Journal of Chromatography, 500 (1990) 395–412
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 105

OPIOID AND TACHYKININ PEPTIDES, AND THEIR PRECURSORS AND PRECURSOR-PROCESSING ENZYMES, IN HUMAN CEREBROSPINAL FLUID

DANXIA LIU

The Charles B. Stout Neuroscience Mass Spectrometry Laboratory, University of Tennessee, Memphis, TN 38163 (U.S.A.)

CHHABIL DASS

The Charles B. Stout Neuroscience Mass Spectrometry Laboratory and Department of Neurology, University of Tennessee, Memphis, TN 38163 (U.S.A.)

GEORGE WOOD

Department of Orthopedics, University of Tennessee, Memphis, TN 38163 (U.S.A.) and Campbell Clinic, 869 Madison Avenue, Memphis, TN 38104 (U.S.A.)

and

DOMINIC M. DESIDERIO*

*The Charles B. Stout Neuroscience Mass Spectrometry Laboratory and Departments of *Neurology and Biochemistry, University of Tennessee, 800 Madison Avenue, Memphis, TN 38163 (U.S.A.)*

(First received April 25th, 1989; revised manuscript received October 11th, 1989)

SUMMARY

Opioid and tachykinin neuropeptides, which were derived from two biological sources (intact, and released from their corresponding precursors by the action of human cerebrospinal fluid (CSF) neuropeptidases), were characterized in human CSF by using a combination of post-high-performance liquid chromatographic (HPLC) detection techniques. Peptides were separated using gradient and isocratic reversed-phase HPLC. Radioimmunoassay measured immunoreactivity corresponding to several different individual neuropeptides including methionine enkephalin, leucine enkephalin, substance P and β-endorphin. Commercial enzymes (trypsin, carboxypeptidase B) were used to release methionine- and leucine-enkephalin from precursors. Human CSF also served as a source of endogenous neuropeptidases. Mass spectrometry produced fragment ions that corroborated the amino acid sequence of methionine enkephalin and of substance P derived from both sources (intact, from precursors). These results demonstrated the presence of endogenous intact neuropeptides, several different neuropeptide-containing precursors and appropriate precursor-processing enzymes in human CSF for precursors of methionine enkephalin, leucine enkephalin, β-endorphin$_{1-31}$ and substance P.

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INTRODUCTION

Endogenous neuropeptides in human cerebrospinal fluid (CSF) are important indicators of homeostasis and of pathophysiological conditions[1–24], and it is therefore crucial to analyze those peptides with a high degree of detection sensitivity and molecular specificity. Although radioreceptor assay (RRA), radioimmunoassay (RIA) and bioassay detection methods all provide sensitivity, they cannot provide amino acid sequence data, which are the only acceptable criteria for specificity. Therefore, we developed a comprehensive analytical system that combined several different post-high-performance liquid chromatographic (HPLC) detection systems, which included UV, RRA, RIA, mass spectrometry (MS) and MS–MS to increase significantly our level of confidence to detect a specific neuropeptide[25].

For human lumbar CSF, which contains extremely low levels of neuropeptides, we obtained corroborative amino acid sequence data for methionine enkephalin [ME; TyrGlyGlyPheMet (YGGFM), $C_{27}H_{35}N_5O_7S$ = 573.2257] and substance P (SP; RPKPQQFFGLM-NH_2, $C_{63}H_{98}N_{18}O_{13}S$ = 1346.7282), from two different sources (intact; from precursors). These novel corroborative data are critical and timely for our studies of human low back pain[13,23,25]. Reversed-phase (RP) HPLC, RIA, fast atom bombardment (FAB) MS, and MS–MS methods characterized these neuropeptides. Also, the presence of precursor-processing enzymes in human CSF, for example the specialized carboxamide enzyme required for SP synthesis, was demonstrated in this study. These enzymes were not purified or characterized, but rather were used as tools to produce target neuropeptides from corresponding precursors.

Although modern four-sector tandem mass spectrometers are ideal for sequencing oligopeptides, the number and availability of those instruments are extremely limited. Alternatively, two-sector, forward-geometry MS instruments can also provide corroborative amino acid sequence data for endogenous peptides, but with less confidence[22,25]. Our corroborative amino acid sequence data were obtained with the latter type of instrument.

EXPERIMENTAL

CSF

Individual human CSF samples were obtained by lumbar puncture from patients undergoing clinical evaluation to determine the cause of their low back pain[13,23]. After standard laboratory tests and checks were performed on all human CSF samples, the remainder of each sample was stored (−70°C) and pooled before analysis. Patients were categorized according to their physiological response to injection of a spinal anesthetic (lidocaine) in terms of pain reduction or alleviation[13,26–28]. Sample volumes collected were generally 4 ml before and 4 ml after injection of spinal anesthetic.

Peptides, inhibitors and enzymes

Synthetic ME, ME–Lys, leucine enkephalin [LE; TyrGlyGlyPheLeu (YGGFL)], LE–Arg, ME–Arg-Phe, α-neo-endorphin, ME–Arg–Gly–Leu, SP and six dynorphin A fragments (1–6, 1–7, 1–8, 1–9, 1–10, 1–13 and 1–17) were purchased from Sigma (St. Louis, MO, U.S.A.), and dynorphin A fragment$_{1-12}$ and β-endor-

phin$_{1-31}$ (BE$_{human}$ = YGGFMTSEKSQTPLVTLFKNAIIKNAYKKGE) from Peninsula Labs. (Belmont, CA, U.S.A.).

The enzyme inhibitors thiorphan, bestatin and leupeptin were purchased from Peninsula Labs. and captopril was a generous gift from Squibb (Princeton, NJ, U.S.A.). A mixture of bestatin, leupeptin, captopril and thiorphan (400 pmol μl^{-1} of each inhibitor) was used. Carboxypeptidase B (CPB; Type 1; 340 units of activity ml^{-1}) was purchased from Sigma and immobilized trypsin (T; 14 units ml^{-1} settled gel) from Pierce (Rockford, IL, U.S.A.).

Enzymolysis

Trypsin (T) and carboxypeptidase B (CPB) were used to treat appropriate HPLC fractions. Immobilized T beads were washed with Tris buffer and centrifuged (4 times) before use. Tris buffer (pH 7.4) was adjusted to pH 8 with dilute ammonia. Sample was incubated with T (pH 8), and was centrifuged (twice). The supernatant was incubated with CPB, and then injected immediately onto an RP-HPLC analytical column.

