column was connected directly to the ion spray LC–MS interface [27]

An additional filtration of the CSF samples through 0 2-μm nylon-66 microfilterfuge tubes (Rainin, Woburn, MA, U S A) before injection into the micro-HPLC system proved to be advantageous The absence of possible carryover effects was verified by solvent blank injections between each sample

Capillary zone electrophoresis

The CZE system, which has been described in more detail [26,28], utilized a 0–60-kV voltage-regulated power supply (Model RHR60P30/EI, Spellman, Plainview, NY, U S A) to establish the electrical field across the capillary Untreated 90–100 cm $\times$ 100 μm I D fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, U S A) were used for all studies The cathode end was connected to the ion spray LC–MS interface [27] via a liquid junction coupling described elsewhere in more detail [28] A net CZE voltage of 30 kV was applied to the capillary for all separations reported, and the current (40–60 μA) passing through the CZE column was measured with an analog meter (Model 260, Simpson, Chicago, IL, U S A) Mixtures (50 50) of acetonitrile and 20–30 mM ammonium acetate buffer (pH 6 8) were used as the separation medium unless indicated otherwise Sample volumes of approximately 15–25 nl were introduced into the capillary at the anode end by hydrostatic injection (5 cm for 10–15 s)

Mass spectrometry

A Sciex (Thornhill, Ontario, Canada) TAGA 6000E triple quadrupole mass spectrometer with a 1400-dalton mass range and equipped with a standard atmospheric pressure ionization (API) source was used to sample ions produced from the ion spray LC–MS interface Both mass analyzers were always operated under unit-mass resolution conditions (10% valley definition) The high vacuum in the analyzer region was $7 \cdot 10^{-6}$ Torr under MS operation and $2 \cdot 10^{-5}$ Torr under MS–MS conditions Ultrapure argon was used for collision-induced dissociation (CID) in the second quadrupole at $250 \cdot 10^{12}$ atoms/cm^2 target gas thickness, the applied collision gas energy used for all MS–MS experiments was 50 V

The ion spray LC–MS interface was floated at 3–4 kV for positive ion op-

eration of the mass spectrometer The ion evaporation mass spectra exhibit only the singly protonated, singly charged molecular ions for LE at m/z 556, ME at m/z 574 and the IS at m/z 570 Selected ion monitoring (SIM) detection was based on the observation of these ions with an acquisition period of 300 ms each The fragment ions chosen for selected reaction monitoring (SRM) in the MS–MS mode were m/z 120, 136, 278 and 556 for LE, m/z 120, 136, 278 and 574 for ME and m/z 120, 136, 292 and 570 for the IS (see Figs 2 and 7b); the dwell time for each selected ion was 100 ms.

RESULTS AND DISCUSSION

Sample preparation

The isolation procedure for endogenous enkephalins in CSF (Fig 1) is similar to that described by Desiderio and Yamada [29] The use of an internal standard method is recommended to compensate for biological matrix effects and to improve the mass spectral quantitative reproducibility The internal standard selected, (D-Ala^2)-leucine enkephalin, is stable, available commercially at high purity and is an enkephalin homolog similar to LE and ME in its chromatographic, electrophoretic and mass spectral behavior (see below, Figs 2, 3 and 5–7) Since peptide-free CSF is not available for a "blank" matrix, the quantitative result relies on a standard addition approach The concentrations of the IS and the spiked LE and ME were near the expected endogenous levels [14] in the low ng/ml range All efforts described here aimed towards an overall detection sensitivity of 1 ng/ml (1 ppb) of LE and ME in equine CSF.

The defrosted sample was spiked with IS (10 ng/ml) and divided into two equal fractions After the standard addition of LE and ME to one of these fractions, protein precipitation by acetonitrile minimized the loss of neuropeptides by peptidase activity The influence of this deproteination step on the overall recovery was investigated using water and CSF samples fortified with IS at the 10 ng/ml level, recovery losses of 5 8% ($n=5$) for water and 16 3% ($n=5$) for CSF were observed The procedure, however, was maintained, because the lifetime of the microbore columns was increased significantly and fewer back-pressure problems occurred when higher molecular weight peptides and proteins were removed from the sample by precipitation before injection into the HPLC system

The subsequent partial evaporation of organic solvent was necessary for reliable solid-phase extraction on C_{18}-bonded silica Proper conditioning of the extraction cartridges and optimization of the volumes for washing and peptide elution are critical for high recoveries The often-preferred acid conditions in the solid-phase extraction procedure for enkephalins and endorphins proved to be disadvantageous for ME recovery with respect to this procedure and the above-mentioned materials As will be discussed later (analysis of CSF samples), most of the recovery variability and imprecision of the method, espe-

cially for ME, is due to the sample pretreatment step, which should be the subject of further investigation

Mass spectrometry

The ion spray LC–MS interface utilizes pneumatically assisted electrospray ionization at atmospheric pressure, where gaseous ions are produced by the mechanism of field-assisted ion evaporation [27] Dominant features of these mass spectra are cationized molecular ions $(M+nX)^{n+}$, where X = H, NH_4, Na, K, etc , and n is the number of positive charges

