Journal of Chromatography, 202 (1980) 500–503
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,259

Note

Development of a combined high-performance liquid chromatographic–fluorometric quantitative assay for enkephalins

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(First received June 24th, 1980; revised manuscript received August 13th, 1980)

After the isolation and identification of 5-methionine–enkephalin (met–enkephalin) and 5-leucine–enkephalin (leu–enkephalin) by Hughes *et al.*[1], quantitation of these opioid peptides has been made possible by using traditional bioassay, immunoassay or receptor binding assay. High-performance liquid chromatography (HPLC) has been used for the identification of met– and leu–enkephalin[2,3]. Several techniques have been employed for detecting these peptides in HPLC eluates. Lewis *et al.*[4] derivatized the HPLC eluates with fluorescamine using a spectrophotofluorometer as a detector. Meek and Bohan[2] used an electrochemical detector for detecting column eluates. Nice and O'Hare[5] and Loeber *et al.*[6] used a UV spectrophotometer for detecting met– and leu–enkephalin. Here we are reporting a sensitive method for identifying opioid pentapeptides quantitatively by using pre-column derivatization with fluorescamine and subsequently detecting reacted met– and leu–enkephalin in the HPLC eluates using fluorometry and a flow-through cell.

EXPERIMENTAL

The HPLC assembly (Waters Assoc., Milford, MA, U.S.A.) consisted of two pumps (Model 6000), a programmer for gradient elution (Model 660) and a universal liquid chromatograph injector (Model U6K), coupled to a Schoeffel GM 970 fluorometer with a flow-through 5-μl cell and a Linear Instrument chart recorder. Chromatography was performed using a reversed-phase μBondapak C_{18} column, 30 × 0.39 cm I.D. (Waters Assoc.). Fluorescamine, leu–enkephalin and met–enkephalin were purchased from Pierce (Rockford, IL, U.S.A.) and D-Ala^2–methionine–enkephalinamide from Peninsula (San Carlos, CA, U.S.A.). Reagent-grade acetic acid, formic acid, sodium phosphate, sodium acetate and ammonium acetate were from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Propanol, methanol, pyridine and acetonitrile were from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Tris(hydroxymethyl)aminomethane·HCl and its base were from Sigma (St. Louis, MO, U.S.A.). Glass-redistilled water was used. Solvents were filtered through a Millipore Pyrex filtration apparatus (Bedford, MA, U.S.A.) before use. Gradient elution was accomplished with Tris buffer, 0.05 *M* pH 7.4 and 100% methanol. Two gradients were employed. In the first, the starting concentration ratio of Tris to methanol was 65:35 and the final ratio was 0:100. The second differed

0021-9673/80/0000–0000/$02.25

from the first in that the starting Tris–methanol ratio was 60:40. Starting at the injection of the sample, linear gradient elution program 6 was performed for 60 min at ambient temperature (21 ± 1°C), and a flow-rate of 2 ml/min. The retention times of the fluorescamine derivatized peptides were determined by an in-line fluorescence detector with excitation at 390 nm and emission at 470 nm. Formic acid, 0.5 *M* adjusted to pH 3.0 with pyridine and propanol (propanol: 0 → 20% in 120 min) solvent system was used for checking the purity of the standards. Sodium acetate–acetonitrile[7], ammonium acetate–acetonitrile[8], formic acid–pyridine–propanol[4], methanol–water[9], methanol–water with acetic acid[9] and propanol–water solvent systems were also evaluated.

Aqueous enkephalin standards, 0.5 ml, plus 1 ml of 0.05 *M*, pH 7.4 phosphate buffer were transferred to polypropylene tubes and reacted with 0.5 ml of 0.02% fluorescamine dissolved in acetone. A 20-μl volume of this solution containing fluorescamine labelled enkephalins was applied to the HPLC column. Standard response curves were established by calculating the area (1/2 base × height) underneath the peak. A spectrophotofluorometer (Aminco, Silver Spring, MD, U.S.A.) with excitation at 390 nm and emission at 480 nm was occasionally used to check fluorescence intensity.

RESULTS AND DISCUSSION

Enkephalins for use as standards

Commercially obtained leu– and met–enkephalin have each shown single peaks in formic acid–pyridine–propanol solvent systems using HPLC and have been successfully separated. A single peak has also been confirmed for each of the fluorescamine derivatized enkephalins using a Tris–methanol solvent system.

Fluorescamine reaction

The effect of pH on enkephalins reacted with fluorescamine was studied. At pH 7.4, the reaction gave the highest response and lowest blank value for both enkephalins.

Chromatography

Various isocratic conditions (such as methanol–Tris 10:90, 20:80, 25:75 and 60:40) and various gradients (gradient program either number 2, 4, 5, 6, 7 or 10) were systematically investigated. The methanol fraction was changed from 10→70%, →90% or →100%; or from 20 or 30→70%, →80% or →100%; or from 40→60%, →70%, →80% or →100% and from 50→100%. The two gradients described in the Experimental section and presented in Table I yielded the best results.

