Journal of Chromatography, 562 (1991) 573–584
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5538

Analysis of methionine enkephalin in human pituitary by multi-dimensional reversed-phase high-performance liquid chromatography, radioreceptor assay, radioimmunoassay, fast atom bombardment mass spectrometry, and mass spectrometry–mass spectrometry

JERRY LEE LOVELACE

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

JOZEF J. KUSMIERZ

Charles B. Stout Neuroscience Mass Spectrometry Laboratory, University of Tennessee, Memphis, 800 Madison Avenue, Memphis, TN 38163 (U.S.A.)

and

DOMINIC M. DESIDERIO*

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

ABSTRACT

Methionine enkephalin (ME = YGGFM) was measured in five individual human post-mortem pituitaries using four different analytical methods, with the objective of comparing the molecular specificities of the methods. Radioreceptor assay (RRA) used a receptor-rich preparation from brain and [^{3}H]etorphine as radioligand to determine ME-like receptoractivity (ME-LR). Radioimmunoassay (RIA) measured ME-like immunoreactivity (ME-LI). Pituitary samples analyzed by RRA and RIA were purified first with a high-performance liquid chromatography (HPLC) gradient on a polymer analytical column. Fast atom bombardment mass spectrometry (FAB-MS) in two different detection modes quantified ME using the protonated molecular ion MH^+ of ME at 574 a.m.u. and B/E linked-field selected reaction monitoring (SRM) to monitor the specific unimolecular metastable transition that produced the unique amino acid sequence-determining tetrapeptide fragment ion $YGGF^+$ from the MH^+ precursor ion. Both FAB-MS methods used the deuterated internal standard YGG[$^2H_5$-F]M. Samples analyzed with FAB-MS were purified first with multi-dimensional reversed-phase HPLC. The first dimension was an ODS gradient, and the second dimension was a polymer isocratic elution. The following ME amounts were measured (mean ± standard error of the mean): ME-LR, 7.0 ± 1.9 $\mu g\ g^{-1}$ tissue; ME-LI, 1.8 ± 0.7 $\mu g\ g^{-1}$ tissue; MH^+, 2.7 ± 0.6 $\mu g\ g^{-1}$ tissue; SRM, 3.0 ± 0.8 $\mu g\ g^{-1}$ tissue. The FAB SRM method provided the highest level of molecular specificity amount these four analytical methods used to measure picomole amounts of endogenous ME in a human pituitary.

0378-4347/91/$03.50 © 1991 Elsevier Science Publishers B.V.

INTRODUCTION

Neuropeptides play a role in several different areas of neurochemistry [1–3]. Opioid and tachykinin peptides are important in, for example, chronic low back pain [4–8] and in psychiatric disorders [9,10]. Other pathophysiologies involved a metabolic defect in neuropeptide processing [3]. In our opinion, it is crucial for an accurate understanding of a pathophysiology, clinical diagnosis and treatment to know the amino acid sequence of a peptide during its qualitative and quantitative analysis [11,12]. This important experimental factor is missing in most of today's research publications; from our perspective, molecular specificity is more important than detection sensitivity. Therefore, a multi-dimensional analytical method used different types of reversed-phase high-performance liquid chromatography (RP-HPLC) [13] combined with radioreceptor assay (RRA), radioimmunoassay (RIA), and fast atom bombardment (FAB) mass spectrometric (MS) and FAB selected reaction monitoring (SRM) methods [7,11,12,14–16] to quantify endogenous neuropeptides and to overcome some of those limitations in molecular specificity.

The purpose of this study was to compare the molecular specificity of four of these analytical methods by analyzing the opioid pentapeptide methionine enkephalin (ME = YGGFM) in a human pituitary. RIA, RRA and MS in two different detection modes: molecular ion (MH^+) quantification, and B/E linked-field SRM of a unimolecular metastable transition to produce the unique amino acid sequence-determining tetrapeptide fragment ion $YGGF^+$ from MH^+ were used. Both FAB-MS methods used the deuterated internal standard YGG[$^2H_5$-F]M (d_5-ME).

This analytical comparison was necessary to test accurately our hypothesis that opioid neuropeptides play a role, for example, in neuroendocrinologically silent pituitary tumor formation [17], drug overdose deaths [18], chronic low back pain [4,5,7,19] and senile dementia of the Alzheimer's type [20].