Ion spray mass spectra of the enkephalins and dynorphins 1–3 to 1–9 are characterized predominantly by singly charged, protonated molecular ions (LE, m/z 556, ME, m/z 574, IS, m/z 570) and/or multiply charged molecular ions, depending on the available protonation sites and the pH of the eluent [26]. The simplified mass spectra produced under these conditions provide the opportunity to operate the mass spectrometer in the selected ion monitoring (SIM) mode with high sensitivity because the ion current is concentrated into only one or a few species, which are characteristic of the analyte (see Fig. 3a and 5) In addition, when the molecular ion carries most of the ion current, one has an ideal situation for MS–MS experiments By monitoring the $(M+H)^+$ ion of LE at m/z 556, m/z 574 for ME, and m/z 570 for the IS with the first quadrupole, performing collision-induced dissocation (CID) in the second quadrupole and scanning the third quadrupole from m/z 50 to 600, the MS–MS daughter ion mass spectra of both enkephalins and the IS were recorded. CID daughter ion mass spectra for LE, obtained by on-line HPLC–MS–MS separation, and for the IS, obtained by on-line CZE–MS–MS separation, are shown in Figs 2 and 7b, respectively The structurally significant sequence ions are labelled following the nomenclature proposed by Roepstorff and Fohlman [30] and are in full agreement with earlier tandem mass spectrometric data [31] The IS differs from LE in its amino acid sequence at the second position, the glycine is replaced by D-alanine The sequence ions B2, B3 and B4 are consequently shifted to m/z values that are 14 mass units higher owing to the additional methyl group in the molecule

The increased molecular specificity from MS–MS is essential in biomedical research and has been discussed thoroughly by Desiderio et al. [8] Monitoring one or several unique amino acid sequence-determining fragment ions in the selected reaction monitoring (SRM) mode, as presented in Figs. 3b and 6, is regarded as the optimum methodology that can currently be obtained for this kind of problem

HPLC-MS

The suitability of microbore HPLC–ion spray MS for the determination of peptides has recently been demonstrated [22] Fig 3 shows a total SIM and SRM ion current chromatogram for a standard mixture of the two target pep-

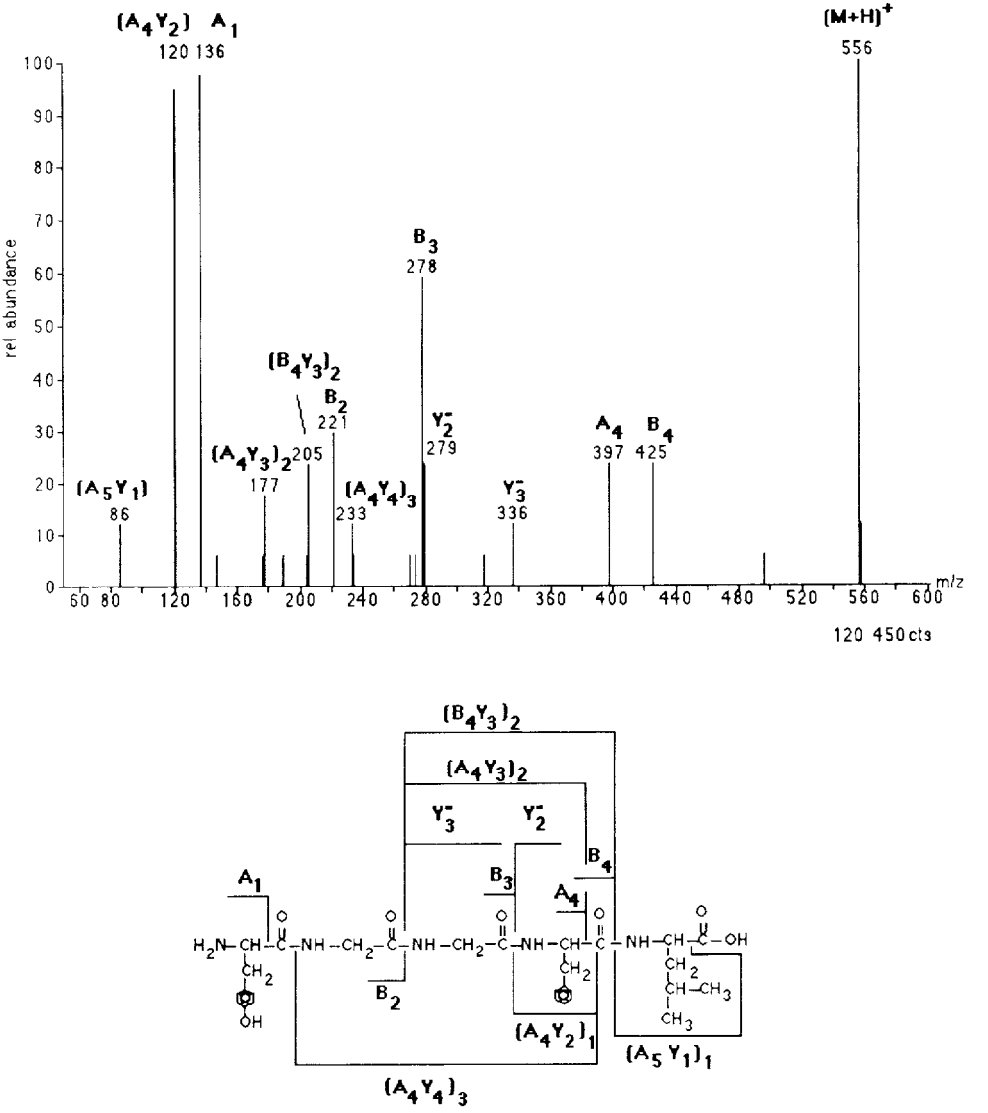

Fig 2 CID daughter ion mass spectrum of leucine enkephalin and its fragmentation interpretation concerning the structurally significant ions obtained by LC-MS-MS of 100 pmol standard material injected on-column

tides and the IS The LE peak shown in Fig 3a exhibits a separation efficiency of approximately 3000 theoretical plates, based on the peak width at half-height, and a peak asymmetry of 1 2 measured at 10% of the peak height. To clarify the limit of detection of the LC–MS system, an aqueous dilution series of LE and ME ranging from 20 ng/ml (20 ppb) to 2 μg/ml (2 ppm) was analyzed,

