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High-performance liquid chromatographic determination of an arginine-containing octapeptide antagonist of vasopressin in human plasma by means of a selective post-column reaction with fluorescence detection

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SUMMARY

A novel high-performance liquid chromatographic method was developed for the determination in plasma samples of the synthetic octapeptide SK&F 105494 {O-ethyl-D-tyrosyl-L-phenylalanyl-L-valyl-L-asparaginyl-5-[1-(carboxymethyl)cyclohexyl]-L-norvalyl-L-arginyl-D-arginamide, cyclic (5–1) peptide}, an active aquaretic agent which significantly increases water excretion in experimental animal models through competitive antagonism of renal epithelial vasopressin receptors. The method involves isolation of Sk&F 105494 from plasma samples by solid phase extraction prior to chromatographic analysis. Following chromatographic separation, in-line fluorescence detection was accomplished via a selective-post-column reaction of the guanidino group of the arginine moiety of the peptide with alkaline ninhydrin to generate a highly fluorescent product. Optimization of the post-column reaction conditions and the use of high-performance liquid chromatography columns of reduced internal diameter (2.1 mm), resulted in an on-column detection limit of 50 pg (45 fmol). The limit of sensitivity in plasma was 0.5 ng/ml. The assay was linear over the range 0.5–100 ng/ml. Precision and accuracy were within 11% across the calibration range. The assay was shown to be suitable to study the pharmacokinetics of SK&F 105494 in human subjects and animals. In addition, the methodology developed has general applicability in the detection of arginine-containing peptides in biological matrices.

INTRODUCTION

The increasing interest in developing pharmacological agents based upon synthetic analogues of naturally occurring peptides necessitates the development of sensitive and specific analytical methodology for their measurement in biological matrices. SK&F 105494 {O-ethyl-D-tyrosyl-L-phenylalanyl-L-asparaginyl-5-[1-(carboxymethyl)cyclohexyl]-L-norvalyl-L-arginyl-D-arginamide, cyclic (5–1) peptide; Fig.

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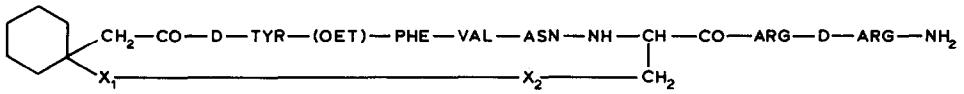

PEPTIDE	SK & F #	X_1	X_2
I	105494	CH_2	CH_2
II	104146	S	S

Fig. 1. Chemical structures of SK&F 105494 (I) and SK&F 104146 (II).

1, I}, a synthetic octapeptide analogue of vasopressin (AVP), is a parenterally active aquaretic agent which significantly increases water excretion in experimental animal models[1]. The compound selectively blocks renal water reabsorption via competitive antagonism of renal epithelial vasopressin (V2) receptors and is currently undergoing clinical trials. The peptide is highly potent and administered in low doses (5–10 μg/kg). The anticipated sensitivity requirement of 1 ng/ml in plasma made the development of analytical methodology for this compound a difficult problem due to a lack of a native chromophoric or common derivatizable functional group in the molecule. Although I has a blocked carboxy and amino terminus and generally non-reactive amino acid residues, the guanidino group in the arginine moiety of the peptide provided a potentially useful site for selective chemical modification to improve the detectability of I and develop a high-performance liquid chromatography (HPLC) assay with fluorescence detection.

Previous reports concerning HPLC analysis of arginine-containing peptides with fluorescence detection via pre- and post-column derivatization[2–5] have suffered from a lack of routine applicability or sensitivity in measuring such peptides at physiological levels. This report describes a novel, rapid, selective and highly sensitive HPLC method for the quantification of I in plasma. The approach involved isolation of the peptide from plasma samples by solid phase extraction and quantitative analysis by HPLC using a selective post-column reaction. Selective detection using the arginine moiety was accomplished through a post-column reaction of the guanidino side chain with ninhydrin under basic conditions to generate a fluorescent product which was measured at high sensitivity with an in-line fluorometer. The use of HPLC columns of reduced internal diameter and careful optimization of the post-column reaction conditions resulted in an on-column limit of detection of 50 pg (45 fmol, signal-to-noise ratio = 3) for this peptide. The methodology developed has general applicability in the analysis of arginine-containing peptides and peptide drugs.

