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Determination of a cyclic heptapeptide, a novel fibrinogen receptor antagonist, in human plasma by high-performance liquid chromatography with automated pre-column derivatization, column switching and fluorescence detection

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ABSTRACT

A sensitive high-performance liquid chromatographic (HPLC) assay for the determination of the cyclic heptapeptide Ac-Cys-Asn-Dtc-Amf-Gly-Asp-Cys-OH (Dtc = β,β-dimethylthioproline, Amf = *p*-aminomethylphenylalanine) in human plasma has been developed. The key steps in the assay include: solid-phase extraction of the drug from plasma, chemical derivatization of the primary amino group with naphthalene-2,3-dicarboxyaldehyde in the presence of N-acetyl-D-penicillamine as a nucleophile to form a fluorescent benzo[*f*]isoindole derivative, and HPLC with column switching to provide the necessary chromatographic separation of the derivative from endogeneous plasma components. The assay has been validated in the concentration range 1–10 ng/ml of plasma.

INTRODUCTION

The cyclic heptapeptide Ac-Cys-Asn-Dtc-Amf-Gly-Asp-Cys-OH (Dtc = β,β-dimethylthioproline, Amf = *p*-aminomethylphenylalanine) (I-a, Fig. 1) is a novel fibrinogen receptor antagonist and is being evaluated for its ability to inhibit intravascular platelet aggregation without precipitating hemorrhagic diathesis [1]. If successful, the drug will be used to prevent reocclusion following thrombolysis in acute myocardial infarction, preventing early occlusion following angioplasty, and in the treatment of unstable angina [2–5].

The development of a highly sensitive and specific assay in biological matrices was required to study the pharmacokinetics of I-a. In addition to radioimmunoassay, several other approaches to the detection of I-a were initially considered. High-performance liquid chromatography (HPLC) with ultraviolet (UV) detection was not sensitive enough to reliably achieve low ng/ml limits of quantification (LOQ) from biological matrices. The presence of a sulfur–sulfur bond in I-a created the possibility of electrochemical detection (ED) in the dual-electrode (reduction–oxidation) mode as described for vasopressin and analogous small peptides [6–8]. However, using this HPLC–ED approach, it was highly unlikely to achieve the desired LOQ below 10 ng/ml with adequate precision (coefficients of variation, C.V. $<10\%$) required for human pharmacokinetic studies. In addition, long-term performance of

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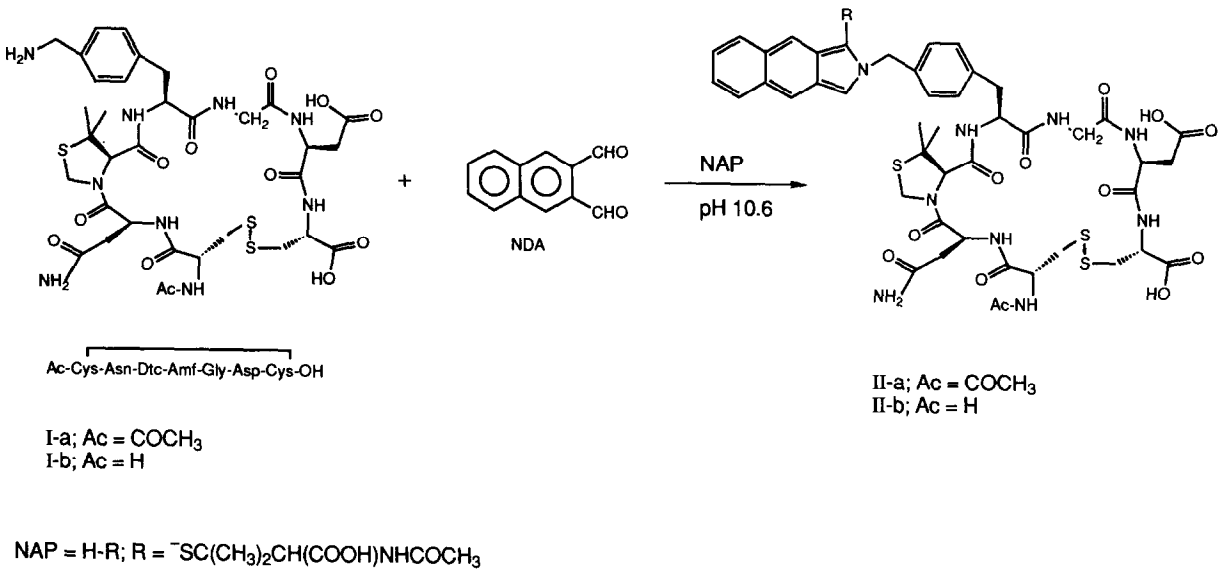

Fig. 1. Structures of I-a and its product II-a of derivatization with naphthalene-2,3-dicarboxaldehyde–N-acetyl-D-penicillamine reagent.

this type of dual-electrode HPLC–ED assay was uncertain.

