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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF ARGININE-CONTAINING PEPTIDES IN BIOLOGICAL FLUIDS BY MEANS OF A SELECTIVE POST-COLUMN REACTION WITH FLUORESCENCE DETECTION

GERALD R. RHODES* and VENKATA K. BOPPANA
Department of Drug Metabolism, Smith Kline and French Laboratories, P.O. Box 1539, King of Prussia, PA 19406-0939 (U.S.A.)

SUMMARY

Highly sensitive and specific high-performance liquid chromatographic methodology for the fluorescence detection of arginine-containing peptides in biological matrices is described. Following HPLC separation, a post-column reaction of the guanidino group of the arginine moiety with ninhydrin under basic conditions is utilized to generate a fluorescent peptide product which can be measured at high sensitivity. Careful optimization of the post-column reaction conditions, and the use of HPLC columns of reduced internal diameter, resulted in on-column detection limits as low as 50 fmol. Application to the determination of synthetic arginine-containing vasopressin antagonists in human plasma resulted in a quantitative response which is linear over the range 0.5–100 pmol/ml. The assay method is sufficiently sensitive, accurate, and precise for use in pharmacokinetic studies of these synthetic peptides. The methodology also has general applicability in the detection of naturally occurring arginine-containing peptides.

INTRODUCTION

Synthetic analogues of naturally occurring peptides are currently of increasing interest in the development of novel therapeutic agents. The quantitative measurement of such species in biological fluids, in support of pharmacokinetic studies, requires the development of sensitive and specific assay methodology. Efforts are currently under way to discover structural analogues of arginine vasopressin that will act as V_2-receptor antagonists of this nonapeptide hormone[1], which is involved in water reabsorption by the kidney. These peptide antagonists are highly potent and administered in low doses (10–20 μg/kg). Plasma levels of these therapeutic agents were anticipated to be as low as 1 pmol/ml, which made development of a high-performance liquid chromatographic (HPLC) assay for this class of compounds an extremely difficult problem. The structural analogues that are currently under study have a blocked carboxy and no amino terminus but contain arginyl residues in the molecule (Fig. 1). Consequently, the arginine residue was selected as a potential site

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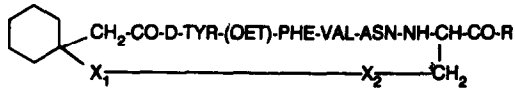

PEPTIDE	SK&F #	X_1	X_2	R
I	105494	CH_2	CH_2	ARG-D-ARG-NH_2
II	104146	S	S	ARG-D-ARG-NH_2
III	104241	S	S	LYS-ARG-NH_2
IV	103784	S	S	PRO-GLN-ARG-NH_2
V	104327	S	S	ARG-GLN-NH_2
VI	101498	S	S	PRO-ARG-GLY-NH_2
VII	103937	S	S	ARG-GLY-OH
VIII	101926	S	S	PRO-ARG-NH_2

Fig. 1. Structures of synthetic analogues of vasopressin.

of chemical modification in order to improve the detectability of these synthetic peptides and develop general, specific, and rapid HPLC methodology with fluorescence detection. Previous reports concerning HPLC analysis of arginine-containing peptides with fluorescence detection via pre- and post-column derivatization[2–4] have suffered from a lack of routine applicability or sensitivity in measuring such peptides at physiological levels.