EXPERIMENTAL

Materials

SK&F 104146 (Fig. 1, II) was used as the internal standard. I and II (purity > 98%) were obtained from Peptide Chemistry, Smith Kline and French Laboratories (Swedeland, PA, U.S.A.). HPLC-grade water (Ultrapure Water Systems, Durban, NC, U.S.A.) was used in the preparation of solutions, buffers and in the mobile phase. Monochloroacetic acid and sodium hydroxide were purchased from Mallinckrodt (Paris, KY, U.S.A.). HPLC-grade methanol and disodium ethylenediaminetetraacetic

acid (disodium EDTA, 99.5%) were purchased from EM Science (Cherry Hill, NJ, U.S.A.). Ninhydrin and trifluoroacetic acid (TFA, 99%) were obtained from Pierce (Rockford, IL, U.S.A.). All other chemicals were reagent grade and obtained from local sources.

Weak cation-exchange (carboxymethylhydrogen form, CBA) solid-phase extraction columns (1 ml) were purchased from Analytichem International (Harbor City, CA, U.S.A.).

Monochloroacetate buffer, pH 3.2, was prepared by dissolving monochloroacetic acid (5.7 g), sodium hydroxide (2.0 g) and disodium EDTA (0.2 g) in 1 l of deionized water to give a molarity of approximately 65 m*M*.

Extraction procedure

An aliquot of plasma (1 ml), containing I as standard or as an unknown, was mixed with 50 μl of methanol–water (50:50, v/v) solution of the internal standard II (1 μg/ml). A CBA column was conditioned by successive washings with 1 ml of 1% TFA in methanol, 1 ml of methanol and 2 ml of water. The plasma sample was applied to the column, the sample tube was rinsed with 1 ml of water and the washings were also transferred to the column. The column was washed successively with 1 ml of 1% TFA in water, 2 ml of water and 2 ml of methanol. The sample was then eluted with 2 ml of 1% TFA in methanol and collected into a 75 × 10 mm borosilicate tube. The eluate was evaporated under a gentle stream of nitrogen at 40°C and the residue was reconstituted in 100 μl of methanol–monochloroacetate buffer (50:50, v/v) and transferred to an autosampler vial. Volumes of 5–50 μl were injected into the HPLC system for analysis.

HPLC

The HPLC system consisted of a Hitachi 665A-12 high-pressure gradient semi-micro solvent delivery system (EM Science), 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 × 2.1 mm I.D., 5-μm octyl silica column (Ultrasphere, Beckman, Palo Alto, CA, U.S.A.), maintained at 60°C, at a flow-rate of 300 μl/min. The initial mobile phase composition was monochloroacetate buffer (pH 3.2)–methanol (45:55, v/v). Following injection, the methanol concentration was raised to 70% over a period of 10 min, held for 5 min and cycled back to initial conditions in 2 min. The system was equilibrated at the initial mobile phase composition for 13 min before injecting the next sample. Mobile phase eluents were filtered through a 0.2-μm Nylon-66 filter and degassed before use. Samples were injected by using an HPLC autosampler (WISP, Model 710B, Waters Associates, Milford, MA, U.S.A.). The post-column reactor module contains two independently heated zones which are used, in this case, as a column heating chamber and a reaction coil heating block. Two additional pumps (Model 114, Beckman) were utilized to deliver the sodium hydroxide (0.4 *M*) and ninhydrin reagent (0.05% w/v) solutions, at flow-rates of 150 and 50 μl/min, respectively, to the post-column reactor where they were mixed with the column effluent utilizing low dead volume mixers. The optimization of the ninhydrin post-column reaction for arginine-containing peptides has been described previously[6]. Following formation of the fluorescent reaction product, detection was accomplished

by utilizing excitation at 390 nm while monitoring the fluorescence emission with a 470-nm cut-off filter. Although the emission maximum for the fluorescent product is 500 nm, utilization of a 470-nm cut-off filter enhanced the fluorescence signal without sacrificing the specificity. The chromatographic data were collected with an automated laboratory system (CIS, Beckman, Waldwick, NJ, U.S.A.).