EXPERIMENTAL

Fig. 1 contains the analytical scheme used in this study and illustrates the allocation of pituitary tissue among the four different detection methods: RRA (9%), RIA (1%) and MS (90%). The MS and SRM analytical scheme has been described [13].

Step 1: tissue acquisition

Post-mortem human pituitaries were obtained during autopsy, which was performed as soon as feasible after death. After tissue was obtained for pathology, the remaining tissue was frozen immediately and kept at −70°C until processed [17]. Anterior and posterior pituitary lobes were obtained from five males: average (± S.D.) age was 27 ± 5 years; four died from thoracic gunshot wounds and

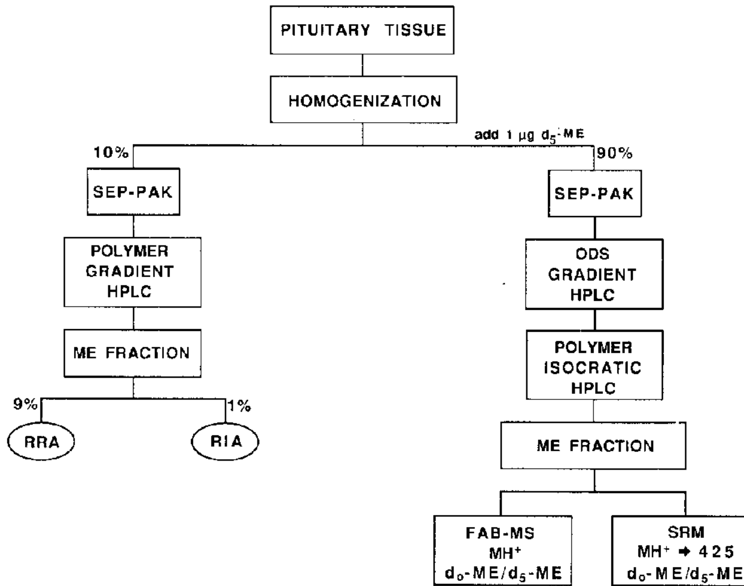

Fig. 1. Analytical scheme to measure endogenous ME in an individual human pituitary by RIA, RRA, FAB-MS using MH^+ ion of ME at 574 a.m.u. and FAB-MS using the SRM method for the $MH^+ \rightarrow YGGF^+$ transition (574 → 425 a.m.u.). The MS methods measured the ratio d_0-ME/d_5-ME of ion currents from MH^+ and from $MH^+ \rightarrow YGGF^+$, respectively. The percentages indicate the amount of tissue used for RIA (1%), RRA (9%) and MS (90%).

one from a upper left forehead gunshot wound that did not involve the pituitary.

Canine limbic tissue used for receptor assay was obtained under NIH guidelines in the Department of Comparative Medicine.

Step 2: tissue homogenization

To minimize peptide enzymolysis, frozen tissue was weighed, placed immediately into cold (4°C) acetic acid (20 ml; 1 *M*; 1:40, w/v), and homogenized for 30 s in a Polytron (setting 6) (Brinkmann, Westbury, NY, U.S.A.). The homogenate was centrifuged (31 000 *g*, 30 min), and a portion (2 ml) of the 20-ml supernatant was used for the RIA and RRA measurements.

Step 3: sample preparation for MS

The d_5-ME (1 μg) was added to the remaining 18 ml (supernatant and precipitate), which was to be analyzed by FAB-MS, and that mixture was vortexed (*ca.* 1 min) to resuspend the precipitate. The mixture was equilibrated (*ca.* 30 min) to allow the exogenous d_5-ME to mix thoroughly with the endogenous d_0-ME, and then was centrifuged. The supernatant was applied to an octadecylsilyl (ODS) disposable cartridge (Sep-Pak®, Waters; Milford, MA, U.S.A.), which had been activated [21] by eluting in sequence methanol (4 ml), HPLC-grade water (4 ml) and trifluoroacetic acid (TFA, 0.1%, 8 ml) [19].

