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Short Communication

Quantification of a decapeptide anticoagulant in rat and monkey plasma by high-performance liquid chromatography

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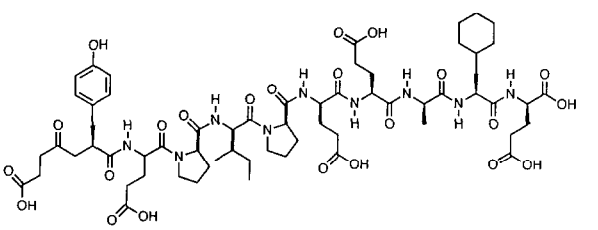
ABSTRACT

A reversed-phase high-performance liquid chromatographic method was developed to quantify a decapeptide anticoagulant in rat and monkey plasma. The compound and internal standard, a nonapeptide analogue, were extracted from plasma with an amino solid-phase extraction column with an extraction efficiency in the range 75–90%. A C_{18} analytical column was used to separate the analytes by gradient elution followed by ultraviolet detection at 215 nm. Quantification of the decapeptide over the concentration range 0.1–10.1 μg/ml resulted in an assay relative error and relative standard deviation both less than 10%. The anticoagulant decapeptide was stable in both rat and monkey plasma frozen at −20°C.

INTRODUCTION

Succinyl-L-tyrosyl-L-glutamyl-L-prolyl-L-isoleucyl-L-prolyl-L-glutamyl-L-glutamyl-L-alanyl-L-β-cyclohexylalanyl-D-glutamic acid (MDL 28 050, I) is a synthetic decapeptide analogue of the C-terminal region of the leech protein hirudin (Fig. 1). The compound is an effective antithrombin agent that could be used therapeutically as an anticoagulant [1,2]. By design, I was synthesized with six carboxyl groups because the anionic groups of the C-terminal region of hirudin are important contributors to the interaction between hirudin and α-thrombin. In addition, the decapeptide has a number of unnatural modifications at its C- and N-termini to prevent enzymic degradation by exopeptidases [2].

Determinations of hirudin in plasma have been indirect measurements based on antithrombin



Succinyl-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-DGlu-OH
M.W. 1328

Fig. 1. Structure and amino acid sequence of compound I.

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action [3–6]. In preparation for both toxicological and pharmacokinetic studies of I, an assay specific for I was necessary. The routine quantification of a peptide that is administered therapeutically can be achieved by methods based on high-performance liquid chromatography (HPLC) but prior to HPLC the peptide must be separated from endogenous proteins present in the biological matrix. Separation techniques that have been used previously for other peptides include solid-phase extraction with C_{18} columns [7,8] and precipitation of proteins [9,10].

This paper describes the development and validation of an assay for the decapeptide I which involves solid-phase extraction of the peptide from plasma by weak anion exchange followed by gradient HPLC separation on a C_{18} column with UV detection at 215 nm.

EXPERIMENTAL

Materials

All organic solvents were HPLC grade and were from Burdick and Jackson (Baxter, Muskegon, MI, USA). Ammonium acetate and phosphoric acid were from EM Science (Cherry Hill, NJ, USA), ammonium hydroxide was from J. T. Baker (Phillipsburg, NJ, USA), sodium phosphate was from Mallinkrodt (Paris, KY, USA) and trifluoroacetic acid (TFA) was from Pierce Chemicals (Rockford, IL, USA). The Bakerbond amino (200 mg size) solid-phase extraction (SPE) columns were purchased from J. T. Baker. Compound I and the internal standard (a nonapeptide analogue of I) were synthesized at the Marion Merrell Dow Research Institute (Cincinnati, OH, USA) [2]. The amino acid sequence of the internal standard is Suc-Tyr-Glu-Pro-Ile-Glu-Glu-Ala-Cha-Glu-OH.

