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DETECTION AND QUANTITATION OF FLUORESCAMINE-LABELED BRADYKININ, ITS ANALOGUES AND METABOLITES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

A sensitive technique is described for detecting and quantitating fluorescamine-labeled kinins and their usual metabolic products using reversed-phase high-performance liquid chromatography (HPLC) linked with a fluorescence detector. Kinins and their enzymatic products were labeled with fluorescamine, subjected to HPLC, and scanned for the fluorescence signal with excitation at 390 nm and emission at 476 nm. The fluorescence signal was linear with bradykinin, Lys-bradykinin and Met-Lys-bradykinin in amounts upward from 2.5 ng. Separation of the fluorescamine-labeled kinins using HPLC was carried out with a solvent system of methanol—triethylammonium formate buffer. Labeled kinins were eluted in the following order: bradykinin, Lys-bradykinin, and Met-Lys-bradykinin.

When native (unlabeled) kinins were subjected to HPLC using a solvent system of acetonitrile—triethylammonium formate buffer, the minimum amount of native kinin detected at 210 nm was 1 μg. All three kinins showed linearity at 210 nm in amounts upward from 1 μg. Kinins were eluted in the following order: Lys-bradykinin, bradykinin and Met-Lys-bradykinin.

The different elution patterns of kinins by means of these two separation techniques provide a useful method for identification of purified kinins. The fluorescamine label provides a 400-fold more sensitive detection technique than ultraviolet absorbance of the native kinins and may be used to identify the metabolic products of kinins.

INTRODUCTION

Different analytical procedures such as paper chromatography, paper and gel electrophoresis, gel chromatography and ion-exchange chromatography have

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been used in conjuction with bioassay on isolated tissues to detect and differentiate bradykinin and its analogues Lys-bradykinin and Met-Lys-bradykinin [1—9]. Stewart and Morris [10] used reversed-phase high-performance liquid chromatography (HPLC) to separate and identify kallikrein products. The absorbance was monitored by UV at 210 nm and elution was carried out using a methanol—ammonium formate mixture. Kinins were detected in the microgram range. Geiger et al. [11], Powers and Nasjletti [12] and Dizdaroglu et al. [13] also separated kinins by HPLC and used detection by UV absorbance at 215—225 nm or by radioimmunoassay or by fluorescamine.

The purpose of the present paper is to report a method of direct separation, identification and quantitation of fluorescamine-labeled kinins and their usual metabolic products at nanogram levels using reversed-phase HPLC. This method, in conjunction with reported separation techniques of native kinins on HPLC provides an excellent tool for identification and quantitation of kinins.

MATERIALS AND METHODS

Ile-Ser-Bradykinin, synthetic bradykinin, Lys-bradykinin and Met-Lys-bradykinin were from Peninsula Labs. (San Carlos, CA, U.S.A.). Rat plasma T-kinin (Ile-Ser-bradykinin) was a gift of H. Okamoto, Medical College of Georgia, Department of Pharmacology.

Triethylamine was from Aldrich (Milwaukee, WI, U.S.A.). Converting enzyme (E.C. 3.4.15.1) and carboxypeptidase-B (E.C. 3.4.17.1) were from Calbiochem (La Jolla, CA, U.S.A.). α-Chymotrypsin (E.C. 3.4.21.1) was from Worthington (Freehold, NJ, U.S.A.). Carboxypeptidase-A (E.C. 3.4.17.1), carboxypeptidase-M (E.C. 3.4.11.2) and fluorescamine were from Sigma (St. Louis, MO, U.S.A.). Disposable extraction columns (Bond-Pak column) packed with reversed-phase octadecylsilane (C_{18}) bonded to silica gel (40 μm average particle diameter) were from J.T. Baker (Phillipsburg, NJ, U.S.A.). The Ultrasphere ODS (C_{18}) (75 mm × 4.6 mm I.D.; with 5 μm mean particle diameter) was a prepared column from Beckman (Concord, CA, U.S.A.). HPLC grade solvents were from J.T. Baker. Deionized glass-distilled water passed through a Bond-Pak C_{18} 3-ml column was used for the preparation of all HPLC buffers. The buffer was degassed for 20 min before applying to the HPLC column.

A Beckman Model 110A dual pump HPLC instrument linked to a Perkin-Elmer 650-10S fluorescence spectrophotometer with a 20-μl flow cell was used for this study.