Radioimmunoassay

Commercial RIA kits (IncStar, Stillwater, WI, U.S.A.) were used for measuring LE-like immunoreactivity (LI), ME–LI, SP–LI and BE–LI.

Reversed-phase high-performance liquid chromatography (RP-HPLC)

A Varian HPLC system with a guard column and a C_{18} reversed phase steel analytical HPLC column (85 Å pore diameter; 10 μm particle diameter was used[29]. UV absorption of the peptide bond at 200 nm was monitored. One-minute fractions were collected at a flow-rate of 1.5 ml min^{-1} during either a 90-min or a 120-min gradient[30] consisting of a set of linear segments of increasing percentage of acetonitrile (see Fig. 1). Triethylamine–formic acid (TEAF, pH = 3.15) was the volatile buffer[31]. A mixture of synthetic peptides was separated with a 90-min HPLC gradient in a separate experiment to calibrate (within ± 1 min) the retention time of each peptide. Isocratic chromatographic conditions were used for elution of ME (15% acetonitrile) and of LE (18% acetonitrile).

Mass spectrometry (MS)

A VG (Manchester, U.K.) 7070E-HF mass spectrometer was used to analyze peptides in HPLC fractions. Experimental details of FAB-MS analysis of peptides were published elsewhere[25,32–34].

Briefly, a lyophilized HPLC fraction was redissolved in a few microliters of methanol and deposited onto the FAB probe tip that contained an appropriate FAB matrix [glycerol for ME and LE; dithiothreitol–dithioerythritol (3:1, v/v) for SP and tryptic fragment β-endorphin$_{20-24}$]. Glutathione and ammonia were added to the probe tip for negative ion FAB-MS experiments[34]. The sample was bombarded with a beam of high energy (7 keV) xenon atoms, and ions were accelerated through a potential of 6 kV.

A mass region corresponding to the appropriate molecular ion [$(M-H)^-$ at m/z 572.2 for ME; $(M+H)^+$ m/z 556.2 for LE, 557.2 for β-endorphin$_{20-24}$ and m/z 1347.7 for SP] was monitored by scanning the magnet over a limited mass range of 10–15

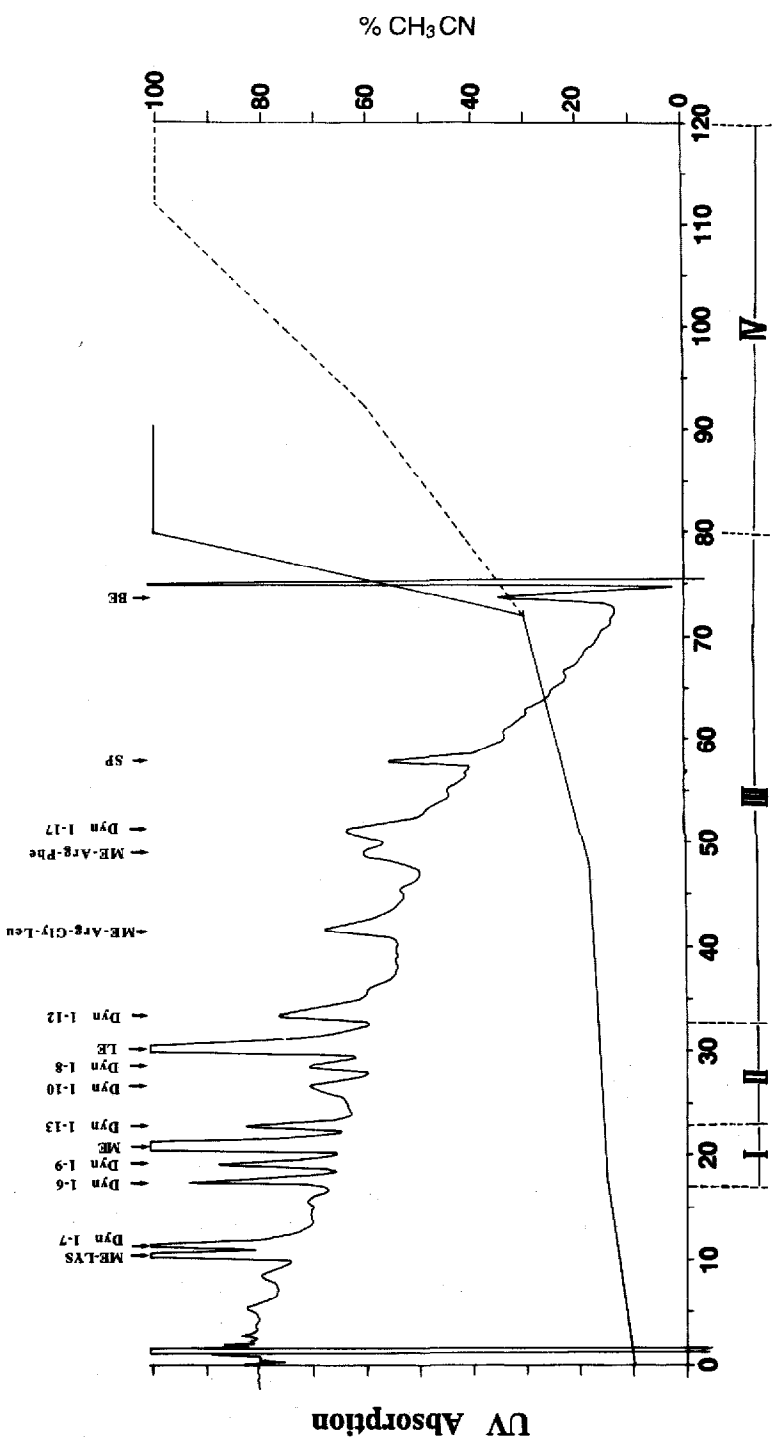

Fig. 1. RP-HPLC gradient chromatogram of a mixture of fifteen synthetic neuropeptides. Abscissa, UV absorption at 200 nm; ordinate, fractions (min) and sections (I–IV). The right-hand abscissa denotes percentage of the organic modifier, acetonitrile; straight lines across the chromatogram denote the gradient. See text for further experimental details.

a.m.u. to bracket the molecular ion. Using the multi-channel acquisition (MCA) mode, 7–10 spectra were accumulated. Product ion mass spectra were obtained[35] following collisionally activated decompositon (CAD) of the molecular ion (36,37) using a constant B/E (B = magnetic field, E = electric field) linked-field scan to collect product ions arising from those respective precursor ions.

Nomenclature has been defined[38] for amino acid sequence ions observed in a peptide's mass spectrum.