The supernatant (18 ml) was applied to an ODS cartridge, the cartridge was washed (4 ml of 0.1% TFA), and a peptide-rich fraction was eluted with 4 ml of acetonitrile–TFA (50:50). A new ODS cartridge was used for each extraction. The volume of the collected effluent was lyophilized, reconstituted in 150 μl of mobile phase, and that volume was injected onto the HPLC column.

Gradient RP-HPLC separation was performed on an ODS analytical column, and the ME fraction was purified further with an isocratic elution from a synthetic hydrocarbon analytical column [13]. These two elutions are described below.

Step 4: sample preparation for RRA and RIA

The supernatant (2 ml) from Step 2 above was applied to an ODS cartridge, the cartridge was washed (4 ml of 0.1% TFA), and a peptide-rich fraction was eluted with acetonitrile (4 ml, 100%). A new ODS cartridge was used for each extraction. The volume of the collected effluent was reduced with a stream of nitrogen to *ca.* 1 ml, and that volume was injected onto the HPLC column.

Step 5: ME recovery

HPLC-purified [^{3}H]ME (New England Nuclear, Boston, MA, U.S.A.) (39 Ci mmol^{-1}) was added (*ca.* 45 000 total counts) to a separate pituitary tissue homogenate (2 ml). Those samples were subjected to the same separation scheme used for RRA and RIA analysis. After one step of HPLC separation and lyophilization, 92 ± 5% (mean ± S.D.; $n=3$), and after a second step of lyophilization, 85 ± 5%, ($n=3$) of [^{3}H]ME was recovered. Those recovery data were used to correct the RRA and RIA measurements in Table I.

Step 6: Synthetic ME

Synthetic ME was purchased from Sigma (St. Louis, MO, U.S.A.) and HPLC-purified before use.

Step 7: RP-HPLC analyses

RP-HPLC analyses used either a Waters (for the two FAB-MS methods) or a microprocessor-driven Varian 5000 (for RRA and RIA) HPLC system. A synthetic polymer (polystyrene–polyvinylbenzene; Polymer Labs. Amherst, MA, U.S.A.) analytical column (PLRP-S, 5 μm bead, 100 Å pore size, 15 cm × 0.46 cm) or an ODS μBondapak (10 μm bead diameter, 30 Å pore size, 30 cm × 0.4 cm, Waters) was used on each instrument. The volatile ion-pairing buffer was triethylamine formate (TEAF) (40 m*M*, pH 3.2) [22], and acetonitrile (HPLC grade; Burdick and Jackson, Muskegon, MI, U.S.A.) was the organic modifier used for elution.

Gradient elution of ME from a polymer analytical column for RRA and RIA. The following gradient [23] was used: 10% acetonitrile at 0 min, 18% at 48 min, 30% at 72 min, 30% at 80 min, 100% at 80 min and 100% at 90 min. UV

absorption at 200 nm was monitored, and the flow-rate was 1.5 ml min^{-1}. Fractions (1 min) eluting from the polymer analytical column were collected with a fraction collector. For RRA and RIA, ME fractions 19–25 were pooled after the gradient elution and lyophilized; the residue was reconstituted into 1 ml of water. An aliquot (100 μl) was used for RIA, and the remainder for RRA. The RIA aliquot was lyophilized, and the residue was dissolved in the RIA phosphate buffer (1 ml).

Gradient (ODS) and isocratic (polymer) elution of ME for MS and SRM. The ODS column gradient was 10% acetonitrile at 0 min, 10% at 10 min, 15% at 30 min, 18% at 48 min, 34% at 72 min, 34% at 80 min, 100% at 80 min and 100% at 90 min. The mobile phase flow-rate was 1.5 ml min^{-1}. Synthetic ME eluted from this gradient at 24 min. The column was washed with the same gradient to minimize memory effects [6]. Sample was applied, and the fractions eluting between 23 and 25 min were collected manually and lyophilized. That residue was reconstituted in 150 μl of isocratic mobile phase and subjected to isocratic separation on a polymer analytical column.

Isocratic elution of ME was performed with acetonitrile–TEAF (16:84, v/v) following an ODS gradient elution. The mobile phase flow-rate was 1.2 ml min^{-1}. The elution time of synthetic ME (6.5 min) was determined in a separate experiment, after which the HPLC column was washed rigorously to avoid any memory effect [6]. The isocratic ME fraction (1 min) was collected manually, lyophilized to dryness, and the residue was reconstituted into 50 μl of TFA (0.1%). That volume permitted several separate FAB-MS measurements (see Step 11 below).