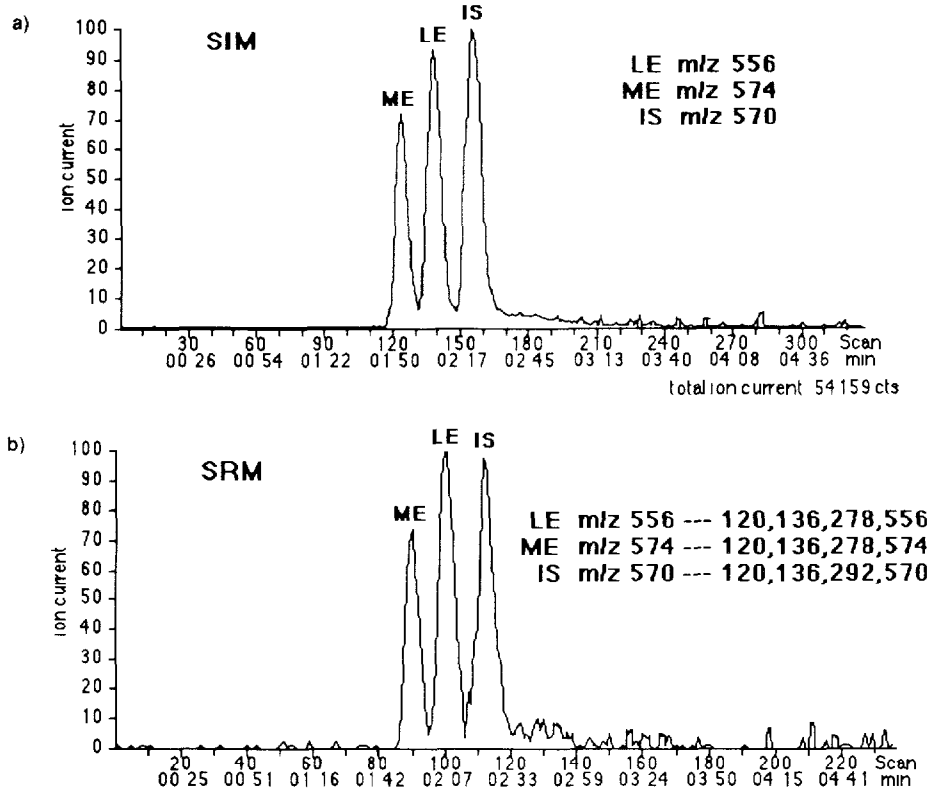

Fig 3 (a) SIM and (b) SRM chromatograms of ME, LE and the IS Experimental conditions 100 mm × 1 mm I D analytical column packed with 3-μm Spherisorb ODS II, eluent, acetonitrile–water (50 50, v/v) with 0 05% TFA at 40 μl/min, 5 μl of standard solution containing 5 ng of each peptide were injected, corresponding to ca 10 pmol on-column

corresponding to 100 pg (ca 200 fmol) and 10 ng (ca 2 pmol), respectively, injected on-column The tandem mass spectrometer was operated in the SRM mode while triplicate injections were made (Fig 4) Based on a signal-to-noise ratio of 3 1 (peak-to-peak), a limit of detection of 20 ppb (100 pg or 200 fmol) for both enkephalins can be estimated, identical results were obtained in the SIM mode To verify the linearity over this working range from 20 ng/ml to 2 μg/ml, an identical dilution series was prepared, except that a constant amount of 1 ng of IS (200 ppb) was added, the investigation of two orders of magnitude starting from the limit of detection was considered sufficient for the expected levels and the sample enrichment factor of 20 The peak-area ratios of enkephalin versus the IS were calculated, and a linear regression of the mean values (triplicate injections) was executed These LC–MS–MS experiments produced correlation coefficients of 0 998 for LE and 0 999 for ME

