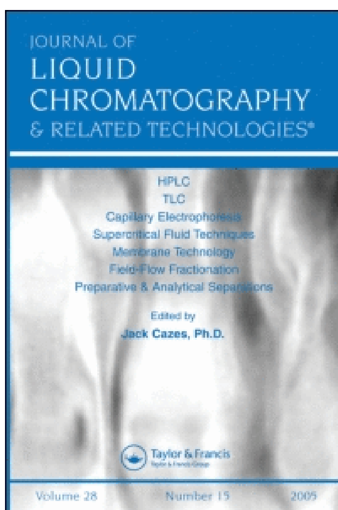
This article was downloaded by:
On: *24 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of Picomole Levels of Neurotensin, Bombesin, and Related Peptide Fragments Using Gradient Elution High Performance Liquid Chromatography Coupled with Electrochemical Detection

Andrea L. Drumhellerl[a]; Helene Bachelard[b]; Serge St-Pierre[b]; François B. Jolicoeur[c]
[a] Department of Nutrition Faculté de Medecine, Université de Sherbrooke, Sherbrooke, Canada [b] Department of Physiology and Pharmacology Faculté de Medecine, Université de Sherbrooke, Sherbrooke, Canada [c] Department of Psychiatry Faculté de Medecine, Université de Sherbrooke, Sherbrooke, Canada

To cite this Article Drumhellerl, Andrea L. , Bachelard, Helene , St-Pierre, Serge and Jolicoeur, François B.(1985) 'Determination of Picomole Levels of Neurotensin, Bombesin, and Related Peptide Fragments Using Gradient Elution High Performance Liquid Chromatography Coupled with Electrochemical Detection', Journal of Liquid Chromatography & Related Technologies, 8: 10, 1829 — 1843

To link to this Article: DOI: 10.1080/01483918508074098

URL: http://dx.doi.org/10.1080/01483918508074098

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(10), 1829-1843 (1985)

DETERMINATION OF PICOMOLE LEVELS OF NEUROTENSIN, BOMBESIN, AND RELATED PEPTIDE FRAGMENTS USING GRADIENT ELUTION HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH ELECTROCHEMICAL DETECTION

Andrea L. Drumheller[1], Helene Bachelard[2], Serge St-Pierre[2], and François B. Jolicoeur[3]

[1]*Department of Nutrition*
[2]*Department of Physiology and Pharmacology and*
[3]*Department of Psychiatry*
Faculté de Médecine
Université de Sherbrooke
Sherbrooke (Québec)
Canada J1H 5N4

Downloaded At: 16:30 24 January 2011

ABSTRACT

A method for the separation and detection of two neuroactive peptides, neurotensin (NT) and bombesin (BB) as well as selected fragments of these peptides including NT 1-8, NT 1-10, NT 9-13, BB 1-8, BB 1-11 and BB 8-14 using gradient elution high performance liquid chromatography coupled with electrochemical detection (HPLC-ECD) is described. Several mobile phase compositions and gradients were tested and compared in terms of resolving power and sensitivity. The gradient method outlined here is 200 to 500 times more sensitive than HPLC-UV detection. A method for separating neurotensin isocratically is also described, and results indicate that the sensitivity of the assay is in the fentomolar range and approaches the sensitivity of radioimmunoassay techniques. Means of ameliorating the sensitivity of the HPLC-ECD assay when using a gradient are discussed. Results of preliminary experiments using this technique to detect the presence of these peptides in various biological media are presented.

Copyright © 1985 by Marcel Dekker, Inc.

0148-3919/85/0810-1829$3.50/0

INTRODUCTION

High performance liquid chromatography coupled with electrochemical detection (HPLC-ECD) has become the method of choice for the determination of catechol and indole amine levels in biological material due to the simplicity, rapidity and sensitivity of the technique (1,2,3). Because the electroactivity of these compounds resides primarily in the structure of their precursors, tyrosine and tryptophan respectively, it was not unreasonable to assume that other substances such as peptides containing these amino acid residues would be electroactive. To investigate this possibility, Bennett et al. (4) determined the electroactivity of a variety of peptides and reported that neurotensin, enkephalins, B-endorphin, somatostatin and cholecystokinin among others were indeed oxidizable at specific potentials. Using this information several investigators have tested the applicability of HPLC separation with electrochemical detection for the analysis of neuropeptides with encouraging results. To date cholecystokinin 4 and 8 (5), angiotensin, Lys-vasopressin (6), Des Tyr-Leu-enkephalin (6,7), B-endorphin, Tyrosylglycylglycine and Tyrosylglycine (7) have been separated and detected by this method and the reported sensitivity of this technique approaches that of radioimmunoassay and greatly surpasses that of HPLC-UV detection.