Experimental procedures

Fig. 1 shows a gradient RP-HPLC trace to demonstrate the chromatographic resolution of a mixture of fifteen synthetic peptides, and defines sections I–IV. A peptide name located above an arrow indicates the calibrated retention time for each synthetic peptide, but not that the particular peptide was always found at that retention time or that the amino acid sequence was established for a peak found in a biological extract at that elution time.

Pooled CSF was lyophilized, dissolved in TEAF and separated by gradient RP-HPLC; 120 fractions (one fraction per minute) were collected. Each injected sample was equivalent to 20 ml of CSF. The protocol used to analyze CSF peptides is illustrated in Fig. 2.

It was necessary to collect later-eluting fractions (80–120 min) containing larger sized precursors that produce peptides, and therefore disposable octadecylsilyl (ODS) cartridges (Sep-Pak) were not used in this study to remove salts from CSF. Because the retention time of an endogenous peptide could shift slightly between injections, a phenomenon noted in the RP-HPLC separation of biological samples, a mixture of opioid peptide standards recalibrated the retention times and a subsequent blank elution guaranteed that injected peptides were eluted and that no peptide immunoreactivity was eluted in that blank[30].

Individual gradient HPLC fractions in sections I and II were combined, lyophilized separately, dissolved in TEAF and re-chromatographed by isocratic HPLC (i-HPLC) to enrich preferentially endogenous ME and LE, respectively. Twenty 1.5-ml fractions were collected and 50 μl from each fraction were used to measure ME (section I) and LE (section II) immunoreactivity (see flow chart and sample nomenclature in Fig. 2). Similarly, the combined HPLC fractions in section III were purified by gradient HPLC (90 min) into three new sections, IIIA, IIIB and IIIC.

Section IIIA contains C-terminally extended ME and LE peptides (Fig. 1) such as ME–RGL, ME–RF and dynorphins, and therefore was treated with T and CPB to produce the pentapeptides ME and LE. The resulting solution was separated by gradient HPLC (90 fractions) to yield sections III-A-1 and III-A-2, corresponding to ME and LE, respectively. An aliquot (50 μl for ME; 100 μl for LE) from each fraction was lyophilized; ME–LI (III-A-1) and LE–LI (III-A-2) were measured, respectively. SP-LI and BE-LI were measured in sections IIIB and IIIC (50 μl each), respectively.

To maximize the amount of analytical information, the contents of all remaining tubes located between the elution of ME and LE, LE and SP and SP and BE, which might also contain opioid- or tachykinin-containing peptides (see retention times, Fig. 1), were combined and treated with T and CPB, to release any ME and LE. ME–LI and LE–LI were measured after gradient HPLC, but no immunoreactivity was detected for these two peptides.

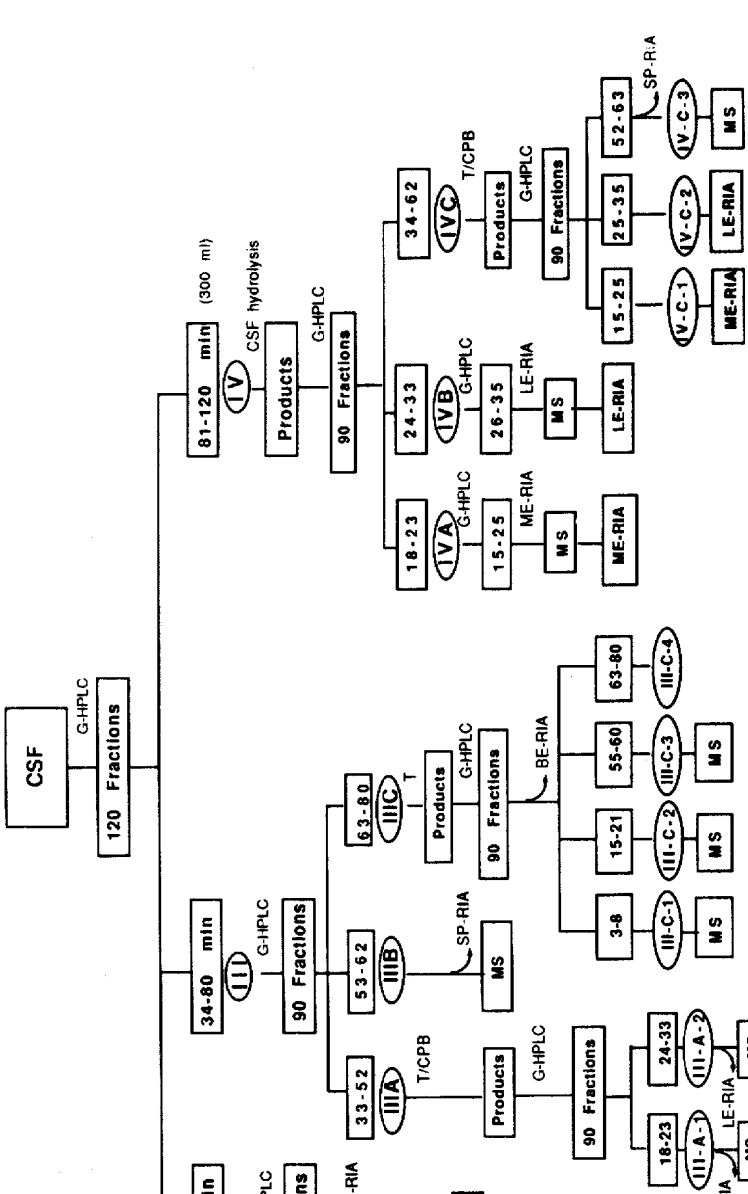

Fig. 2. Flow chart of sample nomenclature, treatment and analysis of sections I–IV. See text for description of experimental techniques and abbreviations.

Fractions from section IV (from an equivalent volume of 300 ml of CSF), which contains larger peptide precursors, were combined, lyophilized and hydrolyzed with human CSF as the source of specific endogenous opioid and tachykinin peptidases. Human CSF (30 ml), lyophilized fractions 81–120, and the mixture of enzyme inhibitors (27 μl) were mixed, incubated (37°C, 5 h) and then lyophilized. Endogenous human CSF neuropeptidases produce enkephalins and substance P from their corresponding precursors, and inhibitors halt the degradation of enkephalins to inactive metabolites. Enzymatic products were separated by HPLC, and 90 fractions were collected to give three sections (IVA, IVB and IVC).

Sections IVA and IVB were separated by gradient HPLC (90 min), and 50 μl of each fraction were taken from fractions 15–25 to measure ME–LI and 100 μl from fractions 26–35 for LE–LI. The fractions containing the highest amount of immunoreactivity were combined and analyzed by FAB-MS.