This report describes a rapid, specific, and highly sensitive HPLC method for the detection of arginine-containing peptides in biological fluids. The approach involves isolation of the peptide from plasma, by solid-phase extraction, prior to quantitative analysis by HPLC with a selective post-column reaction, followed by in-line fluorescence detection. The selective detection of arginine-containing peptides is accomplished through a post-column reaction of the guanidino side chain with ninhydrin under basic conditions to generate a fluorescent product, which can be measured at high sensitivity. Previous work concerning the fluorescence detection of small molecules containing a guanidino group, such as creatinine and methylguanidine, with ninhydrin in basic media[5], indicated that the detection of arginine-containing peptides at high sensitivity might be possible. However, a previous effort concerning the use of ninhydrin to measure bradykinin provided only limited detectability (25 pmol)[4]. In the work reported here, the use of HPLC columns of reduced internal diameter and careful optimization of the post-column reaction conditions resulted in an on-column detection limit in the range 100–150 fmol (signal-to-noise ratio = 3) for these synthetic peptides. Although we developed this methodology primarily for pharmacokinetic studies of synthetic peptide analogues of vasopressin, the method, owing to its extremely high sensitivity, has general applicability in the detection of several naturally occurring arginine-containing peptides, such as bradykinin (detection limit of 50 fmol) and bombesin.

EXPERIMENTAL

Materials

The synthetic vasopressin antagonists (Fig. 1) were supplied by Drug Substances and Products, SK&F Laboratories (Swedeland, PA, U.S.A.). Naturally occurring peptides, such as bradykinin, angiotensins etc., were purchased from Sigma (St. Louis, MO, U.S.A.). HPLC-grade water (Millipore Corp., Bedford, MA, U.S.A.) was utilized in mobile phases and in the preparation of buffers and standard solutions. Ninhydrin and trifluoroacetic acid (TFA, 99%) were obtained from Pierce (Rockford, IL, U.S.A.). Methanol was HPLC-grade, obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Sodium 1-octanesulfonate was obtained from Regis (Morton Grove, IL, U.S.A.). All other chemicals were reagent-grade and obtained from local sources. The monochloroacetate buffer (pH 3.2) was prepared as follows: monochloroacetic acid (5.7 g), sodium hydroxide (2.0 g), and Na_2EDTA (0.2 g) were dissolved in 1 l of HPLC-grade water. The molarity of the buffer was approximately 65 m*M*. Stock standard solutions of the synthetic vasopressin antagonists as well as the naturally occurring arginine-containing peptides were prepared by dissolving the appropriate amounts of the compounds in methanol to obtain a solution with a concentration of 1 mg/ml. Working standard solutions were prepared by diluting a portion of the stock standards with monochloroacetic acid–methanol (1:1) to give a final solution concentration of 1 μg/ml. The working solutions were used in the generation of chromatographic data and standard curves. The stock and working standard solutions were stored at 4°C.

Extraction of synthetic arginine-containing vasopressin antagonists from plasma

An aliquot of plasma (1 ml), containing the appropriate amount of synthetic peptide and 50 μl of internal standard solution (1 μg/ml), were mixed in a 100 × 17 mm polypropylene tube. Weak cation-exchange (carboxymethylhydrogen form, CBA) solid-phase extraction cartridges (1 ml) and the Vac-Elut manifold were purchased from Analytichem (Harbor City, CA, U.S.A.). An extraction cartridge was conditioned by successive washings with 1 ml of 1% TFA in methanol, 1 ml of methanol and 2 ml of water. Following application of the plasma sample to this cartridge, the tube was rinsed with 1 ml of water, and the washings were applied to the cartridge also. The CBA column was then washed successively with 1 ml of 1% TFA in water, 2 ml of water and 2 ml of methanol. The peptide was then eluted from the column with 2 ml of 1% TFA in methanol and collected in a 75 × 12 mm borosilicate tube. The methanol was evaporated under a gentle stream of nitrogen and the residue was reconstituted in 100 μl of monochloroacetate buffer–methanol (50:50, v/v). The sample was transferred to a 200-μl autosampler tube, and 5–75 μl were injected for HPLC analysis.