Downloaded At: 16:30 24 January 2011

In the course of our work on structure activity relationships of neurotensin (8), it became important to us to follow the distribution of the peptide following intraventricular administration and to investigate the metabolic consequences resulting from the injection of structural analogues. Because the number of assays required precluded the use of radioimmunoassay (RIA), an alternative method of detecting and quantifying neurotensin and fragments was sought. The purpose of the present study was to investigate the potential of using HPLC-ECD for the separation and quantification of fentomole amounts of neurotensin, a 13 amino acid peptide containing two tyrosine residues at positions

3 and 11. At the same time the utility of this method for the detection of a tryptophan containing peptide, bombesin, (position 8) was explored. Furthermore, the feasibility of using a modest gradient to separate selected fragments of the parent compounds in a single run was examined for each peptide. In preliminary experiments the applicability of this approach for studying the localization and metabolism of these peptides in biological samples was assessed for neurotensin in whole brain and bombesin in liver perfusates of rats.

EXPERIMENTAL

Chemicals

HPLC grade acetonitrile and methanol were purchased from BDH Chemical Co. Ammonium acetate, and acetic acid were obtained from Fisher Scientific. Trifluoracetic acid was redistilled in glass from a reagent grade product also from Fisher. Distilled water was redistilled in glass and passed through a Water's Norganic cartridge system prior to use. Neurotensin, neurotensin fragments 1-8, 1-10 and 9-13, bombesin and bombesin fragments 1-8, 1-9, 1-10, 3-14, 4-14 and 8-14 were synthesized by the solid phase technique (9).

Downloaded At: 16:30 24 January 2011

Chromatographic Conditions

For all experiments the HPLC apparatus consisted of two Beckman 112 pumps, a model 421 controller and an Altex 210 (Beckman Instruments) injector fitted with a 100 µl sample loop. An LC 2 (Bioanalytic Systems [BAS]) electrochemical detector equipped with glassy carbon working electrode was used to monitor the column effluent. The oxidation potential was set at +0.85 V (tyrosine containing compounds) or +1.00 V (tryptophan containing compounds) with reference to a silver/silver chloride electrode. Peptides were injected onto either a 10 µ Bondapak reverse phase column (Water's, 4 mm x 25 cm) or a 5 µ Ultrasphere ODS reverse phase column (Beckman, 4 mm x 25 cm). Both columns were protected by a short (4 mm x 4 cm) precolumn filled with the appropriate packing. All peptides were dissolved in the equili-

brating buffer and the injection size remained constant at 20 µl. The efficacy of several mobile phases for eluting the peptides and peptide fragments was investigated: 1% trifluoracetic acid, pH 3.0 + acetonitrile, (TFA + ACN); 0.005, 0.01,0.05 and 0.1M ammonium acetate, pH 4.2 + acetonitrile or methanol (NH_4OAC + ACN or MeOH). All solutions were filtered through 0.2 µ Ultipor nylon membranes (Chromatographic Specialties Co) and thoroughly degassed prior to use. The exact composition of the mobile phases and the gradient used are described in Table 1.

Preparation of biological samples

Neurotensin from whole brain tissue was extracted according to the method of Carraway and Leeman (10). Liver perfusates following injection of synthetic bombesin were lyophylised and passed through a SEPPAK (Waters).

Downloaded At: 16:30 24 January 2011

RESULTS

Method Development

Initial attempts to separate and detect the peptides and their fragments by HPLC-ECD were made using a 1% TFA/ACN gradient. This mobile phase has been shown to be adequate for the HPLC-UV detection of bombesin and fragments at 210 nm due to the low UV absorbing quality of this buffer. However the lengthy (150 min) gradient required to resolve the peptides and resulting peak broadening limited the sensitivity of the HPLC-ECD assay to that previously found using HPLC-UV - approximately 5 µg. Further experiments were carried out using various molar solutions of NH_4OAC with either ACN or MeOH as organic modifier. Results indicated that 0.05 NH_4OAC with ACN provided a mobile phase of sufficient ionic strength for optimum sensitivity and resolving power while generating minimum background noise. The various mobile phase compositions tried, as well as gradient details and comments concerning the efficacy of each for the separation and detection of the peptides are shown in Table 1.