Sample IVC was trypsinized (400 μl of T beads, 37°C, 2 h, pH 7.4) and then centrifuged. CPB (200 μl) was added to the supernatant and the mixture was incubated (37°C, 1 h). The reaction mixture was lyophilized and the residue redissolved in HPLC buffer. A 90-min RP-HPLC gradient was used to separate this mixture into three new sections, IV-C-1, IV-C-2 and IV-C-3. A (50 μl) aliquot of each HPLC fraction of IV-C-1 was taken for measuring ME–LI, 100 μl from IV-C-2 for LE–LI and 100 μl from IV-C-3 for SP–LI.

After ME–LI, LE–LI, SP–LI and BE–LI had been measured, tubes containing the maximum amount of immunoreactivity for each peptide were combined separately for FAB-MS corroboration of ME, LE, SP and BE, respectively.

The molecular weight of BE exceeds the upper mass limit of our mass spectrometer and does not yield the highest level of detection sensitivity at that mass. However, the tryptic pentapeptide NAIIK (BE_{20-24}) displays high detection sensitivity[39]. Therefore, fractions contained in IIIC were trypsinized for FAB-MS analysis of BE. T treatment of synthetic BE yields six fragments: YGGFMYSEK, SQTPLVTLFK, NAIIK, NAYK, K and GE. Potential tryptic products from endogenous BE were separated by gradient HPLC (90 min) and three components (III-C-1–3) were analyzed by MS. Fractions 3–8 correlate with the retention time of NAIIK, 15–21 with YGGFMYSEK and 55–60 with SQTPLYTLFK.

After each MS measurement, the tubes were washed and RIA confirmed the retention of the appropriate peptide immunoreactivity to ensure that MS measured the proper immunoreactive peptide.

RESULTS

Analysis of sections I and II for intact endogenous ME and LE

Fig. 3 demonstrates the isocratic HPLC of sections I and II and measurements of intact, endogenous ME–LI and LE–LI in their corresponding HPLC fractions. These two chromatograms were obtained from a total of 210 ml of human CSF. RIA results for an HPLC blank are shown at the bottom of each peak.

Intact ME. FAB-MS–MS results for the analysis of intact ME were obtained from an extracted volume corresponding to 380 ml of CSF. No molecular ion was observed above the background in the MCA mode, probably owing to a combination of several experimental factors, including a small amount of endogenous peptide, a

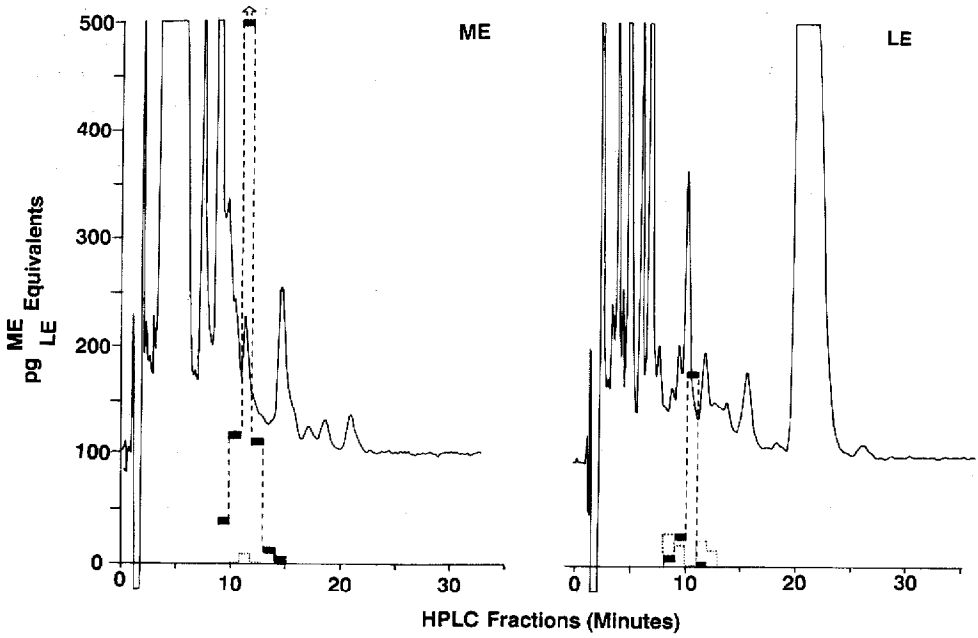

Fig. 3. Isocratic RP-HPLC separation and RIA measurement of sections I (ME–LI, left) and II (LE–LI, right), abscissa, pg enkephalin equivalents; ordinate, time (min).

high level of chemical noise from the FAB matrix and possible suppression of ion formation due to impurities and column bleeding. Nevertheless. a *B/E* linked-field spectrum of $(M-H)^-$ at *m/z* 573.2 contained several amino acid sequence-determining ions that were observed because of the molecular specificity of that MS–MS mode of product ion detection. In a *B/E* linked-field scan spectrum, product ions derive from a selected precursor ion.

Table I collects those amino acid sequence-determining fragment ion masses (calculated and observed) for endogenous ME ions (intact and from precursor). N-Terminus-containing ions were observed at (see Table I; intact column) *m/z* 277.0 ($'B_3$), and C-terminus-containing ions at *m/z* 337.4 (Z_3), 295.5 (Y_2'), 436.7 (X_4) and 321.7 ($'X_2$). The number of apostrophes to the left and right of an indicated ion type denotes the number of hydrogens subtracted or added, respectively[38]. Loss of Tyr and Phe side-chains from $(M-H)^-$ also produces ions at *m/z* 465.2 and 481.7, respectively (data not shown). The fragmentation pattern observed here correlates to general fragmentation features observed for opioid peptides[33]. The small differences in mass observed at low mass values result from the intentional decrease in resolution used for obtaining *B/E* spectra of synthetic (1000 resolution) *vs.* biological ($\leqslant$500 resolution) samples.

Intact LE. LE fractions corresponding to the RP-HPLC separation of 380 ml of CSF did not demonstrate an LE $(M+H)^+$ ion at 556.2 in the positive-ion FAB-MS multi-channel acquisition (MCA) mode or sequence ions in the *B/E* mode. However, when remaining contents from that tube were analyzed for LE–LI, the upper limit of the LE RIA curve (1000 pg) was exceeded, suggesting that the amount of LE, if

TABLE I

AMINO ACID SEQUENCE-DETERMINING FRAGMENT IONS OBSERVED IN THE *B/E*–CAD SPECTRA OF THE $(M-H)^-$ ANION OF ENDOGENOUS METHIONINE ENKEPHALIN (INTACT AND FROM PRECURSORS)

Signal-to-noise ratio ranges from approximately 2:1 to 5:1. A, B, C, X, Y and Z calculated as described in ref. 38. Apostrophes (on the right) indicate hydrogens added.