Typical tracings of these results are shown in Fig. 1. Fig. 2 shows the standard concentration–fluorescence relationship. The least detectable amount is 2.5 ng. No response was observed when the fluorescamine-reacted buffer blank was injected under similar conditions (Fig. 1). Mixtures containing fluorescamine-labelled leu–enkephalin, met–enkephalin and D-Ala^2–methionine–enkephalinamide were completely separated from each other (Fig. 3). D-Ala^2–methionine–enkephalinamide has two free amino groups available for fluorescamine reactions which probably accounts for its enhanced fluorescence.

With other solvent systems such as sodium acetate–acetonitrile[7] (sodium

TABLE I

RETENTION TIME (min) OF ENKEPHALINS IN TRIS 0.05 *M* pH 7.4–METHANOL HPLC SYSTEM AT A FLOW-RATE OF 2 ml/min

Values in parentheses are the number of determinations.

Linear gradient	*Met-enkephalin (± S.E.M.)*	*Leu-enkephalin (± S.E.M.)*
40 → 100% methanol	10.13 ± 0.08 (7)	12.15 ± 0.04 (7)
35 → 100% methanol	13.89 ± 0.03 (20)	16.01 ± 0.04 (20)

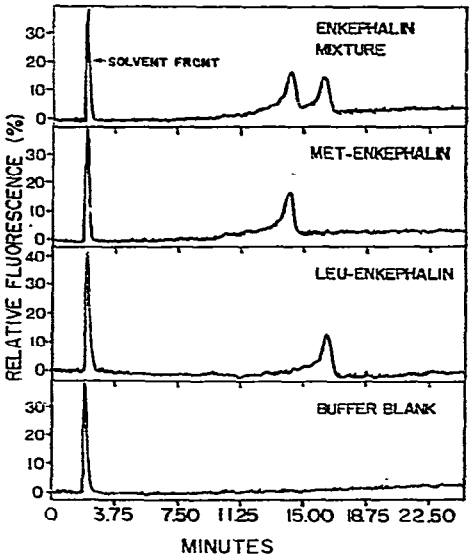

Fig. 1. Separation of fluorescamine reacted leu-enkephalin (8 ng) and met-enkephalin (8 ng) on C_{18} μBondapak HPLC column. Eluting solvent was 0.05 *M*, pH 7.4 Tris, gradient was 35 → 100% methanol at a flow-rate of 2 ml/min for 60 min. Chart speed was 16 in./h. Fluorescence was detected by Schoeffel GM 970 fluorometer.

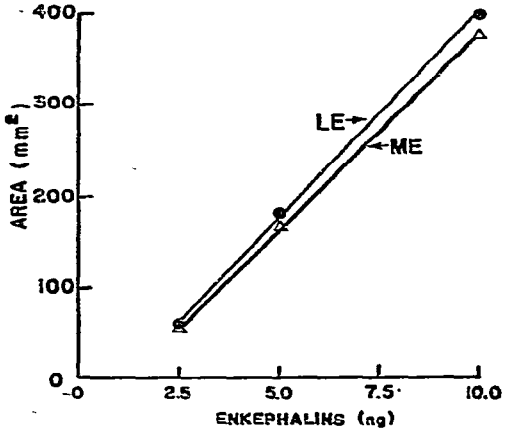

Fig. 2. Standard response curves of met-enkephalin (ME) and leu-enkephalin (LE). Areas underneath the peaks were calculated by 1/2 base × height, when chart speed was 50.8 mm/min.

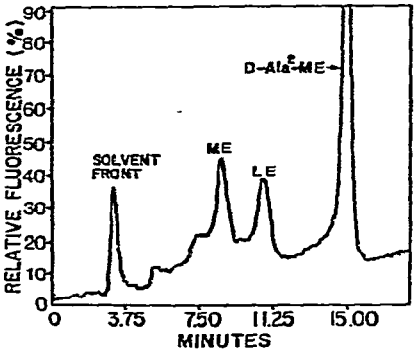

Fig. 3. Elution pattern of fluorescamine-reacted D-Ala²-methionine-enkephalinamide (D-Ala²-ME), leu-enkephalin (LE) and met-enkephalin (ME) mixture containing 15 ng of each peptide. Conditions were the same as Fig. 1 except the gradient was 40 → 100% methanol, flow-rate was 1.5 ml/min.

acetate: 0→30%); formic acid with pyridine–propanol[4] (pyridine: 0→20% or isocratic 20:80 or 25:75); formic acid with pyridine–methanol (20:80); propanol–water (50:50); ammonium acetate–acetonitrile[8] (86:14); methanol–water[9] (50:50, 60:40 or 80:20) and methanol–water with acetic acid[9] (48:52), the column failed to retain the derivatized peptides satisfactorily, which is in contrast with the behavior of the underivatized peptides. The Tris–methanol solvent system provided an optimal condition for the best resolution and retention of the derivatized peptides on the column.

In conclusion, the present results demonstrate that derivatized enkephalins are separated from each other using Tris–methanol solvent HPLC system and that the retention times are reproducible. Advantages of this pre-column labelling technique include economy and simplicity of the method and its sensitivity. The nanogram level of sensitivity of this method compares favorably with other HPLC methods using UV, electrochemical or post-column fluorometry detection.

ACKNOWLEDGEMENT

This research was supported by a grant from the University of Kentucky Tobacco and Health Research Institute.

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