Chromatographic conditions

The HPLC system was composed of a Varian Micropak C_{18} column (150 mm × 4 mm I.D., 5 μm particle size; *ca.* 60 Å pore size; Varian, Sunnyvale, CA, USA) with a 300-Å RP direct connect guard column from Alltech Assoc. (Deerfield, IL, USA), a Varian Model 9010 pump with an in-line filter, and a 250-μl Lee Visco mixer (Westbrook, CT, USA), a Gilson Model 231 autoinjector (Middleton, WI, USA) equipped with a 200-μl sample loop, and a Varian Model 9050 UV detector set at 215 nm. The mobile phase compositions were: A, acetonitrile–water (1:99) containing 0.06% TFA; B, water–acetonitrile (20:80) containing 0.054% TFA. The flow-rate was 1 ml/min. The analytes were eluted with a linear gradient starting at 0% B and increasing to 45% B over 15 min. After holding at 45% B for 5 min, the composition was returned to the initial conditions (5 min) and the column was equilibrated for 10 min before the next injection.

Standard solutions

Solutions of I were prepared in acetonitrile–water (90:10) at 100 and 10 μg/ml. The internal standard solution was prepared in acetonitrile–water (90:10) at a concentration of 100 μg/ml. Calibration curve samples were prepared by fortifying plasma with I at selected levels in the range 0.1–10.1 μg/ml.

Extraction procedure

After the plasma had been pipetted into glass test-tubes, 75 μl (*ca.* 7.5 μg) of the internal standard solution were added and the samples were mixed. To each sample, 2 ml of 0.05 *M* ammonium acetate buffer (pH 8.0) were added. The resulting sample was applied to an animo SPE column that had been conditioned with 3 ml of methanol, 3 ml of water and 3 ml of 0.05 *M* ammonium acetate buffer (pH 8.0). The SPE column was then washed with 3 ml of 0.05 *M* ammonium acetate buffer (pH 8.0) followed by 3 ml of acetonitrile–water (50:50) containing 0.1% H_3PO_4 and 3 ml of 0.1% H_3PO_4. The analytes were eluted with 500 μl of 0.5 *M* Na_2HPO_4 (pH 6.9) and 200 μl were injected into the analytical column.

Data analysis

An HP 3350A laboratory automation system with a Genie integrator (Hewlett Packard, Palo Alto, CA, USA) was used for data handling. Calculations were based on an internal standard

method in which the heights of the I and internal standard peaks were obtained and the peak-heigth ratios were determined. The data were fit to a power curve ($y = ax^b$) by linear regression analysis of the log-transformed data where y is the peak-height ratio and x is the concentration. The amount of I in a sample was obtained by interpolation.

The inter-day relative standard deviation (R.S.D.) and accuracy were determined by analyzing fortified plasma samples (from two to five per day at each concentration) on three separate days. A set of calibration curve samples was analyzed daily with each set of validation samples and used to calculate the concentrations of I.

The accuracy of the assay was assessed by the percentage of the theoretical concentration that was found in fortified plasma samples. The precision of the assay was expressed as the percentage R.S.D.

RESULTS AND DISCUSSION

Chromatography

A linear gradient was used to elute I and the internal standard from the C_{18} analytical column. The retention times were 17 min for I and 18.5 min for the internal standard. Under these conditions two metabolites of I (N-terminal hexapeptide and N-terminal pentapeptide) eluted prior to the parent compound and did not interfere with quantitation of I (Fig. 2). The percentage of TFA in the two mobile phase reservoirs was varied to minimize baseline shifts during the gradient; A contained 0.06% TFA and that in B was 0.054%.

Extraction

Both I and the internal standard could be extracted simultaneously from plasma with an amino SPE column operating in a weak anion-exchange mode because both compounds are negatively charged at pH values greater than 4. The extraction efficiencies of I and the internal standard, which were determined by comparing the peak heights of the analytes after extraction from rat and monkey plasma with those in unextracted standards, are shown in Table I. The extraction efficiencies of I and the internal standard were the same. Moreover, the analytes extracted similarly from both rat and monkey plasma at both 1 μg/ml and 5 μg/ml.