Labeling of kinins with fluorescamine

This was carried out by the method of Prakash et al. [14]. From a stock solution of 1 mg/ml kinin in 0.03 *M* hydrochloric acid, concentrations of standard kinin solutions ranging from 1.25 to 40 μg/ml were prepared by serial dilution with 0.05 *M* sodium borate buffer, pH 8.3. To 100 μl of each concentration, 5 μl of freshly prepared fluorescamine solution (3 mg/ml in anhydrous acetone) were added, mixed vigorously using a Vortex mixer, and kept at room temperature for 10 min. A 0.05 *M* borate buffer blank was similarly treated. Although the pyrollinone kinin derivatives were generally

found to be stable, chromatography was carried out within 2 h after fluorescamine treatment.

HPLC of fluorescamine-labeled peptides and kinins

The reversed-phase column was equilibrated with a minimum of 20 ml of 0.04 *M* triethylammonium formate, pH 4.4—methanol (80:20). For kinin separation, a 2-μl sample (2.5—80 ng) of the fluorescamine-labeled kinin was injected and a linear gradient of methanol from 20% to 100% over 30 min was programmed. The sample was eluted at a flow-rate of 0.5 ml/min at ambient temperature. In the case of separation of kinins and their metabolic products, elution was carried out with a linear gradient of acetonitrile from 20 to 40% over 60 min at a flow-rate of 0.4 ml/min at ambient temperature. Detection was carried out using an excitation wavelength of 390 nm and an emission wavelength of 476 nm.

HPLC of native kinins

The reversed-phase column was equilibrated with a minimum of 20 ml of 0.04 *M* triethylammonium formate, pH 4.4—acetonitrile (88:12). Concentrations of native kinins ranging from 1 to 8 μg were injected and eluted with the same solvent system at a flow-rate of 0.5 ml/min at ambient temperature.

Detection and quantitation were carried out at 210 nm using an on-line Altex Model 155-10/10 UV—VIS spectrophotometer. Between each sample, the sample port was cleaned with 50 μl of acetonitrile followed by 50 μl of buffer.

HPLC of enzymatic products of bradykinin

Incubation of bradykinin with different enzymes was carried out in 1-ml plastic centrifuge cups with a substrate concentration of 10 μg/ml in a final volume of 500 μl of buffer at appropriate pH at 37°C for the time indicated. Following incubation, a 20-μl aliquot of sample was drawn and mixed with 80 μl of 0.1 *M* sodium borate buffer, pH 8.3 and immediately labeled with 5 μl of freshly prepared fluorescamine solution and subjected to HPLC. The conditions of the incubations were as follows:

Converting enzyme: 0.1 *M* sodium phosphate buffer containing 0.3 *M* sodium chloride at pH 7; enzyme concentration 100 μg/ml; incubated for 48 h.

α-Chymotrypsin: 0.1 *M* sodium borate buffer containing 0.2 *M* calcium chloride at pH 7.8; enzyme concentration 10 μg/ml; incubated for 10 min.

Carboxypeptidase-B: 0.025 *M* sodium phosphate buffer containing 0.1 *M* sodium chloride at pH 7.55; enzyme concentration 1 μg/ml; incubated for 10 min.

Carboxypeptidase-B followed by -A: after 10 min of incubation of bradykinin (10 μg/ml) with carboxypeptidase-B (1 μg/ml) in 0.25 *M* sodium phosphate buffer containing 0.5 *M* sodium chloride at pH 7.55, carboxypeptidase-A (10 μg/ml) was added and incubated for an additional 5 h.

Digestion of T-kinin by aminopeptidase-M

T-Kinin (0.8 μg of bradykinin equivalent by the rat uterus assay) was incubated with 2 ng of aminopeptidase-M in 0.05 *M* sodium phosphate buffer

pH 7.0 containing 3 m*M* disodium EDTA in a total volume of 60 μl. After the incubation at 37°C, the enzyme reaction was terminated by addition of 20 μl of methanol. A 50-μl aliquot of the sample was removed and labeled with fluorescamine and chromatographed.

RESULTS

The retention times of the fluorescamine-labeled kinins chromatographed in nanogram quantities were found to be for bradykinin 10.1 ± 0 min (n = 15), Lys-bradykinin 23.3 ± 0 min (n = 22) and Met-Lys-bradykinin 25.1 ± 0 min (n = 15) (Fig. 1B). The retention times of native kinins chromatographed in microgram quantities were found to be for Lys-bradykinin 11.0 ± 0.2 min (n = 16), bradykinin 21.7 ± 0.2 min (n = 16) and Met-Lys-bradykinin 25.1 ± 1 min = 0.1 (n = 16) (Fig. 1A).