Step 8: radioimmunoassay

Commercial (IncStar, Stillwater, MN, U.S.A.) ME RIA kits were used, and the instructions contained therein were followed. [^{125}I]ME was the radioligand used. RIA data are given as ng ME-like immunoreactivity (ME-LI) per g of tissue.

Step 9: radioreceptor assay

The experimental details of this procedure have been published previously [24]. Briefly, a canine limbic system was used to provide an opioid receptor-rich P_2 fraction, and HPLC-purified [^{3}H]etorphine (^{3}H-Et) was used as the broad-based ligand. The precentage displacement of ^{3}H-Et from the receptor preparation by the ME HPLC peak was determined. Because the calibration curve was constructed using synthetic ME as the displacing ligand, the RRA data were described as ng ME-like receptoractivity (ME-LR) per g of tissue.

Step 10: internal standard

The stable isotope-incorporated internal standard, d_5ME, was synthesized by solid-phase peptide methods [13]. A 1-μg amount of HPLC-purified d_5-ME in-

ternal standard was added to that portion of each separate pituitary tissue extract that was analyzed by FAB-MS.

Step 11: mass spectrometry

A Finnigan MAT 731 mass spectrometer (Bremen, F.R.G.) with a microcomputer data acquisition system [25] was used. FAB used 7-KeV xenon atoms from an Ion Tech (Teddington, U.K.) ion gun.

In general, the following experimental procedure was used to quantify ME. Sample (2 μl, 4% of total) was deposited onto the FAB probe tip that contained the glycerol matrix (0.2 μl), and water was evaporated in the MS probe vacuum lock. Ten FAB-MS recordings were obtained (five d_5-ME and five d_0-ME scans) for each sample. Either three or five of these separate MS measurements were obtained for each sample. The same experimental protocol was used for the MH^+ and SRM measurements. Generally, only one half of the sample apportioned to MS was used (see Fig. 1).

For the MH^+ quantitative measurement, the magnetic field (B) was set to center the MH^+ of d_0-ME (574 a.m.u.) on the oscilloscope screen, and in the peak-matching mode, the MH^+ of d_5-ME (579 a.m.u.) was also centered. The microcomputer system acquired each separate MH^+ ion current during alternating scans between d_0-ME and d_5-ME [25]. Similarly, in the SRM quantitation mode, the ion current due to the unimolecular metastable transition $MH^+ \rightarrow YGGF^+$ was monitored in alternate sweeps of the two transitions $574 \rightarrow 425$ for d_0-ME and $579 \rightarrow 430$ for d_5-ME. Ion 425 in d_0-ME is denoted as a B_4 ion [7].

To construct the calibration curve corresponding to the MH^+ and metastable transition data, solutions of synthetic d_0-ME and d_5-ME were prepared and analyzed. The ratio of d_0-ME to d_5-ME was measured over a range of concentrations, and the data for the MH^+ and SRM were both obtained. A solution of d_0-ME (80 ng μl^{-1} in 0.1% TFA) was diluted (1:1, 1:2, 1:4, 1:8 and 1:16) with 0.1% TFA. To 200 μl of each solution were added 200 μl of d_5-ME (40 ng μl^{-1}) to produce individual standard solutions containing 2000, 1000, 500, 250, 125 and 62.5 ng μl^{-1} d_0-ME, respectively. Each dilution also contained 1000 ng of d_5-ME. These amounts of d_0-ME and d_5-ME were chosen to bracket the amount of endogenous ME contained in one human pituitary. The ratio of the area under the FAB desorption curves of d_0-ME/d_5-ME for the MH^+ and SRM ion currents was plotted *versus* the amount (ng) of d_0-ME (see ref. 13).

Of course, due to the low resolution of the precursor ion selection by the electric sector in a forward geometry (E, B) two-sector mass spectrometer, other precursor masses within the range 560–580 a.m.u. could be also selected in the SRM mode. However, that low resolution translated into a higher level of detection sensitivity because all those other ions derive from d_0-ME or d_5-ME, a fact attested to by the linearity of the calibration curve [13].