Under isocratic conditions, superior chromatographic results were obtained with the 5 µ vs. the 10 µ particle size reversed

Downloaded At: 16:30 24 January 2011

TABLE 1. Summary of mobile phase compositions and gradients tested for eluting and detecting neurotensin, bombesin and selected peptide fragments.

Pump	Mobile Phase	pH	Flowrate ml/min	Gradient	Remarks
A: B:	0.1 % TFA + 10% ACN 50% TFA + 50% ACN	3.0	1.5	t = 0, % A = 100 t = 10, % B = 45	Adequate separation but peak broadening results in low sensitivity
A: B:	0.005M NH_4 OAC + ACN 40% 0.005M NH_4 OAC + 60% ACN	4.2	1.5	t = 0, % A = 100 t = 4, % B = 40 in 45 min	Adequate separation, but peaks too broad
A: B:	0.01M NH_4 OAC + 10% ACN 40% 0.01M NH_4 OAC + 60% ACN	4.2	1.5	t = 0, % A = 100 t = 4, % B = 40 in 45 min	Better peak shape, good separation
A: B:	0.05M NH_4 OAC + 10% ACN 40% 0.05M NH_4 OAC 60% ACN	4.2	1.5	t = 0, % A = 100 t = 4, % B = 40 in 45 min	Well defined peaks, excellent separation analysis time too long
A: B:	0.05M NH_4 OAC + 10% MeOH 40% 0.05M NH_4 OAC + 60% MeOH	4.2	1.5	t = 0, % A = 100 t = 4, % B = 40 in 45 min	MeOH results in peak broadening
A: B:	0.10M NH_4 OAC + 10% ACN 40% 0.10M NH_4 OAC + 60% ACN	4.2	1.5	t = 0, % A = 100 t = 4, % B = 40 in 45 min	Well defined peaks, excellent separation, but noise level increased
A: B:	0.05M NH_4 OAC + 12% ACN 20% 0.05M NH_4 OAC + 80% ACN	4.2	1.5	t = 0, % A = 100 t = 4, % B = 20 in 5 min t = 12, % B = 50 in 30 min	Well defined peaks, excellent resolution analysis time:25 min for neurotensin, 37 min for bombesin

phase packing. The peaks eluting from the former column were sharper and better defined than from the latter resulting in decreased detection thresholds thus increasing the sensitivity of the assay approximately 3 fold. However, the 5 μ particle size packing generated excessive column backpressure under gradient conditions which resulted in extreme baseline drifting and elevated noise levels thereby decreasing the sensitivity. Therefore, a 10 μ reverse phase column was chosen for all experiments involving gradient work.

Separation of neurotensin and related fragments

Neurotensin, NT 1-8, NT 1-10 and NT 9-13 were separated within 25 min using a gradient elution system consisting of 0.05 M NH_4OAC, pH 4.5 with ACN:NH_4OAC mixture as the secondary solvent (Fig. 1). Chromatographic conditions including details of the gradient and retention times are given in the legend to Fig. 1. The mean peak heights of 4 separate determinations of NT and fragments were used to construct the calibration curves shown in Fig. 2. For each peptide the detector response was linear up to 1 μg (coefficients of correlations = 0.99). The detection limit with a signal to noise ratio of 5 at 10 nafs is approximately 25 ng for NT, NT 1-8 and NT 1-10 and approximately 10 ng for NT 9-13. Because of the baseline drift generated by the gradient it was not feasible to increase the sensitivity of the detector beyond 10 nafs. For the isocratic elution of neurotensin a 5 μ ultrasphere column was equilibrated with 0.1 M NH_4OAC, pH 4.5 + ACN, 75:25 v/v. Under these conditions less than 250 pg of neurotensin could be reliably detected (Fig. 3).

Separation of bombesin and related fragments

Identical chromatographic conditions were chosen to separate and quantify bombesin and BB 1-8, 1-11, and 8-14 (Fig. 4) except that the oxidation potential used was + 1.00 V. Due to the limited quantity of the peptides made available to us calibration curves based on the mean of 4 separate determination were constructed only for the above mentioned peptides (Fig. 5).