The fluorescent peaks of the fluorescamine-labeled kinins were highly symmetrical. Quantitation was carried out measuring the peak height of the fluorescence signal and plotting it against the amount of kinin injected into the column. The buffer blank did not give any fluorescence signal. As can be seen in Fig. 2, the kinins exhibited different fluorescent sensitivities following labeling with fluorescamine but all showed linearity in terms of fluorescence versus concentration. Fluorescamine-labeled bradykinin could be detected at minimal amounts of 2.5 ng applied to the column. Lys-bradykinin was detected at a minimum of 5-ng quantitites and Met-Lys-bradykinin at 15-ng quantities. Linearity was observed for concentrations at least 10-fold higher than the minimum concentrations without altering the sensitivity of the fluorescence detector. Quantitation of native kinins was carried out by measuring the

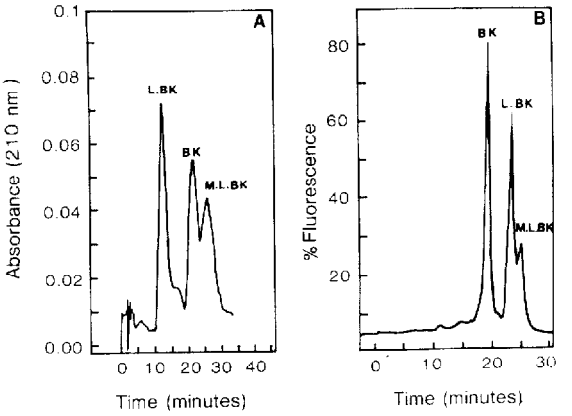

Fig. 1. (A) Elution pattern of a native kinin mixture (2 μg each) on a reversed-phase HPLC column. Kinins were eluted with a solvent system of 0.04 *M* triethylammonium formate buffer, pH 4.4—acetonitrile (88:12) at a flow-rate of 0.5 ml/min at ambient temperature. Kinins were detected at 210 nm. (B) Elution pattern of a fluorescamine-labeled kinin mixture (10 ng each) on a reversed-phase HPLC column. Labeled kinins were eluted with a linear gradient of methanol from 20% to 100% over 30 min at a flow-rate of 0.5 ml/min at ambient temperature. The fluorescence signal was detected at an excitation wavelength of 390 nm and an emission wavelength of 476 nm. Peaks: BK = bradykinin; L.BK = Lys-bradykinin; M.L.BK = Met-Lys-bradykinin.

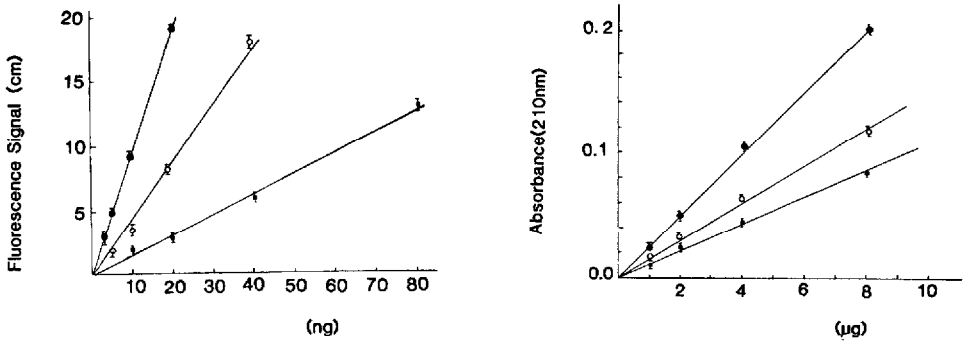

Fig. 2. Fluroescence signal of eluted fluorescamine-labeled kinins from the HPLC column versus concentration of kinin labeled with fluorescamine. Elution was with a linear gradient of methanol from 20% to 100% over 30 min at a flow-rate of 0.5 ml/min at ambient temperature. Fluorescence was determined as in Fig. 1. Each point represents mean ± standard error of quadruplicate experiments: (•) bradykinin (correlation coefficient 0.998; slope 0.954; S.E., 0.018); (○) Lys-bradykinin (correlation coefficient 1.000; slope, 0.370; S.E., 0.003); (▪) Met-Lys-bradykinin (correlation coefficient, 0.998; slope, 0.157; S.E. 0.003).

Fig. 3. Absorbance at 210 nm of eluted native kinins versus concentration injected into the HPLC column. Kinins were eluted with 0.04 *M* triethylammonium formate, pH 4.4,—acetonitrile (88:12). Each point represents the mean ± standard error of quadruplicate experiments: (○) bradykinin (correlation coefficient, 0.998; slope, 0.0145; S.E., 0.0002); (•) Lys-bradykinin (correlation coefficient, 0.997; slope, 0.0253; S.E., 0.0005); (▪) Met-Lys-bradykinin (correlation coefficient, 0.998; slope, 0.0099; S.E., 0.0001).