Step 12: data analysis

The data for the ME measured by the four methods in Table I contain averages ± standard error of the mean (S.E.M.), rather than standard deviation (S.D.), because S.E.M. is used generally to reflect the normal amount of biologic variation observed in the level of ME measured in the five individual human pituitaries. The S.D. of each one of the four analytical methods [13] is much lower (2–5%). The coefficients of variation (100 × S.E.M./mean) of the four analytical methods obtained for these biologic tissue measurements are: RRA; 23%; RIA, 42%; MH^+, 22%; SRM, 26%.

Therefore, importance was attached to a comparison of two sets of RIA data whenever the upper range value of the mean ± S.E.M. of one set of data was lower than the lowest range value of the mean ± S.E.M. of the second set of data. In other words, significance was attached to a measurement when $(\text{mean}_1 + \text{S.E.M.}_1) < (\text{mean}_2 - \text{S.E.M.}_2)$.

RESULTS AND DISCUSSION

Table I contains the measurements of native ME in human pituitary at the ppm level ($\mu g\ g^{-1}$ tissue) by the four analytical methods. The average (± S.D.) pituitary weight was 419 ± 99 mg. The average (± S.D.) measurements were 7.9 ± 1.9 μg ME-LR g^{-1} tissue, 1.8 ± 0.7 μg ME-LI g^{-1} tissue and 2.7 ± 0.6 μg ME g^{-1} tissue for FAB-MS MH^+ and 3.0 ± 0.8 μg ME g^{-1} tissue for the SRM data. The amounts of ME measured by the MH^+ and SRM methods were within the same range as reported elsewhere [13]. It is significant to note that the SRM data represented the highest level of molucular specificity because of the specific link-

TABLE I

ANALYTICAL MEASUREMENTS OF NATIVE METHIONINE ENKEPHALIN IN HUMAN PITUITARY

RRA and RIA data were corrected by the [^{3}H]ME recoveries, which were 92 and 85%, respectively.

Tissue	Weight (mg)	RRA (ng ME-LR g^{-1} tissue)	RIA (ng ME-LI g^{-1} tissue)	MH^+ (ng ME g^{-1} tissue)	SRM (ng ME g^{-1} tissue)
1	503	9655	2324	3089	3164
2	405	4086	479	1973	1567
3	534	7587	1125	2933	4610
4	301	4211	516	1054	902
5	350	14052	4372	4614	4651
Mean	419 ± 99[a]	7918 ± 1860[b]	1763 ± 733[b]	2733 ± 596[b]	2979 ± 768[b]

[a] S.D.

[b] S.E.M.

age provided in the SRM mode between the precursor ion MH^+ and the unique amino acid sequence-determining tetrapeptide fragment ion YGGF-$^+$; furthermore, the five ^2H atoms of d_5-ME are located in the Phe amino acid residue.

Receptor assay data

Fig. 2 contains the plot of the averages of the ME measurement obtained by using RRA, RIA, MS and SRM. Those four averages reflect directly the molecular specificity of the analytical methods used because each pituitary sample was subjected to the four analytical methods.

The average of the RRA measurements was significantly higher than the RIA, MH^+ and SRM data. That trend is not surprising because of several different experimental factors. For example, it must be remembered that the ^3H-Et used in this RRA interacted actually with several different opioid receptors in a receptor-rich preparation, and not only with one receptor. An opioid receptor-rich preparation contains several different receptor sub-types including μ, κ, δ, ε and σ [26]. Second, ^3H-Et interacts with different levels of avidity and affinity with each one of those receptor sub-types. Third, even though endogenous ME was separated with a polymer HPLC gradient, it is possible that one or more other compounds also co-eluted with ME, and also interacted with the ^3H-Et–opioid receptor preparation. Fourth, the synthetic ME used to construct the RRA calibration curve also binds with a different avidity and affinity *versus* ^3H-Et with each one of the