Downloaded At: 16:30 24 January 2011

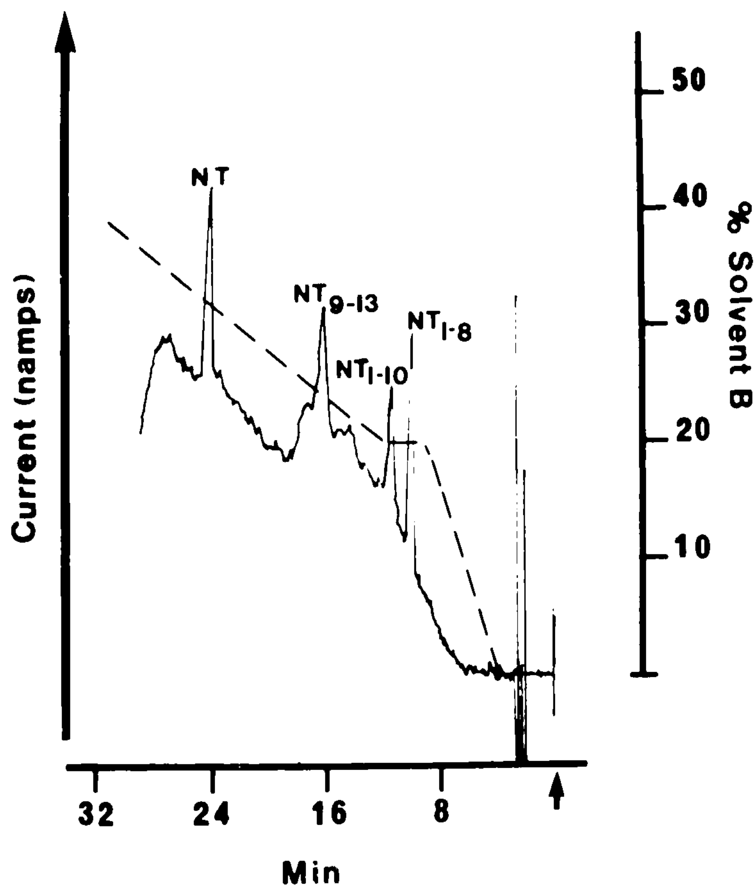


FIGURE 1

Gradient separation of a standard solution of 50 ng neurotensin (rt=23.8 min), NT 1-8 (rt=9.6 min), NT 1-10 (rt=11.2 min) and 25 ng NT 9-13 (rt=15.6 min). Chromatographic conditions: Pump A: 0.05 M NH_4OAC, pH 4.2 + 10% ACN; Pump B: 40% 0.05 M NH_4OAC, pH 4.2 + 60% ACN. Gradient: t=0, %A=100; t=4 min, %B=20 in 5 min; t=12 min, %B=50 in 30 min. Flow rate = 1.5 ml/min; detector potential = + 0.85V; sensitivity = 10 nafs.