Another useful approach for the determination of a disulfide-containing small peptide was used by Boppana and Rhodes [9]. It is based on the reduction of the disulfide bond, followed by derivatization of the thiol group with ammonium 4-chloro-7-sulfobenzofuran at elevated temperatures to form a fluorescent derivative. Before HPLC analysis, the excess of the reducing reagent after derivatization had to be removed from the reaction mixture, using a second solid-phase extraction (SPE) cartridge, considerably extending sample preparation time.

Since compound I-a, in addition to the disulfide bond, contained an easily modifiable primary amino functional group, it was decided to develop a method in plasma based on chemical derivatization of that group in I-a to form a highly fluorescent derivative. This derivative, formed at room temperature, was then analyzed directly by HPLC with fluorescence detection, without the necessity of removal of the excess of derivatizing reagent.

Among several fluorescence derivatizing reagents for primary amines [10,11], the naphthalene-2,3-dicarboxyaldehyde (NDA)–cyanide (CN^-) system was shown to be highly superior [12–14] over *o*-phthalaldehyde (OPA)–thiol, dansyl chloride and other reagents commonly used for the determination of primary amino acids and amines. Contrary to many other derivatizing reagents, where the reagent itself or products of its hydrolysis are fluorescent, the reaction between primary amine and NDA–nucleophile (Nu^-) is fluorogenic leading to the formation of fluorescent derivatives form non-fluorescent analytes and reagents. This type of reaction is most desired for highly sensitive and selective determination of analytes. Because of our familiarity and previous experience [12,14,15] with the NDA–CN^- system, it was decided to examine the NDA reagent for the derivatization of I-a. In order to avoid cyanide salts in the bioanalytical laboratory setting, N-acetyl-D-penicillamine (NAP) was used as the nucleophile in the derivatization reaction. It was found that the reaction of I-a with NDA–NAP produced the desired highly fluorescent derivative, and the assay methodology based on this approach is described in this paper.

The method is based on the isolation of the drug from plasma by SPE, automated pre-col-

umn derivatization of the primary amino group, HPLC column switching and fluorescence detection of I-a derivatized with the NDA–NAP reagent system. The LOQ for the new method is 1 ng/ml of plasma.

EXPERIMENTAL

Materials

HPLC-grade acetonitrile and methanol and analytical-grade acetic acid, sodium acetate, sodium hydroxide, calcium chloride, orthophosphoric acid (85%), sodium mono- and dihydrogenphosphate were from Fisher (Fair Lawn, NJ, USA). Sodium citrate and ethylenediaminetetraacetic acid (EDTA) were from Sigma (St. Louis, MO, USA). NAP was obtained from Fluka (Ronkokoma, NY, USA). The cyclic heptapeptide compound I-a and the desacetylated analogue I-b were obtained from Drs. S. Brady and R. Nutt (Medicinal Chemistry, Merck, Sharp & Dohme Research Labs., West Point, PA, USA). The NDA was purchased from Molecular Probes (Eugene, OR, USA). Deionized water was prepared using a Milli-Q reagent water system (Millipore, Milford, MA, USA).

Instrumentation

A Varian Vista Model 5500 HPLC system with a Varian 9090 autosampler (Walnut Creek, CA, USA) and a Waters Assoc. 6000 A HPLC pump (Milford, MA, USA) were used for all analyses. The column-switching device was an Autochrom solenoid interface and valve module (Milford, MA, USA). The fluorescence detector was a McPherson FL-750 Plus with a xenon–mercury lamp and a high-sensitivity accessory (HSA, Acton, MA, USA). The excitation wavelength was set at 436 nm and a cut-off emission filter with $\lambda > 440$ nm was utilized. The detector output signals were interfaced to a Hewlett-Packard laboratory automation system (HP 3357 LAS, Palo Alto, CA, USA). The analytical columns 1 and 2 were both Polymer Labs. (Amherst, MA, USA) polymeric reversed-phase (PLRP-S, 100 Å 5 μm) columns (150 mm × 4.6 mm). The cyclohexyl SPE cartridges were from Analytichem International (Harbor City, CA, USA).

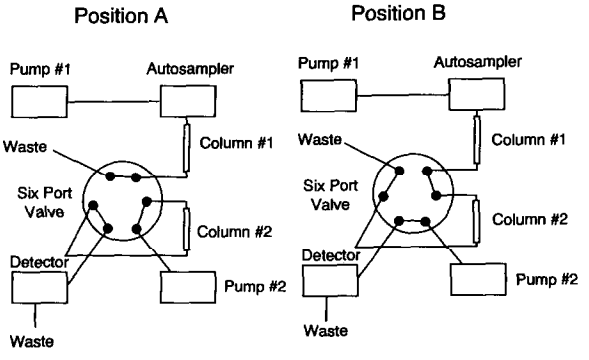

Fig. 2. HPLC system with column switching used for separation and quantification of the derivative I-a.