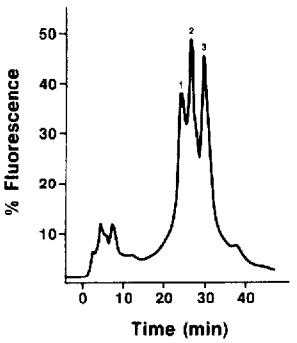

Fig. 4. Elution patterns of a mixture of fluorescamine-labeled bradykinin, des-Arg^9-bradykinin and Phe-Arg dipeptide. A mixture of 20 ng of each peptide was injected into the HPLC column. Elution was with a linear gradient of acetonitrile from 20% to 40% over 60 min at a flow-rate of 0.4 ml/min at ambient temperature. Fluorescence was determined as in Fig. 1. Peaks: 1 = bradykinin; 2 = des-Arg^9-bradykinin; 3 = Phe-Arg-dipeptide.

absorbancy at 210 nm and plotting it against the amount of kinins injected into the column. Native kinins could be detected at a minimum of 1 µg. Linearity was observed for concentrations at least 8-fold higher than the minimum concentrations without altering the sensitivity of the UV detector (Fig. 3).

The elution patterns of fluorescamine-labeled bradykin and its common metabolic products, des-Arg^9-bradykinin and the dipeptide Phe-Arg, using a linear gradient of acetonitrile from 20% to 40% over 60 min are seen in Fig. 4.

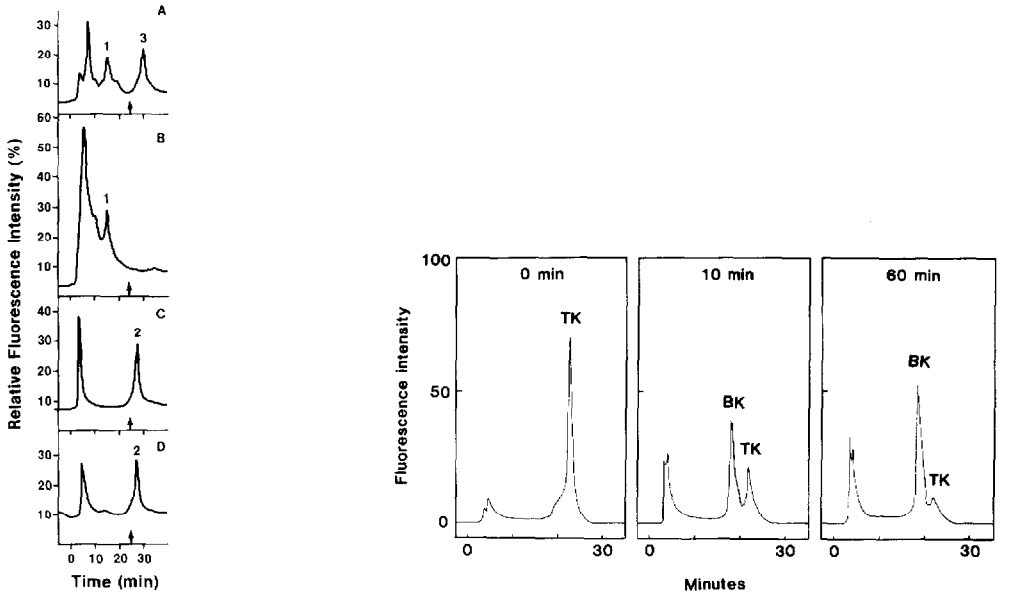

Fig. 5. Elution pattern of fluorescamine-labeled enzymatic products of bradykinin (see text for details of incubations and labeling). The elution time for bradykinin is indicated by an arrow. (A) Converting enzyme products: des-Phe^8-Arg^9-bradykinin (1) and Phe-Arg dipeptide (3); (B) carboxypeptidase-B followed by carboxypeptidase-A: des-Phe^8-Arg^9-bradykinin (1); (C) α-chymotrypsin: des-Arg^9-bradykinin (2); (D) carboxypeptidase-B: des-Arg^9-bradykinin (2).

Fig. 6. T-Kinin was incubated with aminopeptidase-M as described in the text. At 0, 10, and 60 min aliquots were removed, treated with fluorescamine and chromatographed. Labeled kinins were eluted with a linear gradient of methanol from 30% to 100% over 35 min at a flow-rate of 0.5 ml/min at ambient temperature. Peaks: BK = bradykinin, (appears with increasing time of incubation); TK = T-kinin.