Downloaded At: 16:30 24 January 2011

Downloaded At: 16:30 24 January 2011

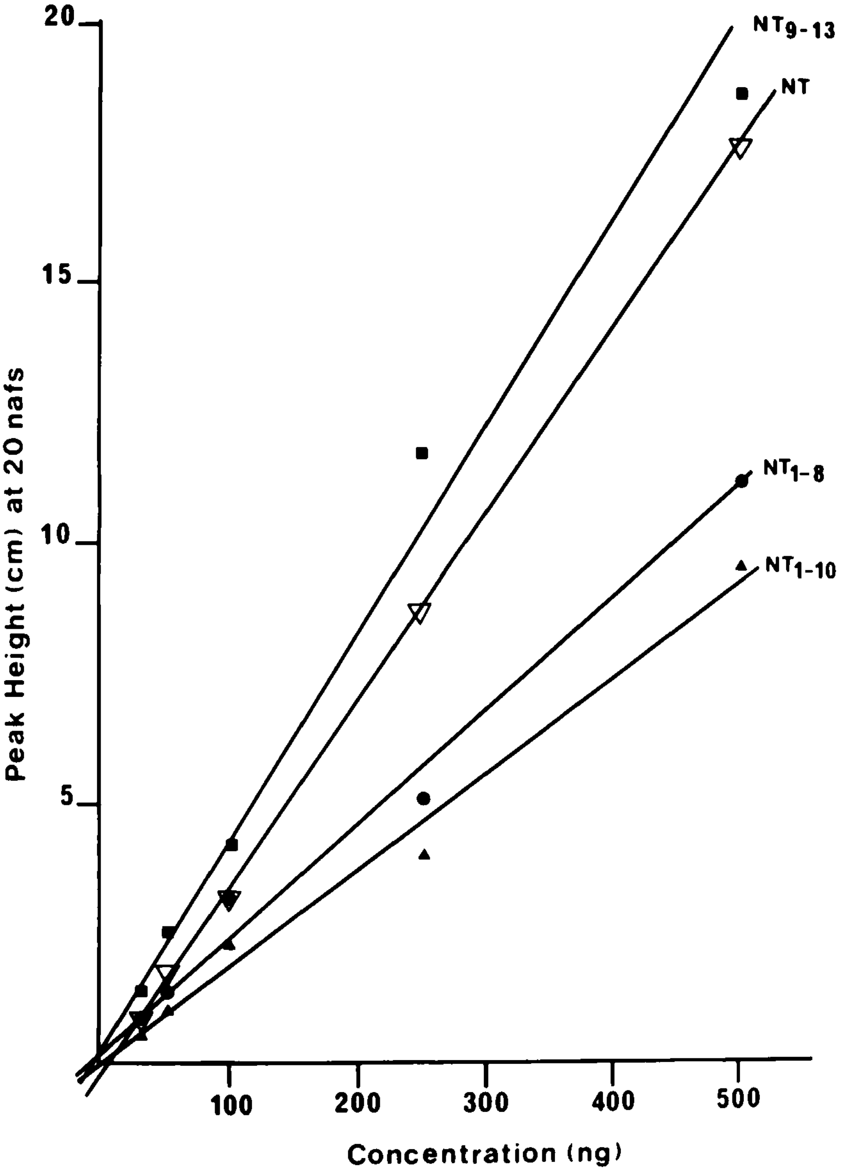
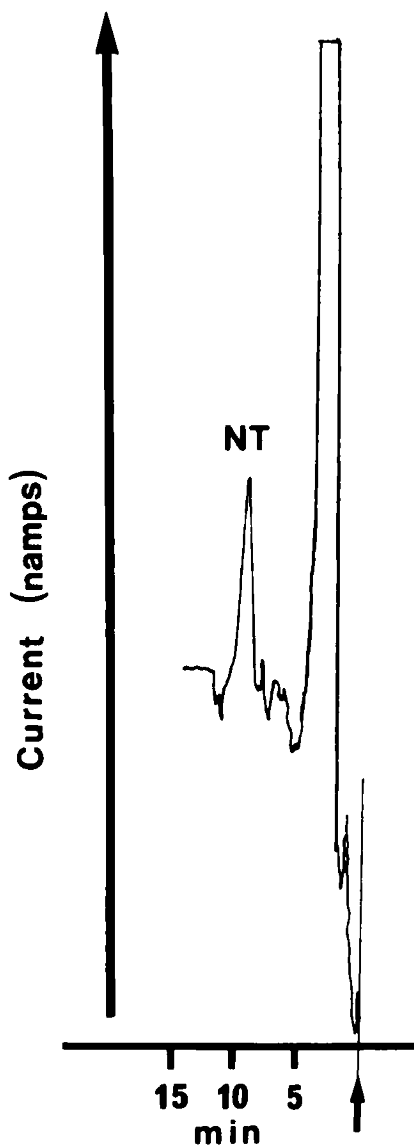


FIGURE 2

Calibration curves of synthetic neurotensin, NT 1-8, NT 1-10 and NT 9-13. Each point represents the mean of 4 separate determinations.



Downloaded At: 16:30 24 January 2011

FIGURE 3

Isocratic separation of neurotensin (250 pg),(rt=11.3 min). Mobile phase was a mixture of 0.1 M NH_4OAC, pH 4.2 + 25% ACN. Flow rate = 1.5 ml/min, detector potential = + 0.85 V;sensitivity = 2 nafs.