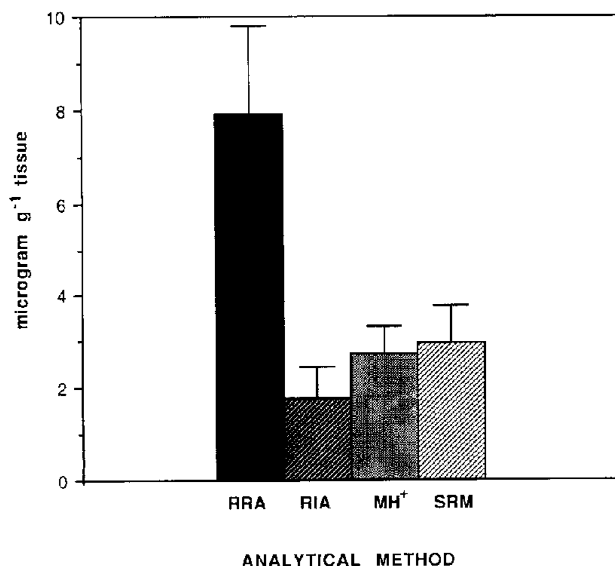

Fig. 2. Plot of the average amount of native ME measured in a pituitary by the four analytical methods RRA, RIA, FAB-MS using MH^+ and FAB-MS using SRM. The four averages (± S.E.M.) are 7.9 ± 1.9 μg ME-LR g^{-1} tissue, 1.8 ± 0.7 μg ME-LI g^{-1} tissue, 2.7 ± 0.6 μg ME g^{-1} tissue for MH^+ and 3.0 ± 0.8 μg ME g^{-1} tissue for SRM.

receptor sub-types. Fifth, RRA data must be corrected by the recovery of [^{3}H]ME. Therefore, RRA analysis is a relatively complex matter, with questionable levels of molecular specificity and of detection sensitivity, at best.

Nonetheless, even though we are aware of the experimental limitations of RRA listed above, the combination of gradient RP-HPLC with an RRA detector is a very powerful method to screen biologic extracts for the presence of several different opioid receptoractive peptides [4,17,20,24]. That HPLC–RRA screen is an important component in our research program because any endogenous peptide that interacted with a receptor has a high probability of being biologically active and clinically important; thus, it merits further detailed study.

Radioimmunoassay data

The data in Fig. 2 demonstrated clearly that the ME-LI average value was lower than the averages obtained with the RRA, MH^{+} and SRM methods. Although one might expect that the molecular specificity of RIA would be between the RRA and the two MS methods, the ME-LI measurement data in Table I do not support that prediction. Clearly, RIA underestimated the amount of native ME quantified in these pituitary tissues. RIA is generally assumed to be specific and to detect only a particular target peptide. However, we disagree with any claims made for the molecular specificity of RIA and RRA measurements [11,12]. RIA is demonstrably more sensitive than most other detection methods, because femtomoles of peptide-like immunoreactivity per tube are listed in the literature for RIA detection sensitivity (however, see ref. 16). However, it must be remembered that many important neuropeptides have a molecular weight less than 4000 Da, are not inherently immunogenic and must be conjugated chemically to a larger protein. Thus, an antibody is raised actually to an ME–protein immunogenic complex (for example thyroglobulin–ME, 1:30 [27]), and not only to the pentapeptide ME. The competition between an antigen, such as ME, and [^{125}I]ME (which is a very different molecule compared to ME) for binding to the antibody involves secondary structure features such as hydrophobicity; thus, a RIA antibody cannot interact unequivocally towards only one peptide or a specific amino acid sequence.

The RIA used in most measurements described in the literature uses either a [^{125}I]peptide tracer molecule or a Bolton–Hunter adduct to add a radiolabel to the N-terminus. Both methods alter drastically the physicochemical properties of the peptide. Iodine is a large atom that adds 22% to the weight of the ME molecule, and thus the iodinated-ME is not the same peptide as the target native ME. All of these factors contribute to the limited molecular specificity of RIA.

The data in Fig. 2 demonstrated unequivocally for the first time that RIA molecular specificity claims cannot be made.