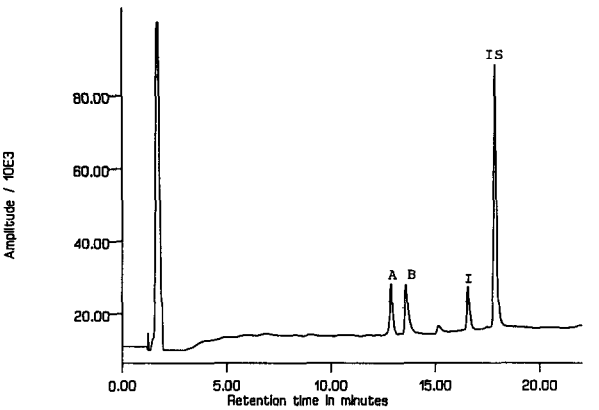

Fig. 2. Chromatographic separation of two metabolites, N-terminal hexapeptide (A) and N-terminal pentapeptide (B), I and the internal standard (IS) contained in an unextracted solution. Chromatographic conditions as described under Experimental.

Selectivity

The extraction procedure provided extracts of both rat and monkey plasma that were free of interfering endogenous substances with the same retention times as I and the internal standard. Typical chromatograms of extracted blank rat and monkey plasma are shown in Fig. 3A and B, respectively. Chromatograms of these two matrices fortified with the compounds of interest are shown in Fig. 3A for rat plasma and Fig. 3B for

TABLE I

EXTRACTION EFFICIENCY OF I AND INTERNAL STANDARD FROM RAT AND MONKEY PLASMA

Plasma	*n*	Concentration of I (μg/ml)	Extraction efficiency (%)	
			Compound I	Internal Standard
Rat	3	1	80.2	81.1
Rat	2	5	74.8	76.2
Monkey	7	1	84.9	83.4
Monkey	2	5	86.4	89.6

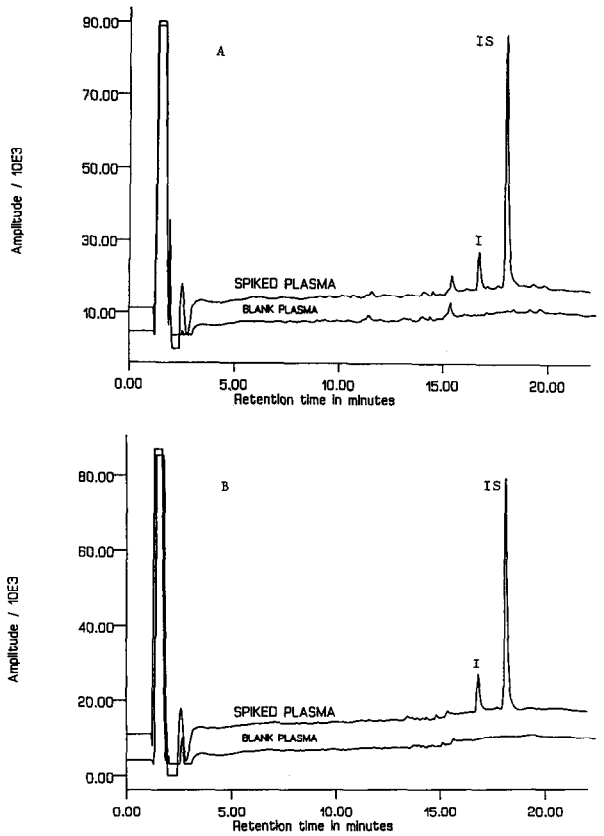

Fig. 3. HPLC resolution of I and the internal standard (IS) extracted from rat and monkey plasma. (A) Blank rat plasma and rat plasma spiked with I (1 μg/ml) and the internal standard. (B) Blank monkey plasma and monkey plasma spiked with I (1 μg/ml) and the internal standard.