High-performance liquid chromatography

The HPLC system (Fig. 2) consisted of a Hitachi 665A-12 high-pressure gradient semi-micro solvent delivery system (EM Science, Cherry Hill, NJ, U.S.A.), a post-column reactor module (PCRS Model 520, ABI Analytical, Ramsey, NJ, U.S.A.) and a Hitachi F-1000 fluorescence detector (EM Science). Chromatographic separations were carried out on a 25 cm × 2.0 mm I.D. Ultrasphere 5-μm octyl silica

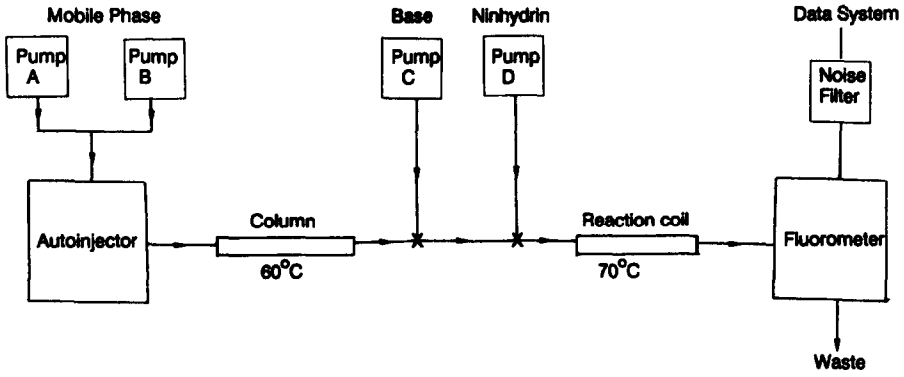

Fig. 2. Schematic diagram of the chromatographic system.

column (Beckmann Instruments, Palo Alto, CA, U.S.A.), maintained at 60°C, at a flow-rate of 300 μl/min. The mobile phase eluents utilized were monochloroacetate buffer (pH 3.2) and methanol. In some cases, 10 m*M* sodium octanesulfonate was added to both eluents as an ion-pairing agent. Eluents were filtered through a 0.2-μm Nylon-66 filter and degassed before use. Samples were injected using an HPLC autosampler (WISP 710B, Waters Assoc., Milford, MA, U.S.A.). The post-column reactor module contains two independently heated zones which were used, in this case, as a column heater chamber and as a reaction-coil heating block. Two additional pumps (Model 114, Beckmann Instruments) were utilized to deliver sodium hydroxide and ninhydrin reagent solutions to the post-column reactor, where they were mixed with the column effluent, utilizing low-dead-volume vortex mixers. Following formation of the fluorescent reaction product, detection was accomplished by excitation at 390 nm while monitoring the fluorescence emission with a 470-nm cut-off filter. Optimization of the post-column reaction conditions was achieved using peptide I as a model (SK&F 105494). A fixed amount of this peptide (10 ng) was injected into the column under varied reaction conditions, keeping the mobile-phase composition (monochloroacetate buffer–methanol, 35:65) and column flow-rate (300 μl/min) constant. The fluorescence intensity was monitored by measuring the resultant chromatographic peak height.

A CIS laboratory automation system (Beckmann/Computer Inquiry Systems, Berkely, CA, U.S.A.) was used for data acquisition and processing. Chromatographic peak height data were collected and utilized in the generation of standard curves.

RESULTS AND DISCUSSION

Optimization of the post-column reaction conditions

Initial off-line experiments to examine the ninhydrin reaction, with sodium hydroxide and ninhydrin reagent concentrations of 0.8 *M* and 0.6% (w/v)[5], respectively, indicated that a reaction time of approximately 2 min was required to obtain maximum fluorescence intensity. Preliminary post-column optimization experiments were conducted in-line with a 1-ml reaction coil (12 m × 0.33 mm I.D.). In order to minimize the dilution effects and to provide a reaction time of ca. 2 min, the flow-rates of base and ninhydrin reagent solutions were chosen to be 150 and 50 μl/min,