Mass spectrometric data

MH^{+} method. It is interesting to note that the MH^{+} and the SRM data are

both comparable in the amount of native ME that they have measured (2.8 ± 0.6 and 3.0 ± 0.8 μg ME g^{-1} tissue, respectively), and that the average of both MS measurements is higher than the RIA data (*ca.* 60% higher) and lower than the RRA data (MS data are *ca.* 36% of the RRA data). These differences are rationalized readily. On one hand, it must be remembered that the RIA and the RRA data were both obtained after one stage of gradient RP-HPLC separation (see Fig. 1), whereas the MH^+ and the SRM data were both obtained after two stages (gradient ODS and isocratic polymer) of RP-HPLC. That point of chromatography is significant here because, in general, if one performs only one step of gradient HPLC and then quantifies ME based upon only the MH^+ ion, then one could conceivably have a higher measurement value because a co-eluting compound could be present that also has a peak at m/z 574 a.m.u. or another compound with no peak at 574 a.m.u. could alter positively or negatively the desorption of ME (d_0, d_5) from the FAB matrix. On the other hand, the SRM data with their higher molecular specificity (selection of a fragment ion from MH^+) would be quantitatively lower because, even though other peptides could be present in that ME HPLC fraction, SRM supplies an additional level of molecular specificity. The high level of chromatographic purity of the collected ME fraction in this present study is attested to by the fact that the MH^+ and SRM data are so similar.

An internal standard also provides the optimum level of confidence during quantification of a peptide as opposed to the use of a correction factor for recovery that is required for RRA and RIA measurements. For example, d_5-ME is one of the closest chemical structures to ME that is possible, and it also displays very similar chromatographic and MS behaviors, which are especially important factors. A 1-μg amount (1.7 nmol) of d_5-ME may also act as a carrier to improve the recovery of native d_0-ME from the pituitary tissue extracts.

SRM method. However, even the FAB-MS MH^+ data are not unambiguous in terms of determining a known amino acid sequence. For example, ME is a pentapeptide, and therefore 5! or 120 different peptides have exactly the same amino acid composition, accurate mass and elemental composition data. Therefore, what is needed to quantify endogenous neuropeptides in biologic extracts is not an increase in mass resolution, but rather an increase in structure resolution [11,12,15], which is a parameter afforded readily by SRM (tandem MS, three or four sector magnetic sector instruments, triple sector quadrupole or linked-field scanning on forward geometry two-sector instruments, such as on the 731 used in this study).

These present experiments constitute one first step towards unambiguous molecular specificity because here we link an MH^+ measurement with the ion products 425 (N-terminal tetrapeptide) and the C-terminal Met residue.

CONCLUSIONS

Data presented in this paper demonstrated the molecular specificity of four analytical methods: RRA, RIA, MS and SRM used to measure endogenous ME in the human pituitary at the ppm level. The highest level of molecular specificity was achieved when an amino acid sequence-determining fragment ion was measured by SRM.

These data are important because the name of a biologically active peptide cannot be used unless a molecular parameter such as the amino acid sequence or unique fragment ion of that peptide is determined experimentally during each analytical measurement. Immunoreactivity, receptor activity, mRNA measurement, *in situ* hybridization, molecular weight, chromatographic co-elution [28], electroactivity, fluorescence or behavior following enzyme treatment cannot provide sufficient molecular specificity for the measurement of a peptide. The most significant datum is the amino acid sequence. The literature contains examples of the use of non-specific methods to claim amino acid sequence specificity of a biologic peptide. These facts are, of course, trivial to state, but experimentally difficult to achieve. Nevertheless, such a critical experimental parameter is needed in today's research.

The highest level of molecular specificity to measure picomole amounts of native ME in one human pituitary is multi-dimensional RP-HPLC followed by FAB-MS SRM to monitor the ion current from a unique peptide fragment ion, using d_5-ME as the internal standard.

ACKNOWLEDGEMENTS

The authors acknowledge gratefully the financial support of the National Institutes of Health (GM 26666), Drs. J. Francisco, R. C. Haruff and H. Berryman (Department of Pathology) for pituitary tissue, G. Fridland for HPLC assistance, Deanna Reid and Dianne C. Cubbins for typing assistance and Dianne C. Cubbins for data analysis.