Standard curves

To establish calibration curves, three standard solutions of I [10, 1 and 0.1 μg/ml in water–methanol (50:50, v/v)] were used to prepare a series of 1-ml plasma samples at concentrations of 0, 1, 2, 5, 10, 20, 50 and 100 ng/ml. These samples were processed by the extraction procedure described above to generate an eight-point standard curve. The peak height ratios of I and the internal standard were weighed by $1/y$ (based on analysis of residual plots) and plotted against the concentrations of I. Linear regression analysis gave a calibration line that was used to calculate the concentration of I in unknown samples and seeded control samples. Standard solutions were stable for two months stored at 4°C.

RESULTS AND DISCUSSION

Application of the HPLC methodology described here provided a highly sensitive and specific assay for I in plasma samples. In large part, the specificity of the method was due to the selectivity of the post-column reaction chemistry. Although the structure is not presently known, under the strongly basic conditions used here (pH 12) ninhydrin gives a fluorescent product with the guanidino moiety of arginine but not with other common peptide functional groups, including primary amines. Injection of a variety of other peptides lacking arginine resulted in a lack of fluorescence response. Additional specificity was obtained through the use of weak cation-exchange rather than reversed-phase solid phase extraction of the peptide from plasma samples. Moreover, the product formed in the post-column reaction has excellent fluorescence properties in reversed-phase HPLC allowing detection of I at low femtomole levels.

Recovery and stability

The recovery of I and the internal standard from plasma was estimated with five determinations by comparing the peak height obtained with processed samples to that obtained by direct injection of an amount of standard equivalent to 100% recovery. At 75 ng/ml, a mean plasma recovery of 75.6 ± 3.7% was obtained for I. The recovery of the internal standard, II, from plasma, determined at a concentration of 50 ng/ml, was 77.5 ± 4.3%. In addition, I and internal standard were found to be stable in the final extract at room temperature for at least 24 h. Samples reanalyzed up to 24 h later showed no significant variation in peak height. Consequently, injection of prepared samples can be performed on the next day without observable quantitative changes.

Sensitivity, selectivity and linearity

By utilizing a 2.1-mm I.D. HPLC column, the on-column limit of detection (signal-to-noise ratio = 3) was 50 pg. Under the conditions used in this assay, the lowest concentration of I that could be determined quantitatively in 1-ml plasma samples without interference was 0.5 ng, which corresponded to an injected amount of

TABLE I

SUMMARY OF THREE-DAY ASSAY VALIDATION STUDY

Five replicates at three concentrations were analyzed on each of three successive days. At 2.5, 25 and 75 ng/ml, the within-day precision (mean of the daily coefficients of variation) was 10.26, 6.53 and 3.83%, respectively, the between-day precision (coefficient of variation of the daily means) 5.63, 4.59 and 1.87%, respectively and the mean accuracy 95.6, 100.6 and 102.6, respectively. S.D. = Standard deviation; C.V. = coefficient of variation.

Sample (*N* = 5)	*Day 1*	*Day 2*	*Day 3*
2.5 ng/ml			
Mean	2.24	2.43	2.50
S.D.	0.22	0.15	0.37
C.V. (%)	9.80	6.17	14.80
Accuracy	89.60	97.20	100.00
25 ng/ml			
Mean	26.38	24.99	24.09
S.D.	1.03	2.17	1.72
C.V. (%)	3.80	8.70	7.10
Accuracy	105.50	99.90	96.40
75 ng/ml			
Mean	77.99	77.65	75.34
S.D.	2.15	2.86	3.76
C.V. (%)	2.80	3.70	5.00
Accuracy	103.90	103.50	100.50