N-Terminus sequence ions				*C-Terminus sequence ions*			
Symbol	*Calculated, m/z*	*Observed*		*Symbol*	*Calculated, m/z*	*Observed*	
		m/z from intact	*m/z from precursor*			*m/z from intact*	*m/z from precursor*
A_4	397.2	–	395.1	Z_4	394.2	–	394.3
				Z_3	337.1	337.4	–
B_4	425.2	–	425.6	Z_2	280.1	–	280.6
B_3	278.1	277.0	278.1				
				Y_4''	411.2	–	409.1
C_4	440.2	–	439.9	Y_3''	354.1	–	352.1
				Y_2''	297.1	295.5	296.1
				X_4	437.2	436.7	437.4
				X_3	380.1	–	380.2
				X_2	323.1	321.7	322.4

present, was probably below the limit of FAB-MS detection of biological peptides, or that the presence of biological or chromatographic impurities suppressed the $(M+H)^+$ ion signal.

This difference observed in detection sensitivity between MS[34] and RIA involves at least two factors: first, matrix effects (biological, chromatographic contaminants), and second, the RIA antibody may detect immunoreactivity due to sources other than LE[40].

These two sets of data demonstrate clearly the presence in human CSF of the intact ME pentapeptide (based on gradient and isocratic RP-HPLC retention time, immunoreactivity and unique FAB-MS–MS sequence ions), and probably intact LE (gradient and isocratic RP-HPLC, and immunoreactivity).

Analysis of section III for intact SP and BE

A 380 ml volume of CSF was lyophilized, the residue was dissolved in TEAF and this solution was injected (in individual 20-ml batches to avoid any analytical column overload) onto the HPLC column. Fractions collected within the 34–80 min range were combined. Also, similar fractions remaining after a previously completed HPLC–RRA profile study of other low back-pain patients (total $\approx$ 100 ml) was added to section III.

Section III was purified further by gradient HPLC (two portions; each HPLC gradient equaled 210 ml of CSF) to provide sections IIIB and IIIC (see Fig. 2). Fig. 4 shows the gradient HPLC of section III (210 ml of CSF), and demonstrates the presence of SP–LI and BE–LI in their HPLC fractions 51–61 and 71–77, respectively (note the combined SP/BE ordinate label). RIA data were obtained from an equivalent volume of 7 ml of CSF (50 μl from each 1.5-ml fraction).

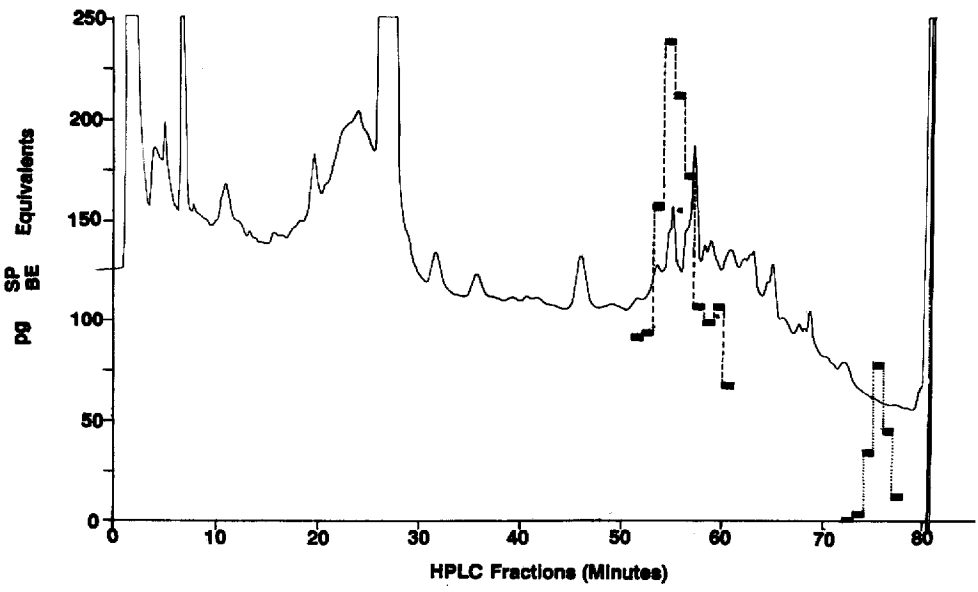

Fig. 4. Gradient RP-HPLC separation of section III, and measurement of SP–LI in fractions 51–61 and BE–LI in fractions 71–77.

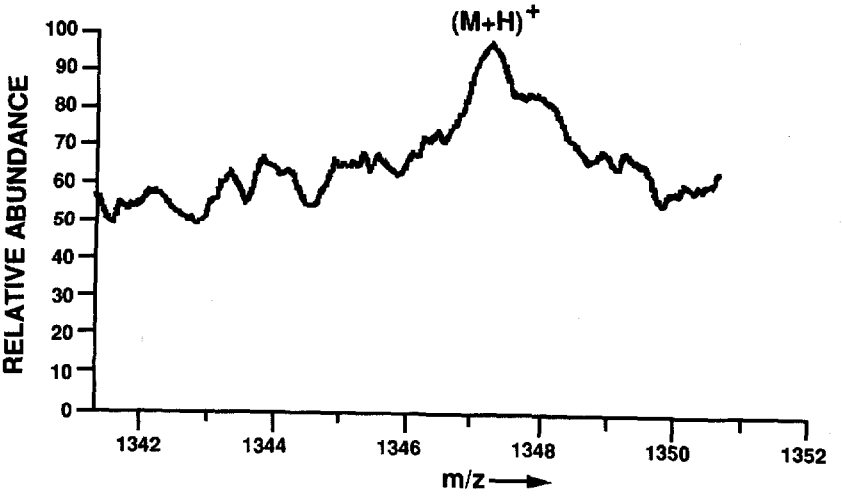

Fig. 5. FAB-MS $(M+H)^+$ MCA data for intact SP. This spectrum is the accumulation of ten scans.