Downloaded At: 16:30 24 January 2011

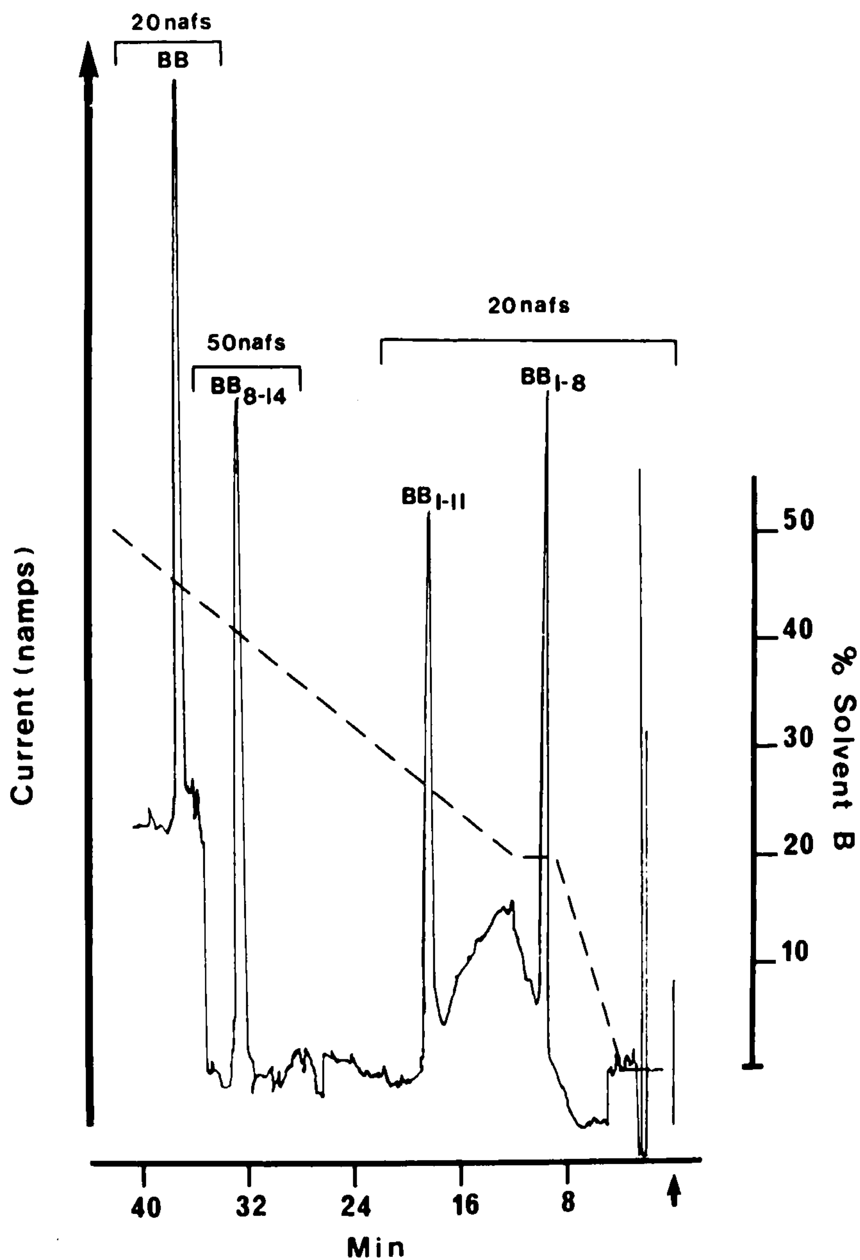


FIGURE 4

Gradient separation of 500 ng bombesin (rt=36.2 min), BB 1-8, (rt=9.2 min), BB 1-11 (rt=17.6 min) and BB 8-14 (rt=31.8 min). Chromatographic conditions as in Fig. 1 except that the detector potential = + 1.0 V and the sensitivity = 20 nafs for all peptides except BB 8-14, where sensitivity = 50 nafs.

However, it is possible, using these conditions, to separate BB 1-9 (rt=10.1 min), BB 1-10 (rt=18.8 min) and BB 2-14 (rt=37 min). For all peptides examined the detector response was linear up to 1 μg with a detection limit of 25 ng for BB, BB 1-8, BB 1-11 and approximately 10 ng for BB 8-14.

Separation of endogenous peptides

In whole brain, peaks having the same retention time as synthetic neurotensin, NT 1-8 and NT 1-10 were observed. In liver perfusates peaks having the same retention time as bombesin, BB 1-10 and BB 6-14 were seen (Data not shown).

DISCUSSION

Downloaded At: 16:30 24 January 2011

The results of this study indicate that neurotensin, bombesin and selected fragments can be detected and quantified amperometrically following separation by gradient elution HPLC. For optimum results, care must be taken to maintain the integrity of the glassy carbon electrode. A rapid decline in electrode sensitivity was observed over days, probably due to the relatively high potentials necessary to oxidize the tyrosine and tryptophan residues in these peptides. As such, daily polishing of the electrode is deemed warranted. Also, these peptides seem to be very sensitive to oxidation in solution, even at 0°C. We found it necessary to freeze the aliquots of our standard solutions at -80°C to insure prolonged stability of the peptides.