Thus, microbore LC–MS shows a separation efficiency that is adequate for

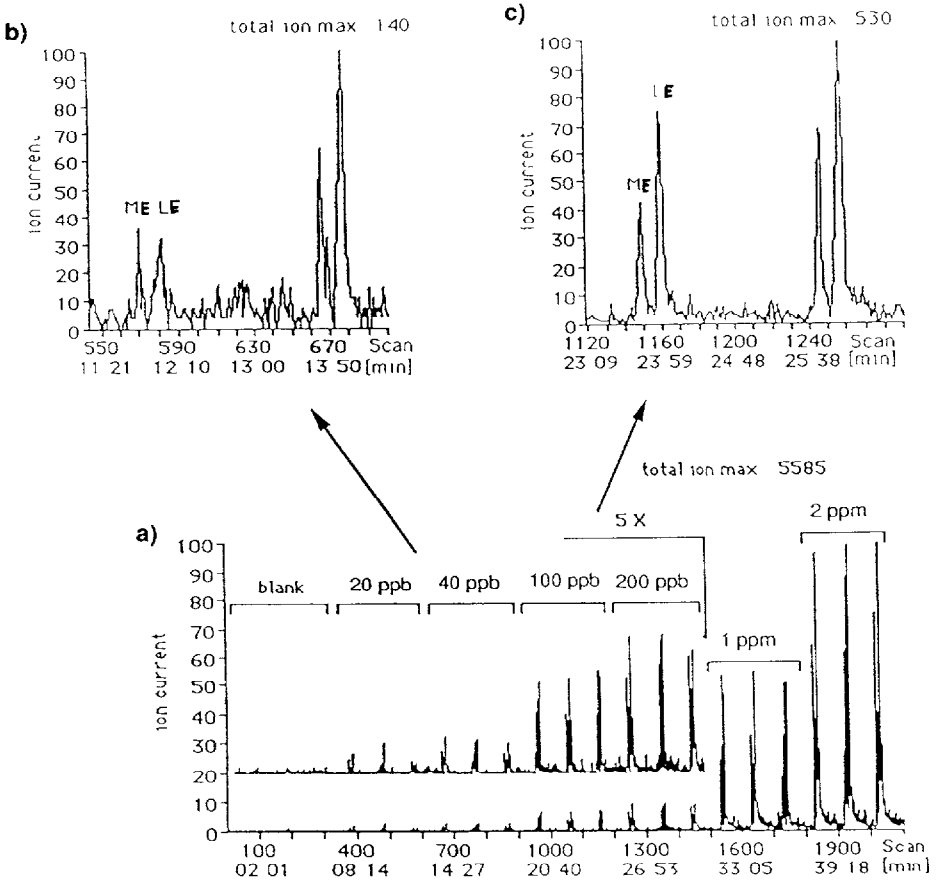

Fig 4 Detection limits of ME and LE by LC-MS-MS (SRM) Experimental conditions as in Fig 3 The aqueous dilution series ranged from 20 ng/ml (20 ppb) to 2 μg/ml (2 ppm), 20 ppb of LE correspond to 100 pg (ca 200 fmol) on-column (a) Triplicate injections of each concentration with a time interval of 2 min, (b) enlarged, peak pairs of 20 and 40 ppb, (c) enlarged, peak pairs of 100 and 200 ppb

a biological sample such as CSF It should be noted that these CSF extracts are relatively "clean", especially after the described sample clean-up procedure The achievable limit of detection allows an initial concentration of 1 ng of neuropeptides per ml of CSF with a linear quantification range up to 100 ng/ml

CZE-MS

The use of CZE-MS for the determination of dynorphins has been reported previously [26] Additional CZE-MS separations are presented in Figs 5–7 Fig 5 shows the SIM electropherograms of LE, the IS and three dynorphins,

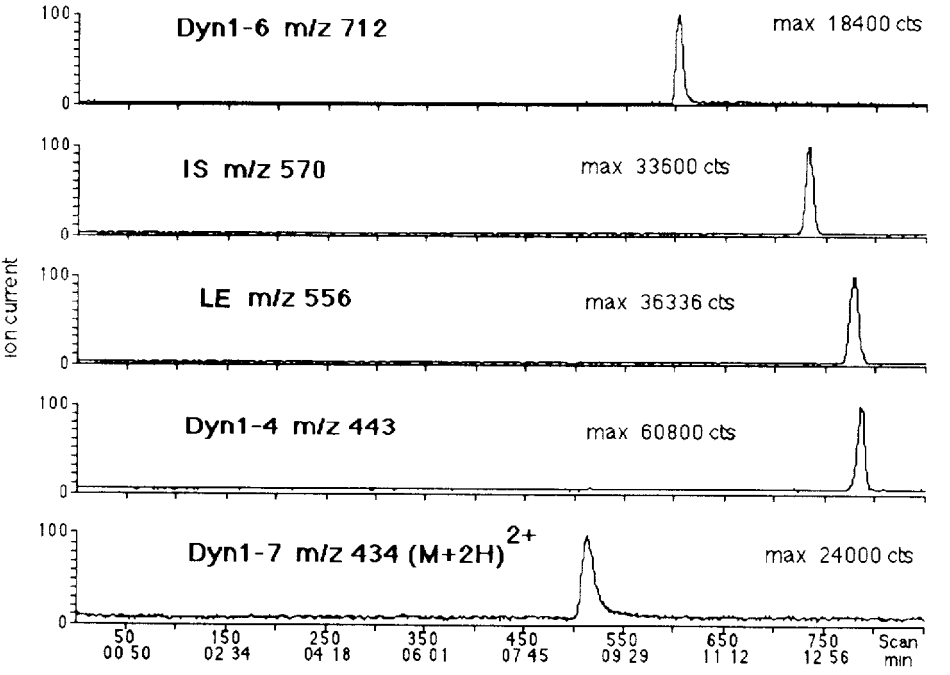

Fig 5 CZE–MS (SIM) of dynorphin standards Experimental conditions 100 cm × 100 μm I D fused-silica capillary, eluent, acetonitrile–25 m*M* ammonium acetate, pH 6 8 (50 50, v/v), applied voltage, 30 kV, ca 15 nl were loaded by hydrostatic injection (5 cm for 10 s) The amount injected was calculated as 2 3 ng each, corresponding to ca 5 pmol

which are known relatives of the enkephalins Figs 3 and 6 demonstrate the conformation and screening capability of CZE–MS–MS operated in the full-scan and the SRM mode, for example, in Fig 6, all peptides are characterized by their common CID daughter ion m/z 136, which is characteristic for the amino-terminal tyrosine