Intact SP. FAB-MS MCA analysis (see Fig. 5) of a CSF sample (150 ml), at low resolution to optimize detection sensitivity, demonstrated the presence of an apparent ion at *m/z* 1347.3, which is compatible with intact endogenous SP (1347.7 a.m.u.). Corroboration of the presence of SP was obtained by acquiring the *B/E* linked-field

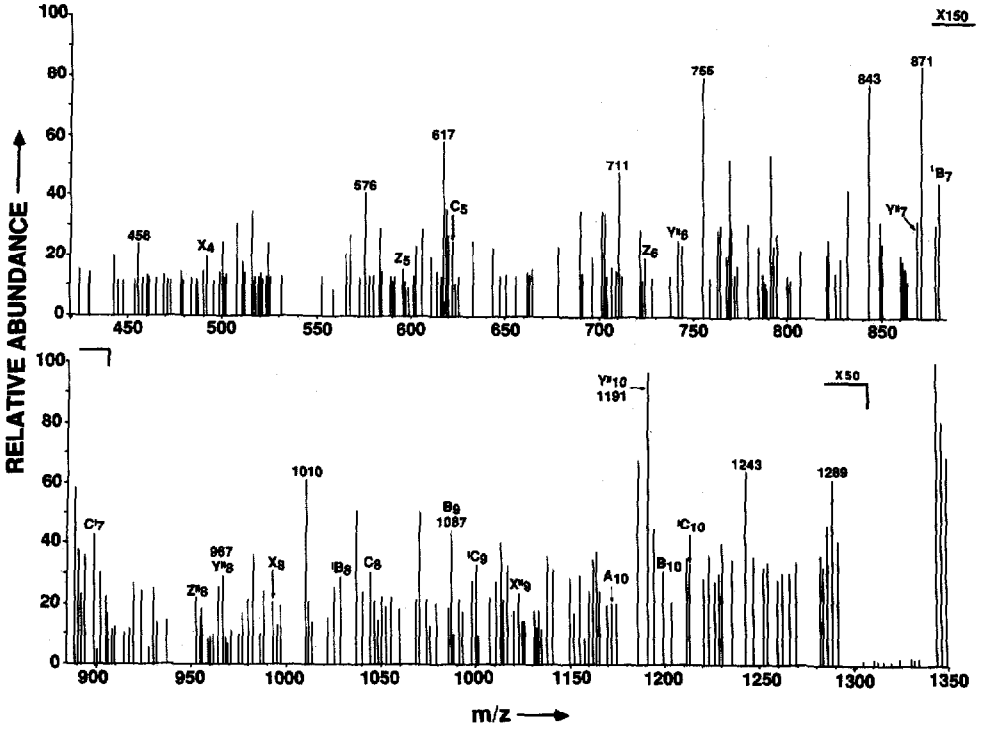

Fig. 6. FAB-MS *B/E* linked-field scan of the $(M+H)^+$ ion of intact SP.

TABLE II

THE AMINO ACID SEQUENCE-DETERMINING FRAGMENT IONS OBSERVED IN THE *B/E*–CAD SPECTRA OF THE $(M+H)^+$ PRECURSOR ION OF SUBSTANCE P (INTACT AND FROM PRECURSOR)

In general, the signal-to-noise ratio of these data ranges from approximately 2:1 to 5:1 (see Fig. 6). Apostrophes on the right indicate added hydrogens. A, B, C, X, Y and Z calculated using the scheme of ref. 38. C_4 and X_4'' are isobaric. We have less confidence in assignment of the sequence ions shown in parentheses because of poor signal-to-noise.

N-Terminus sequence ions				*C-Terminus sequence ions*			
Symbol	*Calculated, m/z*	*Observed*		*Symbol*	*Calculated, m/z*	*Observed*	
		m/z from intact	*m/z from precursor*			*m/z from intact*	*m/z from precursor*
A_{10}	1171.7	1171.7	(1170.5)	Z_{10}	1174.6	1174.5	–
A_9	1058.6	–	–	Z_9	1077.6	–	(1078.5)
A_8	1001.6	–	–	Z_8	949.5	–	–
A_7	854.5	–	–	Z_7	852.4	–	852.7
A_6	707.4	–	–	Z_6	724.4	724.4	723.6
A_5	579.4	–	–	Z_5	596.3	595.8	595.8
A_4	451.3	–	–	Z_4	449.2	–	–
B_{10}	1199.7	1199.1	–	Y_{10}''	1191.6	1191.0	1190.3
B_9	1086.6	1087.1	–	Y_9''	1094.6	–	1094.7
B_8	1029.6	1028.6	1028.6	Y_8''	966.5	966.7	–
B_7	882.5	–	–	Y_7''	869.4	869.2	869.0
B_6	735.4	–	735.4	Y_6''	741.4	(742.2)	–
B_5	607.4	–	–	Y_5''	613.3	–	–
B_4	479.3	–	–	Y_4''	466.2	–	–
C_{10}	1214.7	1213.3	1213.6	X_{10}	1217.6	–	–
C_9	1101.6	–	1103.7	X_9	1120.6	1122.7	–
C_8	1044.6	1044.6	(1043.2)	X_8	992.5	993.1	992.6
C_7	897.5	898.8	899.4	X_7	895.4	–	–
C_6	750.4	–	750.6	X_6	767.4	–	–
C_5	622.4	622.0	–	X_5	639.3	–	640.2
C_4	494.3	–	493.7	X_4	492.2	491.9	493.7

scan spectrum (Fig. 6) from this apparent $(M+H)^+$ ion. Table II lists the sequence-containing fragment ions (calculated; observed). N-Terminus-containing ions were observed (see Table II; intact) at *m/z* 1171.7 (A_{10}), 1199.1 (B_{10}), 1087.1 (B_9), 1028.6 ($'B_8$), 1213.3 ($'C_{10}$), 1044.6 (C_8), 898.8 (C'_7) and 622.0 (C_5), and C-terminus-containing ions at *m/z* 1174.5 (Z_{10}), 724.4 (Z_6), 595.8 ($'Z_5$), 1191.0 (Y''_{10}), 966.7 (Y''_8), 869.2 (Y''_7), 742.2 (Y''_6), 1122.7 (X''_9), 993.1 (X_8) and 491.9 (X_4). Again, the signal level is low, but the observation of several different B, C, X, Y and Z ions indicates strongly the presence of intact SP in that sample. These amino acid sequence ion data corroborate the SP sequence RPKPQQFFGLM-NH_2. The fragmentation pattern observed here correlates well with the corresponding spectrum of synthetic SP[37]. The presence of ions not related to the SP sequence in Fig. 6 reflects clearly the limitations of the *B/E* linked-field technique. The poor mass resolution of the technique may result in transmission of other sample contaminants and matrix ions along with the precursor ion of concern, and thus may contribute to the extraneous ions.