In order to minimize background current generated by oxidation of mobile phase contaminants, the lowest possible oxidizing potential was chosen. Previous reports indicate that the peak oxidation potential for tyrosine and tryptophan at the mobile phase pH used here are 0.84 and 0.88 V respectively (11). At E=+0.85 V neurotensin fragments generated peak heights close to expected values relative to neurotensin, which contains 19% tyrosine vs. 12% for NT 1-10, 15% for NT 1-8 and 25% for NT 9-13. For bombesin and fragments however, the potential had to be raised to + 1.00 V in order to obtain linear results. Even at

Downloaded At: 16:30 24 January 2011

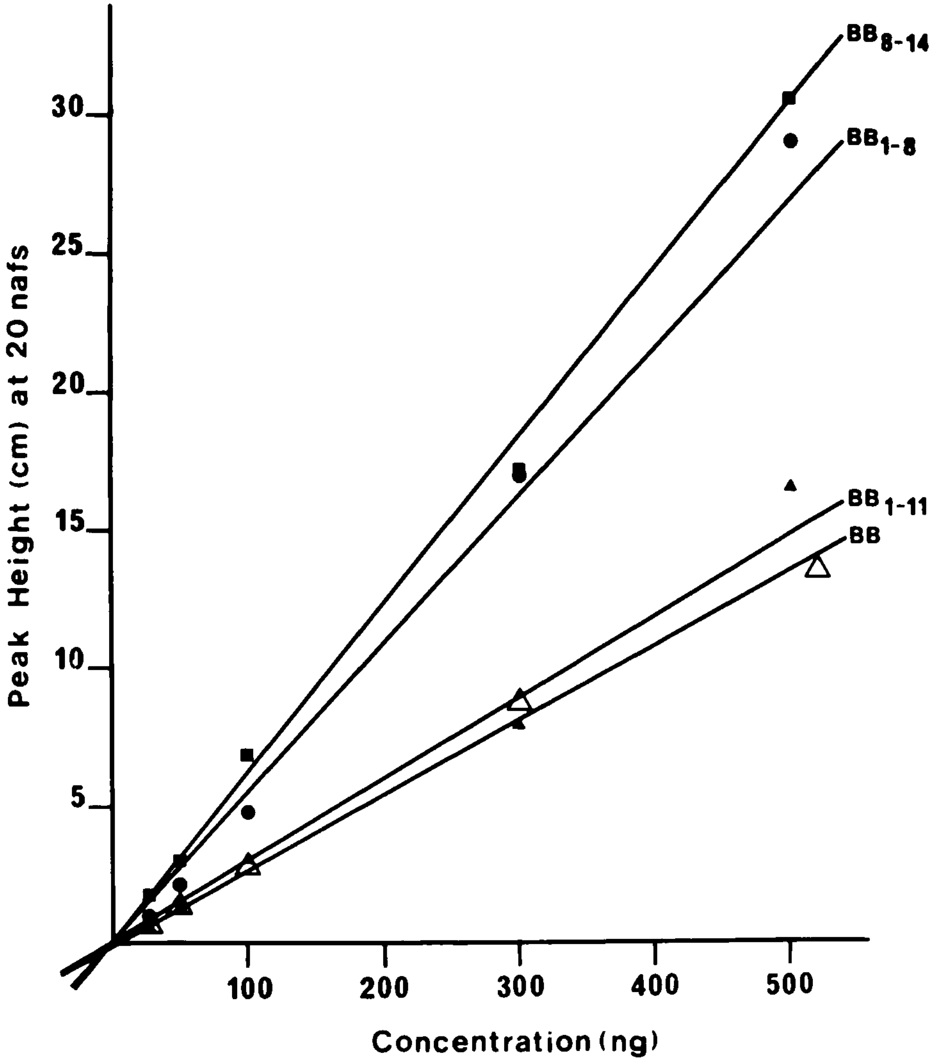


FIGURE 5

Calibration curves of synthetic bombesin, BB 1-8, BB 1-10 and BB 8-13. Each point represents the mean of 4 separate determinations.

this potential BB 1-8 (18% tryptophan) and BB 8-14 (22% tryptophan) generated peak heights higher than expected relative to bombesin (11% tryptophan). The need for a higher oxidation potential for tryptophan containing peptides coupled with the deviation from expected values might reflect the conformation of the peptides in that, depending on chain length and amino acid composition, the tryptophan molecule is more or less accessible to oxidation at the electrode surface. Another possibility involves the so-called "rotational freedom" of tryptophan in protein and peptide molecules, a phenomenon relating to the constantly changing conformation in space of this amino acid in the aforementioned structures (12).