The separation medium is a compromise between CZE requirements, sample behavior and MS constraints, nevertheless, it has already demonstrated its potential in the CZE–MS study of a tryptic digest [28] At the chosen pH of 6 8, the two enkephalins LE and ME (isoelectric point ca pH 5 5 [32]) are weakly negatively charged and migrate slowly to the anode Owing to the small difference in the mass-to-charge ratio, their separation is hardly observed (Fig 9), whereas the IS and the dynorphins 1–3 to 1–7 are well resolved in the ion current electropherogram (Figs. 5 and 6) The use of a lower pH, e g , 4 8, as discussed previously [26], allows an efficient separation of the higher, multiply charged dynorphins 1–7, 1–8 and 1–9 with acceptable peak shape, but eliminates the small electrophoretic differences of the smaller compounds LE and ME can be separated using an eluent consisting of acetonitrile–acetic acid with pH < 4 [33], the broad, tailing peaks, however, suggest a separation based pri-

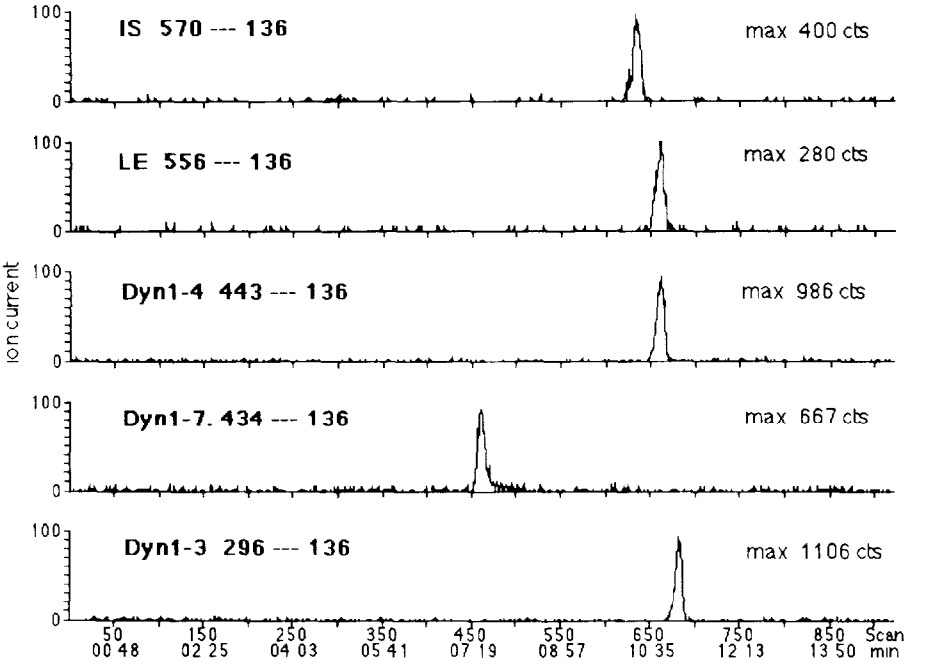

Fig 6 CZE-MS-MS (SRM) of dynorphin standards Experimental conditions 90 cm × 100 μm I D fused-silica capillary, eluent, acetonitrile-30 m*M* ammonium acetate, pH 6 8 (50 50, v/v), applied voltage, 30 kV with a measured current of 60 μA, ca 15 nl were loaded by hydrostatic injection (5 cm for 10 s) The common daughter fragment ion of *m/z* 136 (tyrosine-terminus), taken with a dwell time of 75 ms for each ion, is plotted as the SRM ion current electropherogram for each parent ion

marily on adsorption phenomena at the silica surface, which are favored at low pH for protonated peptides (compare also the tailing peak of dynorphin 1–7 in Fig 5)

The LE peak in the SIM electropherogram of Fig 5 exhibits a separation efficiency of about 32 000 theoretical plates, more than ten times higher than the LC–MS chromatogram (Fig 3a), with a nearly symmetrical peak shape A similar approach as for the LC–MS system was chosen to determine the detection limit of our CZE–MS approach An aqueous dilution series of LE and ME ranging from 1 μg/ml (1 ppm) to 100 μg/ml (100 ppm) was injected in duplicate, corresponding to 15 pg (ca 30 fmol) and 1 5 ng (ca 3 pmol) loaded into the capillary The resulting SIM electropherograms for ME are shown in Fig 8 (identical results were obtained in the SRM mode) In this instance, a concentration limit of detection of 2 ppm (20 pg or 60 fmol) can be estimated, which is a factor of 100 higher than in the corresponding LC–MS experiment Comparing the relevant injection volume of 5 μl in LC–MS with about 15 nl in CZE–MS explains easily this decisive contrast The slightly better perform-