BE. Fractions 63–80 (section IIIC) were combined and trypsinized; the HPLC-separated products were analyzed by MS[39]. Fractions 71–77 exhibited BE–LI (Fig. 4) in 50 μl. Because T treatment of section IIIC occurred before BE–LI was measured (see Fig. 2), the actual amount of BE–LI in untreated CSF is higher than the measurement shown in Fig. 4. FAB-MS data for NAIIK in that HPLC fraction (3–8) did not demonstrate a positive indication of BE. In conclusion, BE–LI was found at the appropriate HPLC retention time.

Analysis of C- and N-terminal extensions of ME and LE

It was mentioned above that ME C-terminally extended peptides such as ME–Arg–Phe, ME–Arg–Gly–Leu and dynorphins, such as dyn_{1-12} and dyn_{1-17} (see Fig. 1 for retention times) exist in human CSF. Therefore, section IIIA (from 480 ml of CSF) was treated with T and CPB, and separated by gradient RP-HPLC to obtain free ME and LE. Sections III-A-1 (for ME) and III-A-2 (for LE) were collected. Although ME–LI and LE–LI were both detected (data not shown), FAB-MS data were not observed for these two peptides.

Analysis of section IV for larger-sized precursors

ME. A peptide, cleaved from HPLC-purified precursor(s) present in a corresponding volume of 300 ml of CSF with its endogenous neuropeptidase enzymes (30 ml of CSF), showed ME-LI. Even though no $(M-H)^-$ of ME was observed by FAB-MS MCA, possibly for the same reasons as outlined above, a *B/E* linked-field scan of that precursor mass was obtained. Those data are listed in Table I. N-Terminus-containing ions (Table I; precursor column) were observed at *m/z* 395.1 ($''A_4$), 278.1 (B_3), 425.6 (B_4) and 439.9 (C_4), and C-terminus-containing ions at *m/z* 394.3 (Z_4), 280.6 (Z_2), 409.1 (Y_4), 352.1 (Y_3), 296.1 (Y'_2), 437.4 (X_4), 380.2 (X_3) and 322.4 ($'X_2$). These sequence data corroborate the presence of ME in that sample, and thus also the presence of ME-containing precursors, and of ME precursor-processing enzymes in the human CSF (after MS analysis, the tube was washed and ME–LI was measured; ME–LI exceeded the 500 pg upper limit of the RIA kit).

LE. The gradient HPLC trace (not shown) of section IVB (100 ml of CSF) contained LE–LI from 100 μl, which was equivalent to approximately 7 ml of CSF. MS results (MCA; *B/E*) for this sample were negative. After MS measurement, the

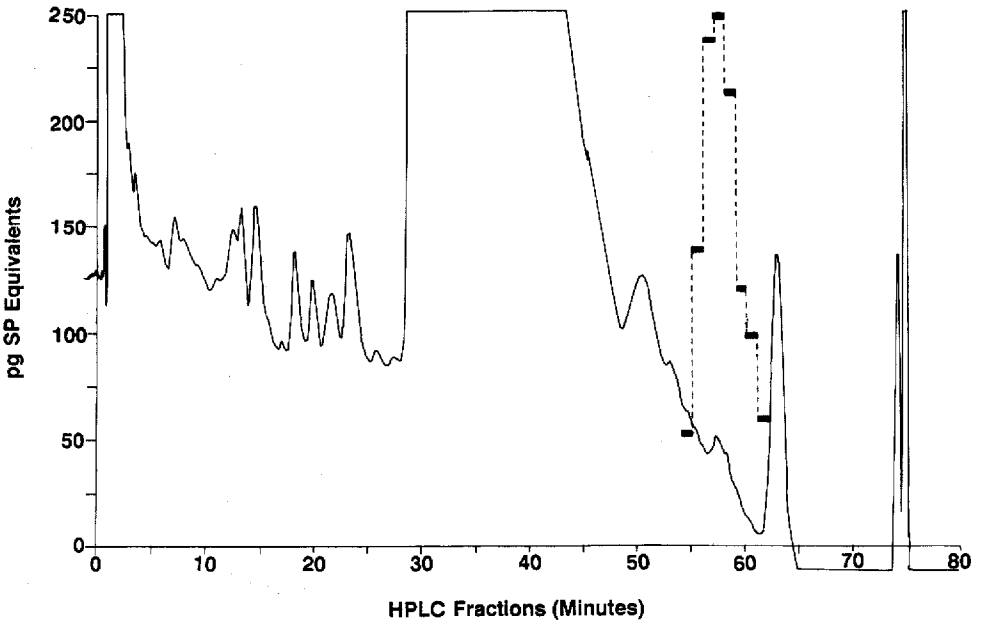

Fig. 7. Gradient RP-HPLC separation of T–CPB-treated sample IVC and measurement of SP–LI in sample IV-C-3.

tube was washed; it still contained >1000 pg of LE–LI. We conclude from these HPLC and LE–LI data that probably the precursor contains the LE sequence, but that the amount is lower than ME.

SP. Fig. 7 shows the HPLC of T and CPB-treated section IVC in 300 ml of CSF and the SP–LI measurement of section IV-C-3. For measuring SP, 100 μl were used, which were equivalent to 20 ml of CSF. SP–LI was detected in fractions 55–61. In addition, an $(M+H)^+$ ion was found, and a *B/E* scan of that precursor ion (Table II; precursor column) contains N-terminus-containing ions at *m/z* 1170.5 ($'A_{10}$), 1028.6 ($'B_8$), 735.4 (B_6), 1213.6 ($'C_{10}$), 1103.7 (C''_9), 1043.2 ($'C_8$), 899.4 (C''_7), 750.6 (C_6) and 493.7 (C_4), and C-terminus-containing ions at *m/z* 1078.5 (Z'_9), 852.7 (Z_7), 723.6 (Z_6), 595.8 (Z_5), 1190.3 (Y'_{10}), 1094.7 (Y''_9), 869.0 (Y''_7), 992.6 (X_8), 640.2 (X'_5) and 493.7 (X_4). These MS–MS data indicate that section IV contains an SP precursor, and that human CSF contains SP precursor-processing enzymes.

DISCUSSION

Our objective in this study was to determine the presence of and to corroborate the amino acid sequence of endogenous intact peptides in human CSF and of those peptides excised from precursors by CSF peptidases. We attained that goal for ME (intact; from precursors) and for SP (intact; from precursors). On the other hand, BE–LI and LE–LI were both found, but sequence data were not corroborated. Another objective was to demonstrate the presence of appropriate precursor-processing enzymes for ME and SP in CSF. We infer the presence of those enzymes because we detected the product of their action. It was not the purpose here to purify or to

characterize those enzymes, but rather to take experimental advantage of their presence in CSF to produce and to characterize ME and SP. To date, no equivalent amino acid sequence data are available for that enzyme–product relationship in human CSF.