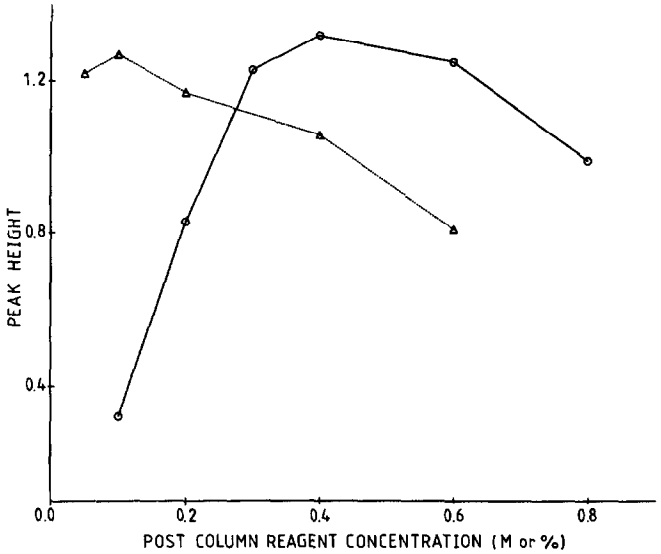

Fig. 3. Effect of post-column reagent concentrations, sodium hydroxide (—O—) and ninhydrin (—△—), on the fluorescence intensity, as measured by the peak height of peptide I.

respectively. Optimization of the post-column fluorescence reaction proceeded from these initial experimental parameters.

The effect of reagent concentration was examined by varying the concentration of base and ninhydrin successively to establish optimal concentrations for each reagent. Initially, the concentration of base was varied from 0.1 to 0.8 *M* while the ninhydrin concentration was maintained at 0.6%. As shown in Fig. 3, maximum fluorescence intensity was observed at a base concentration of 0.4 *M*. Higher base concentrations did not result in an increase in fluorescence. Following base optimization, the ninhydrin concentration was varied from 0.6 to 0.05% while keeping the base concentration at 0.4 *M*. As can be observed in Fig. 3, maximum fluorescence intensity was obtained at a ninhydrin concentration of 0.05–0.1%.In order to minimize the background fluorescence, a ninhydrin concentration of 0.05% was chosen to provide the optimal signal-to-noise ratio. Higher ninhydrin concentrations resulted in increased background noise without increasing the fluorescence signal.

Temperature effects on the post-column reaction were also examined, using the reagent concentrations established earlier, by varying the reaction-coil temperature from 40 to 80°C. Based on the results of this experiment, shown in Fig. 4, a reaction temperature of 70°C was chosen for the post-column derivatization.

The effect of different reaction-coil volumes (1.5 and 2 ml) on the post-column reaction was also examined. Reaction coils having a larger volume, to increase the overall reaction time, resulted in no further increase in chromatographic peak height but did cause a loss in chromatographic resolution due to band-broadening effects. The 1-ml coil utilized in these experiments resulted in no significant chromatographic band broadening and also provided the highest chromatographic peak height.

Studies with several other arginine-containing peptides, both synthetic and naturally occurring, gave similar post-column optimization results. The use of acetate

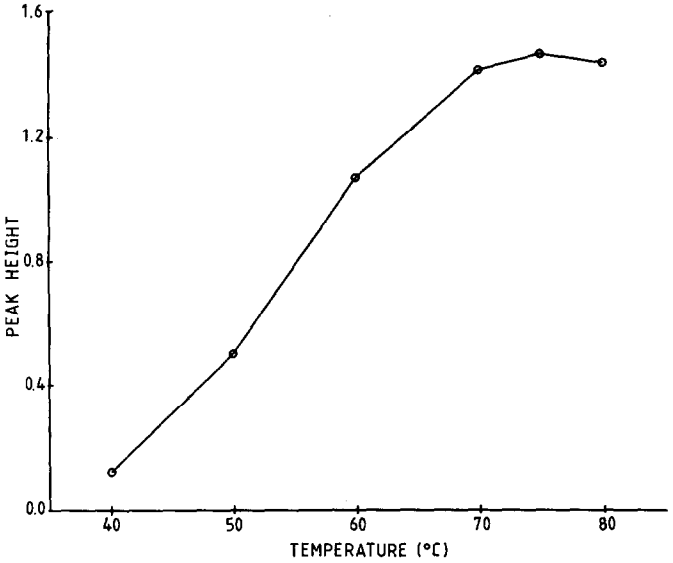

Fig. 4. Effect of post-column reaction temperature on the peak height of peptide I.