TABLE II

PRECISION AND ACCURACY OF THE ASSAY FOR I IN RAT AND MONKEY PLASMA

Samples were assayed on three separate days. The values are the mean for six to nine determinations per concentration.

Theoretical concentration (μg/ml)	Found concentration (μg/ml)	Accuracy (%)	R.S.D. (%)
Rat plasma			
0.10	0.10	100.0	6.9
0.75	0.74	98.7	6.4
1.00	0.94	94.0	4.4
1.52	1.52	100.0	4.6
6.08	6.22	102.3	5.0
10.14	10.60	104.5	6.0
Monkey plasma			
1.00	0.97	97.0	8.3
6.08	6.12	100.7	9.3

monkey plasma. As mentioned above, the metabolites of I [11] eluted before the parent compound on the reversed-phase HPLC system used in this assay, and therefore would not interfere with quantification in samples from dosed animals.

Calibration curves

Calibration curves prepared in monkey or rat plasma over the concentration range 0.1–10.1 μg/ml were linear, with a coefficient of determination (r^2) greater than 0.99. The calibration curves in both matrices were identical; a typical curve for rat plasma was $y = 0.170x^{0.993}$ and that for monkey plasma was $y = 0.174x^{0.985}$. Owing to the difficulty of obtaining monkey plasma, the calibration curve samples were routinely prepared in rat plasma, even when analyzing monkey plasma.

Precision and accuracy

The inter-day R.S.D. (Table II) was less than 10% at concentrations ranging from 0.1 to 10.1 μg/ml in rat plasma and at 1.00 and 6.08 μg/ml in monkey plasma. The inter-day accuracy (Table

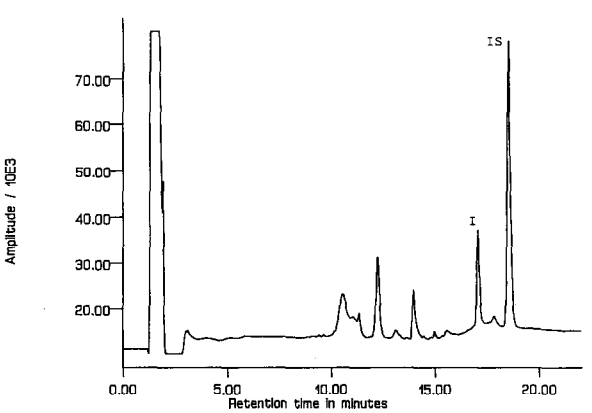

Fig. 4. Chromatographic separation of I (1.97 μg/ml) and the internal standard (IS) extracted from monkey plasma obtained 6 h after administration of 100 mg/kg I to a cynomolgus monkey.

II) ranged from 94 to 104.5%. The intra-day precision and accuracy, which were determined with five replicates at 1 μg/ml in rat plasma, were 2 and 95.9%, respectively. The lowest concentration quantified was 0.1 μg/ml. Concentrations less than 0.1 μg/ml resulted in peak heights that were too close to background baseline perturbations.

Toxicology study samples

This method of analysis has been used to measure the concentrations of I in plasma drawn from cynomolgus monkeys after intravenous administration of 100 mg/kg I. A representative chromatogram from this study is shown in Fig. 4.

Stability

Compound I was added to both rat and monkey plasma, which was then frozen at −20°C. At various times, the samples were thawed and analyzed for I. After 21 days of storage the recovery of I stored in monkey plasma was greater than 90%. The recovery in rat plasma was also greater than 90% even after 79 days of storage at −20°C. Therefore, I appears to be stable when stored in rat or monkey plasma at −20°C.

CONCLUSION

Weak anion-exchange SPE followed by reversed-phase HPLC is suitable for the quantification of I in both rat and monkey plasma over the concentration range 0.1–10.1 μg/ml. The precision and accuracy of the method are adequate for use in toxicological and pharmacokinetic studies. The compound appears to be stable in plasma frozen at −20°C.

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