These peptide data contribute significantly to our understanding of molecular mechanisms involved in neurochemical events. For example, our working hypothesis for this study is that opioid and tachykinin neuropeptide pathways play a homeostatic regulatory role in "normal" human CSF, and that metabolic derangements in these peptide systems are involved in the pathophysiology of patients[13] experiencing pain[41], stress[42,43] or other illnesses. The most direct experimental approach to answer these questions was to combine the RP-HPLC separation of neuropeptides with a battery of several different post-HPLC detectors, each of which possesses a different level of detection sensitivity and molecular specificity towards peptides[22,25].

The ME (intact) sequence was corroborated by one N-terminus- and four C-terminus-containing fragments, the ME (precursor) sequence by four N-terminus- and eight C-terminus-containing fragments, the intact SP sequence by eight N-terminus- and ten C-terminus-containing fragments and SP from a precursor by nine N-terminus- and ten C-terminus-containing fragments (see data summarized in Tables I and II). Because these *B/E* linked-field spectra were obtained at the lowest end of our MS detection threshold, the signal-to-noise ratio was low and not all of the possible sequence ions were observed. Nevertheless, these FAB-MS–MS sequence data for neuropeptides extracted from human CSF are remarkable because of several experimental factors including sub-picomole levels of endogenous peptides in human CSF, a relatively limited volume of human CSF available from each patient for this study, possible loss of peptide during purification steps, unavoidable introduction of HPLC column bleeding to an MS sample, possible other unknown experimental factors and because of the very high molecular specificity of an MS–MS measurement. These data demonstrate the usefulness of *B/E* linked-field mass scanning to detect product ions, even when the precursor ion current is very small, and at least to corroborate the presence of an endogenous peptide.

Corroboration adds structural significance to the HPLC, RIA and RRA combined data. A complete amino acid sequence determination of these endogenous neuropeptides from CSF and other biological sources awaits significant improvements in detection sensitivity of current MS instruments. Corroborating the presence of a known peptide is an important factor in CSF studies, and is clearly different from determining the amino acid sequence of an unknown biological peptide.

The discovery and amino acid sequence determination of a hypothalamic-releasing hormone, thyrotropic-releasing hormone (TRH)[44], prompted intensive research into brain neurochemistry, and since then many other neuropeptides have been discovered[45], including opioid[46,47] and tachykinin peptides[48,49]. It is well established that the cell body of a nerve synthesizes peptide precursors that lead, during axonal transport and metabolism, to opioid and tachykinin peptides[50], among many others. Opioid precursors include proenkephalin A, which produces LE and ME, and other C-terminally extended ME peptides; proenkephalin B, which produces a range of dynorphin peptides[51], which are C-terminally extended LE peptides; and proopiomelanocortin (POMC), which produces β-endorphin$_{1-31}$ (BE), adrenocorticotropin hormone (ACTH), α-melanocyte-stimulating hormone (MSH) and β-lipotropin[52]. Three tachykinin precursors (α, β and γ) are known.

Several papers have reported immunoreactivity or receptoractivity in human CSF corresponding to enkephalins, endorphins, dynorphins and tachykinins. However, no amino acid sequence data have been determined. For example, enzymes that degrade the smaller opioid (especially enkephalin) peptides have been reported in CSF; the use of CSF as a source of enzymes to degrade smaller enkephalins, but not larger precursors, to inactive metabolites was also reported[18]. Endopeptidases that cleave dynorphins A and B and α-neo-endorphin[4] and SP[6] have also been purified from human CSF. Precursors (probably proenkephalins A and B) were found in human CSF[4,7], pituitary and hypothalamus. However, the presence of enzymes that cleave precursors to opioid and SP peptides, which was one of the objectives of this study, has not yet been reported in human CSF.

A unique aspect of this research is the production of SP from an HPLC-purified precursor by an endogenous CSF peptidase. The neuropeptidases mentioned in this paper are not characterized here (that was not the objective of this work), but their presence is indicated because the peptide products arising from their action on HPLC-purified precursors are characterized. Although opioid peptides can be excised directly from precursors with T–CPB, SP cannot be produced in that way. The C-terminal carboxamide in SP requires a glycine residue in the precursor after the SP sequence and a carboxamide-synthesizing enzyme[53]. Thus, to prove the presence of these neuropeptidases in human lumbar CSF, we have met those rigid biosynthetic criteria, and further we have added MS–MS sequence data (Table II) to corroborate the presence of SP produced from an SP precursor and of SP precursor-processing enzyme in CSF.

An abundant $(M+H)^+$ cation or $(M-H)^-$ anion of a peptide is produced with positive or negative FAB-MS, respectively[54]. A molecular ion indicates a peptide's molecular weight, but not its amino acid sequence. As shown elsewhere[35,55], linked-field scan MS methods[35], also referred to as MS–MS[55], provide amino acid sequence-determining, and other fragment, ion information from a specifically selected $(M+H)^+$ or $(M-H)^-$ precursor ion[36,37]. Linked-field scanning at constant B/E is an MS–MS method available to collect product ions from a precursor ion. Even if a molecular ion is not observed by FAB-MS–MCA, the favorable signal-to-noise ratio of a B/E scan could still detect sequence ions. For example, in this study, the mass spectra contained abundant N-terminus-containing ions, due to ^{1}Arg and ^{3}Lys in SP, for example[56], and also numerous C-terminus-containing ions. That rigorous structure correction between a precursor ion and its product ions increases the confidence in corroborating the amino acid sequence of a peptide in a biological peptide. In terms of the use of FAB-MS in this study, it must be realized that the sensitivity of detection of MS methodology for quantifying synthetic enkephalins approaches the detection level sensitivity of radioimmunoassay. Femtomole[32] and attomole[34] levels of detection sensitivity have been achieved for solutions of synthetic LE, but biological samples demonstrate less detection sensitivity, generally by a factor of ten. More recently, tandem four-sector MS instruments with multi-channel array detectors[57] have demonstrated a 100-fold increase in detection sensitivity, which will impact significantly on these types of human CSF studies.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge financial assistance from the NIH (GM 26666 and DRR 01651), typing assistance from Linda Rutherford, Deanna Darling and Dianne Cubbins, and microcomputer assistance from Dianne Cubbins.

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