and citrate buffers in place of monochloroacetate gave similar optimization conditions. Ion pairing agents, such as sodium alkylsulfonates, also did not effect the post-column reaction. Thus, the optimized post-column parameters described above are: 0.4 *M* sodium hydroxide at 150 μl/min, 0.05% ninhydrin at 50 μl/min, and a 1-ml reaction coil, maintained at 70°C. These conditions were utilized to obtain the remainder of the results described in this report.

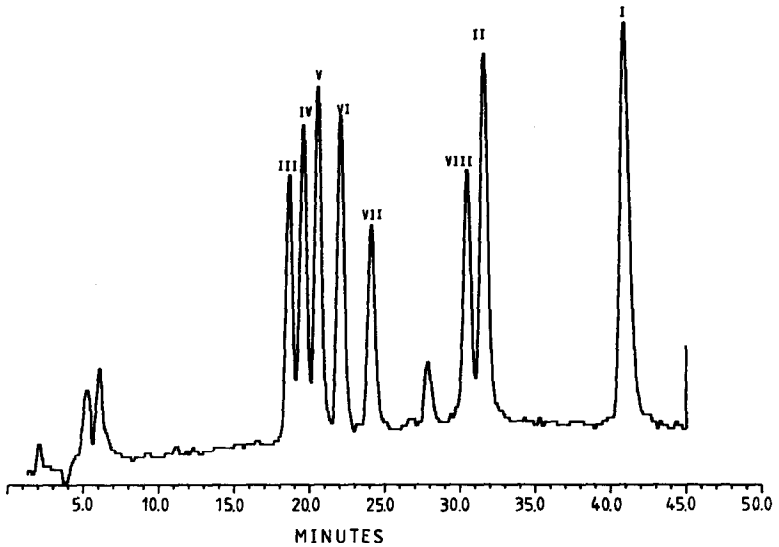

Fig. 5. Chromatogram of an aqueous standard solution of synthetic analogues of vasopressin. See text for chromatographic conditions and peak identification.

Detection of synthetic arginine-containing vasopressin antagonists

Fig. 5 shows the HPLC separation of eight synthetic arginine-containing vasopressin antagonists, by gradient elution on a 2 mm I.D. octyl silica column, followed by fluorescence detection with the post-column reaction conditions described earlier. The initial mobile phase was monochloroacetate buffer–methanol (42:58) with 10 m*M* sodium octanesulfonate added as an ion-pairing agent. Following injection, the methanol concentration was increased to 65% over a period of 20 min. The peptides were well separated, although in some cases the structural differences are only minor. Under these conditions, the limit of detection for these synthetic peptides ranged from 100 to 135 fmol injected on-column.

Application of this methodology to the quantitative measurement of synthetic arginine-containing vasopressin antagonists was exemplified by an HPLC assay, developed to determine the concentration of peptide I in human plasma samples. The internal standard used for this assay was SK&F 104146 (peptide II). The assay involves solid-phase extraction of the peptide from plasma as a preliminary isolation step, followed by quantitative gradient HPLC analysis by the methodology described here. Typical chromatograms of a drug-free plasma sample and a plasma sample, spiked with 20 pmol of SK&F 105494 (approximately 5 pmol injected on-column), are shown in Fig. 6. In this case, the initial mobile phase consisted of monochloroacetate buffer–methanol (45:55). After injection, the methanol concentration was increased to 70% in 10 min and held at this final concentration for 15 min. The retention times for peptide I and the internal standard (peptide II) were 16.1 and 20.5 min, respectively. No interference from endogenous compounds was observed, and the drug and internal standard were well separated. The mean recovery of peptide I and of the internal standard from plasma was 74.0 ± 3.7% and 77.5 ± 4.3%,