The retention times were found to be for bradykinin 26.3 ± 0.3 min (n = 5), des-Arg^9-bradykinin 28.7 ± 0.3 min (n = 5) and Phe-Arg dipeptide 31.8 ± 0.3 min (n = 5). Using this same separation system, the elution patterns of fluorescamine-labeled products of bradykinin incubated respectively with converting enzyme, α-chymotrypsin, carboxypeptidase-B and carboxypeptidase-B followed by carboxypeptidase-A are shown in Fig. 5.

The retention time of fluorescamine-labeled des-Arg^9-bradykinin formed after the hydrolysis of bradykinin by carboxypeptidase-B (Fig. 5D) and by α-chymotrypsin (Fig. 5C) was found to be the same as the standard (see above). Labeled des-Phe^8-Arg^9-bradykinin, generated by the action of converting enzyme (Fig. 5A) and by carboxypeptidase-B followed by -A (Fig. 5B) on bradykinin, eluted with a retention time of 15 min. Labeled Phe-Arg dipeptide, formed by the action of converting enzyme on bradykinin was eluted as above.

The elution patterns of labeled T-kinin and its hydrolytic product, bradykinin, formed by the action of aminopeptidase-M with time are shown in Fig. 6. T-kinin was eluted with a retention time of 22.5 min.

DISCUSSION

As described here, fluorescamine labeling of kinins followed by HPLC provides for rapid identification and quantitation at nanogram levels of bradykinin and its analogues Met-Lys-bradykinin, Lys-bradykinin and Ile-Ser-bradykinin (T-kinin). The magnitude of the sensitivity of detection is the same as the bioassay on the rat uterus with the added advantages of distinct identification and the reproducibility and speed of a chemical procedure. In addition to kinin identification, slight alterations of the chromatographic procedure allow for detection of the enzymatic products of bradykinin when incubated with converting enyzme (des-Phe^8-Arg^9-bradykinin and Phe-Arg dipeptide), carboxypeptidase-B, (des-Arg^9-bradykinin), α-chymotrypsin (des-Arg^8-bradykinin and carboxypeptidase-B followed by carboxypeptidase-A (des-Phe^8-Arg^9-bradykinin). The conversion of T-kinin to bradykinin by aminopeptidase-M is also clearly demonstrable.

The fluorescamine method is much more sensitive (400-fold) than the UV detection of native kinins. Both methods taken together, however, are very useful in providing clear identification of bradykinin and its analogues since the sequence of elution of the kinins differs in the two systems. In the native kinin separation using acetonitrile and triethylammonium formate buffer, the order of elution is Lys-bradykinin, bradykinin and Met-Lys-bradykinin. The separation may be improved by using longer columns (e.g. 25 cm) and increasing the concentration of acetonitrile (e.g. 18%). When fluorescamine-labeled kinins (pyrollinone derivatives [14]) are chromatographed, using methanol and triethylammonium formate, the order of elution is bradykinin, Lys-bradykinin and Met-Lys-bradykinin.

It should be pointed out that attempts to identify a kinin by only one of these two HPLC procedures can be misleading since the hydrophobic nature of the molecule is an important criterion for separation. For example, T-kinin is clearly separated from bradykinin and its known analogues on one system of HPLC but chromatographs the same as Lys-bradykinin in the second system [15, 16]. Gabriel et al. [17] have made similar observations with other peptides. It should also be noted that these procedures are useful for detecting kinins only following purification, since, as pointed out by Geiger et al. [11], proteins may interfere with HPLC.

The fluorescamine–HPLC procedure for studies on kinetics of enzymatic degradation or conversions of purified kinins is simple, quantitative and rapid and needs no bioassay. However, during purification of a kinin from natural sources, the use of chromatography of the native kinin in conjunction with bioassay or radioimmunoassay is necessary since fluorescamine labeling will produce a plethora of peaks which are difficult to identify. However, once purification is moderately achieved, the identification by HPLC of both labeled and non-labeled kinins can be carried out without bioassay.

Radioimmunoassay is still the most sensitive method for quantitation of kinins at picogram levels [18]. A drawback of this method is that it cannot distinguish kinin analogues. Also, the antibody may react with biologically inactive peptides (des-Arg^9-bradykinin) and other degradative products of kinins. Though bioassay is a very useful method for quantitating kinins during

purification procedures, the method also cannot conveniently distinguish kinin analogues.

Finally, by using the fluorescamine—HPLC procedure the amounts necessary are minimal for determining purity as well as for identification, and peptide purification may proceed with a minimal loss of product.

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