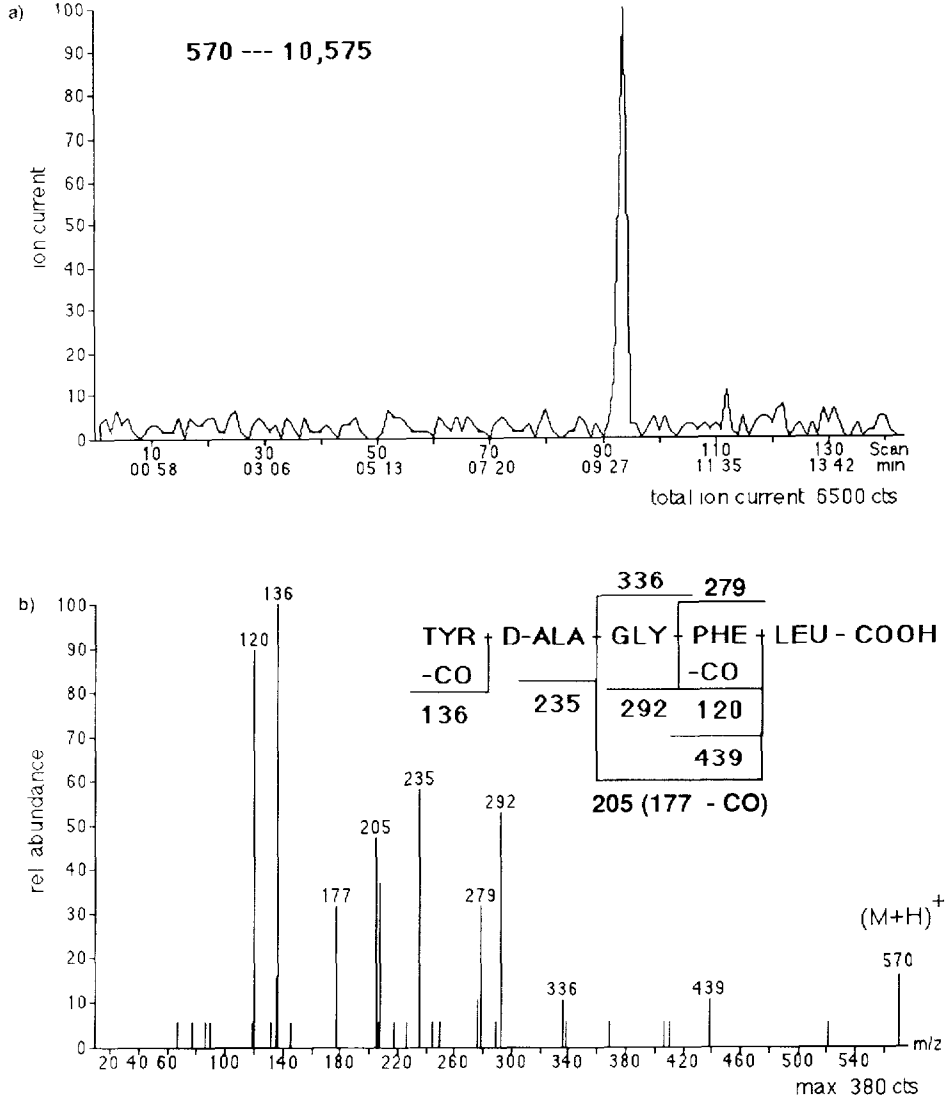

Fig 7 CZE–MS–MS of the internal standard (D-Ala^2)-leucine enkephalin Experimental conditions 100 cm × 100 μm I D fused-silica capillary, eluent, acetonitrile–20 mM ammonium acetate, pH 6 8 (50 50, v/v), applied voltage, 30 kV with a measured current of 40 μA, ca 15 nl were loaded by hydrostatic injection (5 cm for 10 s) (a) Total ion electropherogram, (b) CID daughter ion mass spectrum with suggested fragmentation pathways

ance of CZE–MS (100 pg compared with 30 pg, a factor of 3) is due to the increased peak concentration by the higher separation efficiency of CZE and an improved performance of the ion spray ionization process at lower flow-

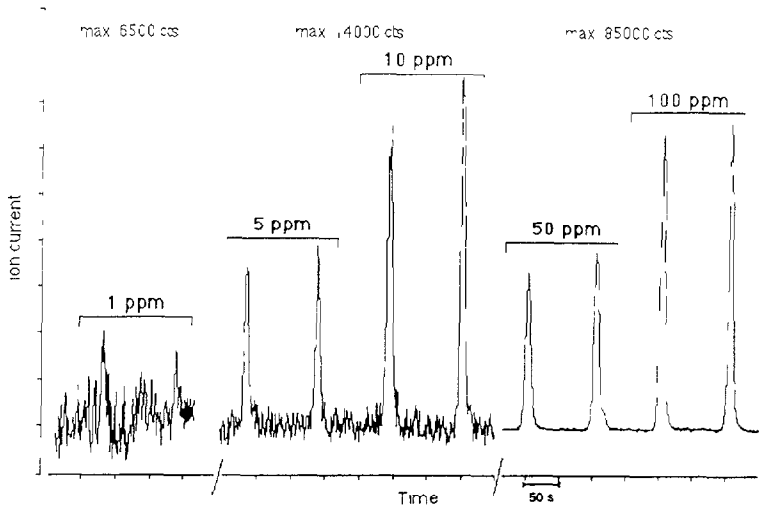

Fig 8 Detection limit for ME by CZE-MS (SIM) Experimental conditions as in Fig 7 The aqueous dilution series ranged from 1 μg/ml (1 ppm) to 100 μg/ml (100 ppm), 1 ppm of ME corresponds to ca 15 pg (about 30 fmol) loaded on the capillary