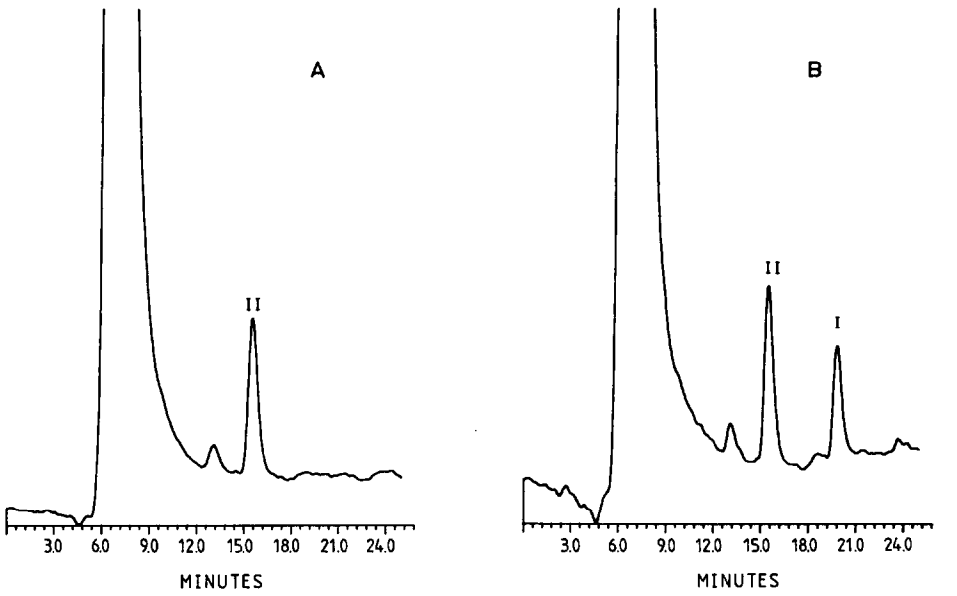

Fig. 6. Chromatograms of extracts of drug-free plasma (A) and plasma spiked with 20 ng/ml of peptide I. See text for chromatographic conditions.

respectively. Under the conditions utilized in this assay, the lowest concentration of I that can be determined quantitatively in 1 ml plasma samples is 0.5 pmol, which corresponds to an injected amount of *ca.* 250 fmol. The assay is linear over the plasma concentration range of 0.5–100 pmol/ml. Correlation coefficients for plasma standard curves were typically greater than 0.99.

Application to naturally occurring arginine-containing peptides

The methodology reported here has also been applied to the HPLC detection of naturally occurring arginine-containing peptides. Fig. 7 shows a gradient-elution HPLC separation of eleven naturally occurring arginine-containing peptides on an octyl silica 2 mm I.D. column. The initial mobile phase was monochloroacetate buffer–methanol (55:45) with 10 m*M* sodium octanesulfonate added as an ion-pairing agent. Subsequent to injection, the methanol concentration was increased to 70% over a period of 30 min and held constant for 60 min. The minimum detection limit with gradient HPLC for the peptides examined was in the range of 100–150 fmol injected on-column. Gradient elution did not effect the post-column reaction or the fluorescence intensity, but it did increase the baseline noise by two-fold. Therefore, slightly improved detection limits were possible by using optimized isocratic conditions for individual peptides. For example, Fig. 8 shows the isocratic separation of angiotensin II and bradykinin on a 2 mm I.D. octyl silica column. The mobile phase was monochloroacetate–methanol (45:55) with 10 m*M* sodium octanesulfonate added. In this case, the minimum detection limits for angiotensin II and bradykinin were 100 and 50 fmol, respectively. With the present reaction conditions, the fluorescence signal obtained from different peptides did not always increase in proportion to the number of arginine residues in the molecule. This may be the result of varying reaction rates for hindered arginine residues in a peptide.