Downloaded At: 16:30 24 January 2011

The use of radioimmunoassay (RIA) for the determination of endogenous levels of peripheral and brain peptides is to date the method of choice. As with most assay methods there are several inherent drawbacks. Though this technique displays more than adequate sensitivity, the problems of cross-reactivity have yet to be resolved. While the specificity of the assay is increased by prior separation of the compounds using HPLC, the procedure becomes even more time consuming and expensive and introduces an additional source of error. The results of this study demonstrate that HPCL-ECD might provide an attractive alternative to conventional RIA techniques for the estimation of minute quantities of peptides in biological samples. For example, under the conditions described above the detection limit for neurotensin standard is approximately 25 ng (13.12 pmoles) using gradient elution and less than 250 pg (0.13 pmoles) when separated isocratically. Whole brain levels of NT-like peptide have been reported to be 12.5 pmole (24 ng), while regional levels range from 0.82 pmoles/g in the cerebellum to 60.0 pmoles/g in the hypothalamus (10), levels readily detected by HPLC-ECD under isocratic conditions. Nonetheless, the method presented above is not sufficiently sensitive to detect levels of neurotensin in sub-nuclei of the above regions. However, there are certain parameters of HPLC-ECD than can be altered to improve measurably

the sensitivity. First of all, using an oxidation potential greater than + 1.00 V will, in most instances, result in increased peak height (unpublished observations). Doing so will elevate the noise level and, under gradient conditions, accentuate the baseline drift. However, since both noise and drift are, in great part, the result of oxidizing mobile phase contaminants, oxidation of the mobile phase, either prior to use or by the addition of a "guard cell" operated at a high potential between the mobile phase reservoir and the injector (11), will eliminate much of the extraneous interference of the detecting electrode. As such, the sensitivity at the detector can be increased, thus lowering the detection threshold.

In preliminary experiments we tested the applicability of HPLC-ECD for the detection of neurotensin in whole brain tissue, and of bombesin in liver perfusates. Peaks exhibiting the same chromatographic behavior as neurotensin, NT 1-8, and NT 1-10 in brain and bombesin, BB 1-10, BB 6-14 and BB 4-14 in liver perfusates were observed. Before it can be unequivocally stated that these peaks really correspond to endogenous peptides it will be necessary to validate the method by analyzing further the composition of the peaks. Only by changing chromatographic conditions and observing the behavior of these compounds relative to standards, by subjecting the eluates to RIA and, ideally by testing the authenticity of these substances by mass spectroscopy will we be able to describe with assurance peptide-like electro-activity.

Downloaded At: 16:30 24 January 2011

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council of Canada (Development Grant No. DG 284 and Operating Grant No. MT-6135).

REFERENCES

(1) Kissinger, P.T., Refshauge, C., Dreiling, R. and Adams, R.N., Anal. Lett., 6 (1973) 465.

(2) Mefford, I.N., Gilberg, M. and Barchas, J.D., Anal. Biochem., 104 (1980) 469.

(3) Shibuya, K., Sato, K. and Salafady, B., Int. J. Clin. Pharmacol. Ther. Toxicol., 20 (1982).

(4) Bennett, G.W., Brazell, M.P. and Marsden, C.A., Life Sci., 29 (1981) 1001.

(5) Sauter, A. and Frick, W., Analyt. Biochem., 133 (1983) 307.

(6) White, M.W., J. Chromatogr., 262 (1983) 420.

(7) Mousa, S. and Couri, D., J. Chromatogr., 267 (1983) 307.

(8) Jolicoeur, F.B., Rioux, F. and St-Pierre, S., in Handbook of Neurochemistry, A. Lajtha, ed., Plenum Press, Vol. 8 (1983) 93.

(9) Merrifield, R.B., J. Am. Chem. Soc., 85 (1963) 2149.

(10) Carraway, R.E. and Leeman, S.E., J. Biol. Chem. 251 (1976) 7045.

(11) Downer, R.G.H., Baily, B.A. and Martin, R.J., Chromatogr. Rev., 11 (1984) 297.

(12) Lakawicz, J.R., Maliwal, B.P., Cherek, H. and Balter, A., Biochem., 22 (1983) 1741.

Downloaded At: 16:30 24 January 2011