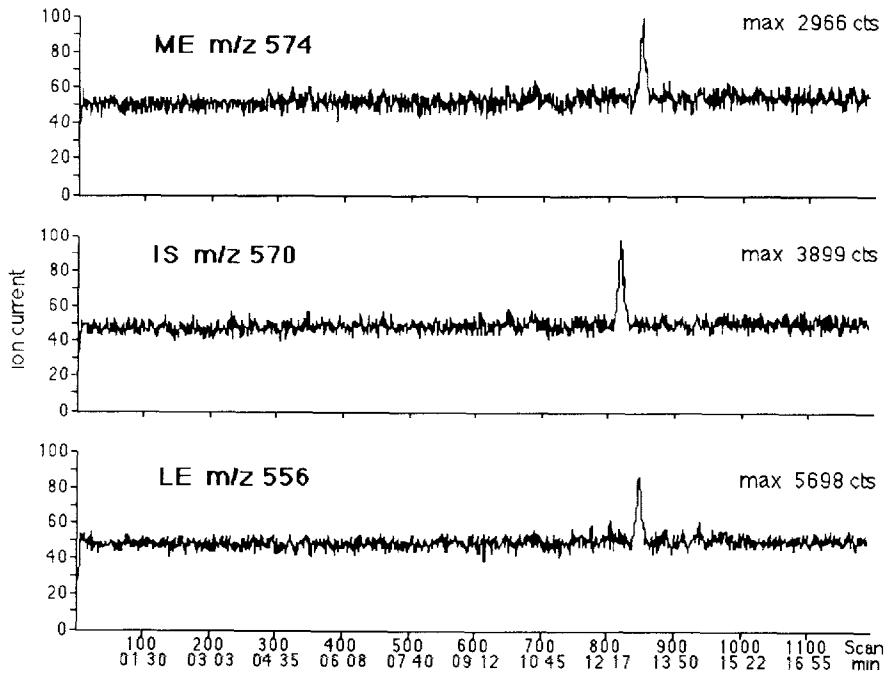

Fig 9 CZE-MS (SIM) of enkephalins extracted from a 100 ng/ml spiked water sample Experimental conditions as in Fig 5 Approximately 20 nl of liquid were hydrostatically injected (5 cm for 15 s) resulting in 60–100 pg (ca 150 fmol) of each enkephalin loaded on to the capillary

rates (micro-LC, 40 μl/min; CZE–liquid junction coupling, 10–20 min μl/min [28]) [27] Another aqueous dilution series ranging from 1 to 100 μg/ml, spiked with a constant amount of 10 μg/ml of the IS, was tested for its linear quantitative behavior (SRM mode, triplicate injections, peak-area ratios), resulting in correlation coefficients of 0.999 for LE and 0 996 for ME

In conclusion, CZE–MS possesses the expected superior separation efficiency The same degree of linearity in the calibration graph, starting from the detection limit through two orders of magnitude, was observed, but its inferiority concerning the concentration sensitivity is strikingly evident The advantage of consuming only very small sample volumes in the pico- or low nanoliter range becomes a crucial problem in trace analysis Although concentration detection limits of 10^{-6}–10^{-9} *M* in the best cases have already been demonstrated for electrochemical and laser-induced fluorescence detection [34], both detection principles lack the universality and molecular specifity of MS. Improvements in the sample enrichment step, the loadability of CZE capillaries and the detection sensitivity of the ion spray/API–MS, which all seem to be possible, will alleviate this present limitation of CZE–MS The general suitability of CZE–MS for this type of analytical problem is reflected in Fig 9, where 100 ng/ml (100 ppb) of three peptides spiked into water was analyzed following extraction.

Analysis of CSF samples for target endogenous peptides by micro-LC–MS

Owing to the insufficient concentration detection sensitivity of CZE–MS, all the following results were obtained by micro-LC–MS Table I presents the

TABLE I

RECOVERY VIA LC–MS–MS (SRM) DETERMINATION OF ENKEPHALINS

All calculations are based on ion counts of peak-area measurements LE was excluded from the CSF experiments owing to its endogenous presence in equine CSF

Spiked sample	Compound	Recovery (%)	
		10 ng/ml	50 ng/ml
Water ($n=5$)[a]	LE	64 4 ± 14 2	63 7 ± 11 4
	ME	62 8 ± 17 5	61 4 ± 20 8
	IS	65 2 ± 10 9	71 3 ± 8 8
CSF ($n=5$)[b]	ME	67 2 ± 29 7	79 4 ± 12 4
	ME[c]	67 1 ± 12 4	79 0 ± 15 7
	IS	74 9 ± 13 0	76 8 ± 7 0
	IS[c]	67 4 ± 9 0	73 4 ± 16 3

[a]All values are evaluated against external standards of 200 ng/ml and 1 μg/ml The LC–MS–MS reproducibilities of the external standard injections at the 200 ng/ml level were 10 3% ($n=5$) for LE, 6 0% ($n=5$) for ME and 12 4% ($n=16$) for the IS

[b]As in *a*, except that ME data are based on ratio calculation with the IS

[c]The second data sets were obtained 8 weeks later

TABLE II

RELIABILITY OF ENKEPHALIN DETERMINATION BY LC-MS-MS (SRM)

Compound	Concentration (ng/ml)[a]				
	1	2	3	4	Mean
ME	9 0	7 2	9 4	17 4	10 7±4 4
IS	8 9	9 3	13 1	7 4	9 7±2 4

[a]Four CSF samples (1-4) were spiked at the 10 ng/ml level and processed as described under Experimental