I of approximately 250 pg. Calibration curves obtained were linear over the range of 0.5–100 ng/ml of I. In this range, no interferences either from endogenous substances or from the known metabolites of I were observed. Weighed $(1/y)$ linear regression analysis of standard curves provided the equation $y = 0.03311x - 0.004373$ and a correlation coefficient greater than 0.99. Standard curves obtained over five successive days provided a composite curve with a correlation coefficient of 0.998. The precision, as measured by the relative standard deviations at each of the seven seeded concentrations, was within 11% across the calibration range. The average concentration back-calculated from the composite curve was within 10% of the seeded value at each concentration.

Accuracy and precision

The accuracy and precision of the assay were within 11% across the calibration range. Table I summarizes the results obtained from a three-day validation study in which five replicate-seeded standards at three concentrations, 2.5, 25 and 75 ng/ml, were analyzed each day by this methodology. The mean accuracy of the assay at these concentrations ranged from 95.6 to 102.6%, whereas the within-day precision, indicated by the mean of the daily coefficients of variation, varied from 3.83 to 10.26%. The reproducibility of the assay was high with between-day precision, indicated by the coefficients of variation of the daily means, ranging from 1.87 to 5.63%.

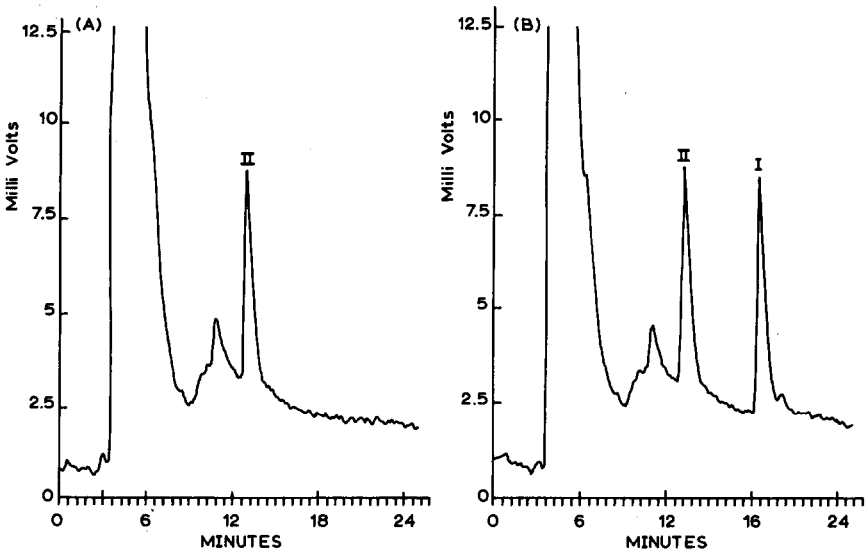

Fig. 2. Chromatograms of plasma extracts from a human subject before (A) and 2 h after (B) intravenous administration of 10 μg/kg of I. The concentration of I was 21.9 ng/ml.

Application of the procedure to plasma samples

Typical results obtained from the analysis of human plasma samples are shown in Fig. 2, which displays HPLC chromatograms of processed samples of drug-free human plasma and a plasma sample obtained from a human subject following an intravenous dose of I. The chromatography is highly reproducible and provides a retention time for I and internal standard of 18.6 and 14.0 min, respectively. To date the procedure has been used successfully in the analysis of biological samples from clinical and pre-clinical studies. The assay methodology developed was sufficiently sensitive for use in the study of the pharmacokinetics of I in humans and animal species.

In conclusion, a novel HPLC assay with fluorescence detection for an arginine-containing synthetic octapeptide antagonist of vasopressin, I, has been developed that is capable of measuring as low as 0.5 ng of I in 1 ml of plasma. The assay has excellent linearity, accuracy and precision over the range 0.5–100 ng/ml and has been shown to be suitable for pharmacokinetic studies of I. In addition, with slight modifications in HPLC conditions, the approach described here has general applicability in the detection of arginine-containing peptides, especially those without other easily modifiable functional groups.

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