REFERENCES

1 D. T. Krieger, M. J. Brownstein and J. B. Martin (Editors), *Brain Peptides*, Wiley, New York, 1983, p. 1032.
2 K. A. Roth, G. Makk, O. Beck, K. Faull, K. Tatemoto, C. J. Evans and J. D. Barchas, *Regul. Peptides*, 12 (1985) 185.
3 D. M. Orth, R. Guillemin, N. Ling and W. E. Nicholson, *J. Clin. Endocrinol. Metab.*, 46 (1978) 849.
4 D. M. Desiderio, H. Onishi, G. Fridland, G. Wood and D. Pagidipati, *Biomed. Chromatogr.*, 2 (1987) 2.
5 D. M. Desiderio, D. Liu and G. Wood, *Life Sci.*, 43 (1988) 577.
6 D. Liu and D. M. Desiderio, *J. Chromatogr.*, 422 (1987) 61.
7 D. Liu, C. Dass, G. Wood and D. M. Desiderio, *J. Chromatogr.*, 500 (1990) 395.
8 T. Higa, G. Wood and D. M. Desiderio, *Int. J. Pept. Protein Res.*, 33 (1989) 446.
9 C. Gillberg, L. Terenius and G. Lonnerholm, *Arch. Gen. Psychiatry*, 42 (1985) 780.

10 G. Salar, S. Mingrino, M. Trabucchi, A. Bosio and C. Semeza, *J. Neurosurg.*, 55 (1981) 935.
11 D. M. Desiderio, *Analysis of Neuropeptides by Liquid Chromatography and Mass Spectrometry*, Elsevier, Amsterdam, 1984, p. 235.
12 D. M. Desiderio, in D. M. Desiderio (Editor), *Mass Spectrometry of Biologically Important Neuropeptides, Mass Spectrometry of Peptides*, CRC Press, Boca Raton, FL, 1990, in press.
13 J. J. Kusmierz, R. Sumrada and D. M. Desiderio, *Anal. Chem.*, in press.
14 F. S. Tanzer, E. Tolun, G. H. Fridland, C. Dass, J. Killmar, P. W. Tinsley and D. M. Desiderio, *Int. J. Pept. Protein Res.*, 32 (1988) 117.
15 C. Dass, G. H. Fridland, P. W. Tinsley, J. T. Killmar and D. M. Desiderio, *Int. J. Pept. Protein Res.*, 34 (1989) 81.
16 E. Tolun, C. Dass and D. M. Desiderio, *Rap. Commun. Mass Spectrom.*, 1 (1987) 77.
17 D. M. Desiderio, R. C. Cezayirli, G. Fridland, J. T. Robertson and H. Sacks, *Life Sci.*, 37 (1985) 1895.
18 D. M. Desiderio, G. H. Fridland, J. T. Francisco, H. Sacks, J. T. Robertson, R. C. Cezayirli, J. Killmar and C. Lahren, *Clin. Chem.*, 34 (1988) 1104.
19 T. Higa and D. M. Desiderio, *Int. J. Pept. Protein Res.*, 33 (1989) 250.
20 M. Muhlbauer, J. C. Metcalf, J. T. Robertson, G. Fridland and D. M. Desiderio, *Biomed. Chromatogr.*, 1 (1986) 155.
21 H. P. J. Bennett, *J. Chromatogr.*, 359 (1986) 383.
22 D. M. Desiderio and D. M. Cunningham, *J. Liq. Chromatogr.*, 4 (1981) 721.
23 G. H. Fridland and D. M. Desiderio, *J. Chromatogr.*, 379 (1986) 251.
24 H. Takeshita, D. M. Desiderio and G. Fridland, *Biomed. Chromatogr.*, 1 (1986) 126.
25 D. M. Desiderio, J. S. Laughter, I. Katakuse, M. Kai and J. Trimble, *Compt. Enhanced Spectros.*, 2 (1984) 21.
26 E. Simon, in R. S. Rapaka and R. L. Hawks (Editors), *Progress in the Characterization of the Opioid Receptor Subtypes: Peptides as Probes, Future Directions in Opioid Peptides: Molecular Pharmacology, Biosynthesis, and Analyses*, NIDA, Rockville, MD, 1986, p. 155.
27 C. Gros, P. Pradelles, C. Rouget, O. Bepoldin, F. Dray, M. C. Fournie-Zaluski, B. P. Roques, H. Pollard, C. Llorens-Cortes and J. C. Schwartz, *J. Neurochem.*, 31 (1978) 29.
28 C. Kim and R. Cheng, *J. Chromatogr.*, 494 (1989) 67.