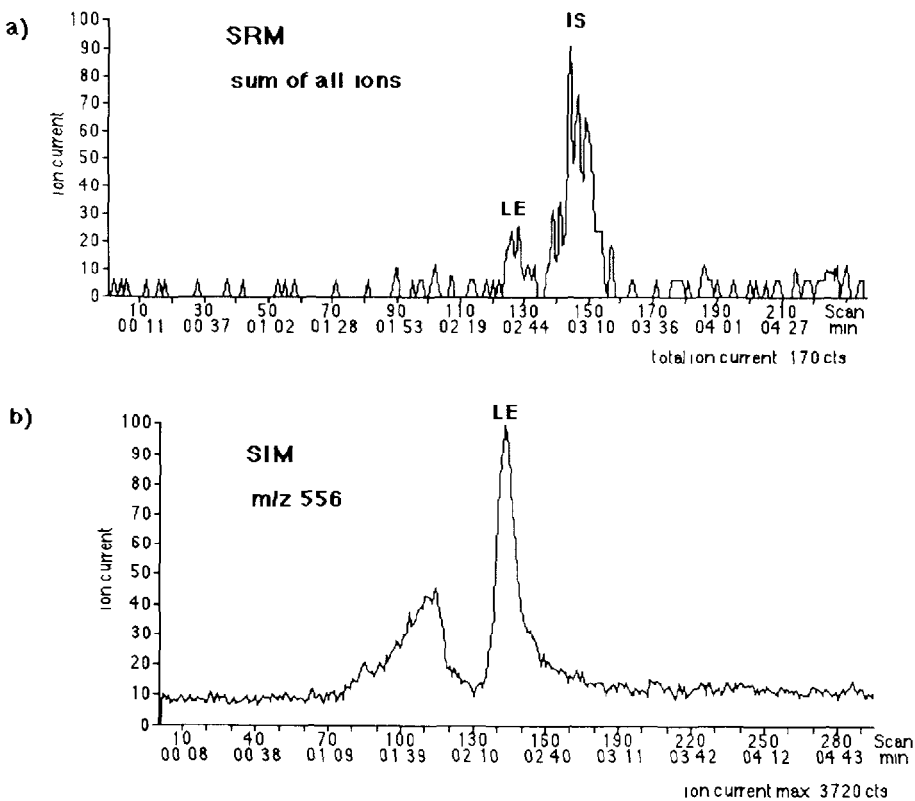

Fig 10 Determination of endogenous LE in equine CSF by micro-LC-MS Experimental conditions as in Fig 3 The different retention times of LE are due to the use of different HPLC columns (a) CSF extract of horse No 1, 3 5 ng/ml of LE detected by SRM of ions of m/z 120, 136, 278 and 556, (b) CSF extract of horse No 3, 4 4 ng/ml of LE detected by SIM of ion of m/z 556

TABLE III

LEUCINE ENKEPHALIN LEVELS DETERMINED IN EQUINE CSF BY LC–MS and LC–MS–MS

ME was not detected in any sample, referring to the available detection limit of 1 ng/ml CSF

Horse	Concentration (ng/ml)					
	1	2	3	4	5	Mean
No 1 (thoroughbred mare, 8 yrs, ca 450 kg)	—	1 3	3 5	1 2[a]	—	2 0
No 2 (thoroughbred mare, 5 yrs, ca 500 kg)	1 2	1 2	2 6	3 7[a]	1 9[a]	2 1
No 3 (thoroughbred gelding, 11 yrs, ca 500 kg)	—	—	3 2	4 4[a]	—	3 8

[a]These data were obtained from SIM chromatograms by peak-area comparison against external standard without use of the IS

overall recovery of the procedure All experiments were executed first with the IS Because ME was not detected in equine CSF, an additional series of analyses were undertaken for ME The recoveries for both compounds were 60–70%, comparable to or better than those reported earlier [6,12], though the variability, especially for ME, needs further clarification The reliability data, obtained for CSF samples spiked with 10 ng/ml ME and presented in Table II, can only be preliminary owing to the limited number of samples ($n=4$) Whereas the accuracy is in good agreement with the fortified amount, the imprecision of 20–40% in the final result is due primarily to the variation in peptide recovery at this low level Other contributing factors to the varying reproducibility are losses occurring during the sample clean-up with several pipetting steps and the increased uncertainty in peak-area measurements due to the low signal-to-noise ratio at very low ion current (see Fig 10)

At the limit of detection of the method of 1 ng/ml, endogenous LE could be detected in equine CSF LE concentrations identified in post-mortem CSF from three different horses are given in Table III, Fig. 10 shows SRM and SIM chromatograms from two different CSF extracts. Because all the values are below the 10 ng/ml level, which is regarded as the limit of quantification of this method, and near the limit of detection of 1 ng/ml, the quantitative results should be considered as preliminary data with an estimated error of $\pm 50\%$ To improve this method with regard to decreased detection limit and increased precision will be the object of future studies.

CONCLUSIONS

Microbore HPLC–MS and CZE–MS have been evaluated for their suitability to determine low levels of endogenous enkephalins in equine CSF Owing to its limited sample capacity, CZE–MS does not reach the concentration detection sensitivity necessary for trace analysis in the low-ppb range Under

identical conditions, microbore LC with on-line tandem MS detection was able to provide usable quantitative data with a high molecular specificity and appears to be a good substitute for off-line HPLC–MS methods for the determination of neuropeptides Although the presented data for leucine enkephalin levels in equine CSF are a good start for detailed investigations to follow, their interpretation from a medical standpoint seems to be difficult, because the influences of the collecting procedure are still unknown

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