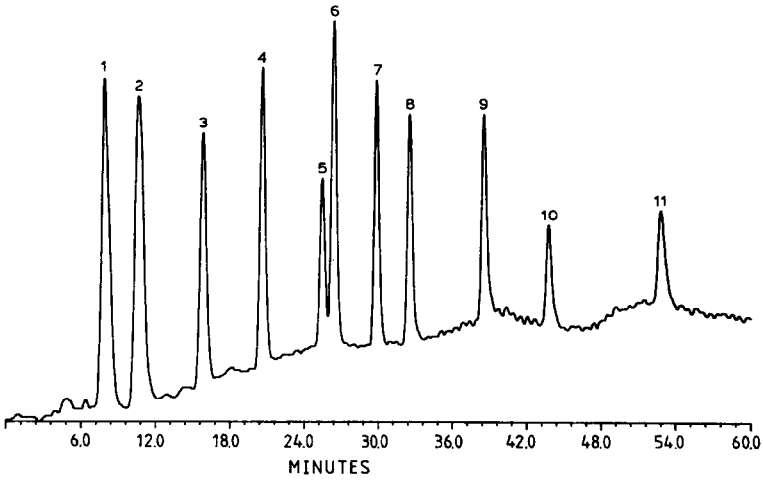

Fig. 7. Chromatogram of an aqueous standard solution of endogenous peptides. See text for chromatographic conditions. The chromatographic peaks and the amount injected are: 1 = desglycylamide vasopressin (39 pmol), 2 = Arg-vasopressin (41 pmol), 3 = luteinizing-hormone releasing hormone (18 pmol), 4 = angiotensin II (20 pmol), 5 = bombesin (13 pmol), 6 = bradykinin (8 pmol), 7 = angiotensin III (22 pmol), 8 = angiotensin I (16 pmol), 9 = atrial natriuretic peptide (7 pmol), 10 = adrenocorticotropin (ACTH) (6 pmol), 11 = growth-hormone releasing factor (4 pmol).

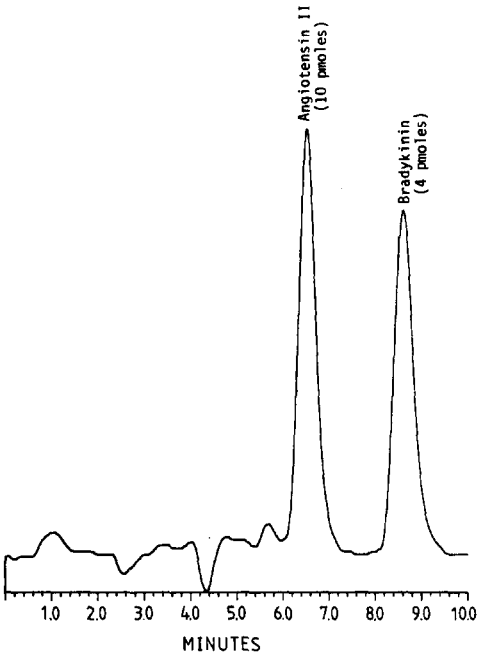

Fig. 8. Chromatogram of an aqueous standard solution of angiotensin II (10 pmol) and bradykinin (4 pmol), separated under isocratic conditions. See text for chromatographic conditions.

The quantitative HPLC methodology described here provided for specific and highly sensitive detection of arginine-containing peptides. Injection of a variety of other peptides which did not contain arginine resulted in a lack of fluorescence response. The detection limits reported here are substantially lower (approximately two orders of magnitude) than those described in previous reports for the post-column detection of arginine-containing peptides. The sensitivity of the method allowed successful application to the quantitative measurement of synthetic vasopressin antagonists in human plasma samples.

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