Chromatographic conditions

The HPLC system (Fig. 2) was composed of two pumps delivering two mobile phases to two HPLC columns. The columns both contained styrene–divinylbenzene co-polymer packing. The columns were connected via a six-port valve which was controlled by a column-switching device. The device consisted of a solenoid activated by a timed event (contact closure) programmed into the HPLC system. The derivatized I-a was injected onto column 1 and eluted with a "weak" mobile phase 1 composed of 25% acetonitrile in a 0.025 *M* sodium citrate–0.025 *M* sodium dihydrogenphosphate solution (pH 7.5 adjusted with phosphoric acid). During the first 27 min the flow from column 1 was directed to waste (Fig. 2, position A). The system was then switched (Fig. 2, position B) to load the derivative from column 1 onto column 2. After 5 min of loading column 2, the system was switched again (Fig. 2, position A) which directed the flow from column 1 back to waste. At the same time the derivatized I-a was eluted from column 2 with a "strong" mobile phase 2 which was composed of 32.5% acetonitrile in a 0.025 *M* sodium citrate–0.025 *M* sodium dihydrogenphosphate solution (pH 5.1 adjusted with phosphoric acid). The effluent from column 2 was monitored by a fluorescence detector. The fluorescent derivative of I-a entered the detector about 37 min after its initial injection onto column 1.

Standard solutions

Stock solutions of I-a were stored at −5°C and kept for a period of up to two weeks. Five working standard solutions with concentrations of I-a equal to 0.01, 0.02, 0.05, 0.10 and 0.20 g/ml were prepared by the appropriate dilution of a stock standard.

Sample preparation

Standard curves were determined by analyzing control human plasma (1 ml) spiked with 50 μl of working standards of I-a. A 1-ml aliquot of plasma containing standard I-a was placed into a 100 mm × 13 mm glass culture tube with 1 ml of 0.5 *M* phosphoric acid. The solution was vortex-mixed, then placed onto a cyclohexyl SPE cartridge. The cartridge was pretreated with 2 ml of methanol and 3 ml of water prior to introducing the spiked plasma solution. After the loading step, the SPE was washed with 3 ml of water, and standard I-a was eluted from the cartridge with 0.5 ml of acetonitrile–buffer [30% acetonitrile–70% 0.025 *M* sodium citrate–0.025 *M* sodium dihydrogenphosphate buffer pH 8.5 (1:1, v/v)]. A 350-μl aliquot of the eluent was placed in an amber sample vial, capped, and the vial then placed on the HPLC autosampler which was programmed to add automatically and sequentially 40 μl of 1 *M* carbonate buffer (pH 10.6), 20 μl of NAP (1 mg/ml in methanol) and 10 μl of NDA (1 mg/ml in methanol) solutions. After allowing the reaction to proceed for 1 min, 50 μl of the sample were automatically injected onto the HPLC system.

Precision, linearity and specificity

The precision of the method was determined by replicate analysis ($n = 5$) of human plasma containing I-a at 0.5, 1.0, 2.5, 5.0 and 10.0 ng/ml. The linearity of each standard line was confirmed by plotting the drug concentration *versus* peak area. Unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by the weighted linear regression of the standard curve. The weight was set to equal the inverse of the variance at each concentration. The specificity of the assay was checked by running blank plasma; endogenous interferences were not observed. The specificity of the assay in the presence of the desacetylated analogue I-b, a potential metabolite of I-a, was investigated. Fig. 3 shows that I-b elutes from column 2 approximately 1 min earlier than I-a, thus demonstrating the specificity of the assay.

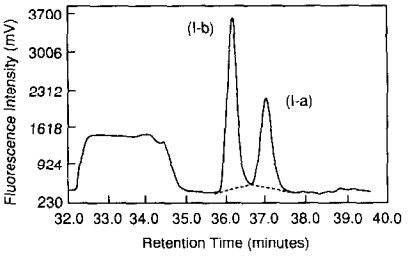

Fig. 3. Chromatogram resulting from the analysis of a solution containing 10 ng/ml of both I-a and the desacetylated analogue I-b.

RESULTS AND DISCUSSION

The development of an analytical procedure for the routine determination of I-a in plasma at low ng/ml concentrations required three key steps: initial isolation of this cyclic heptapeptide from plasma, automated pre-column derivatization to convert this molecule into a highly fluorescent analogue, and HPLC with column switching to separate the derivative from endogenous components present in plasma.

Isolation of I-a from plasma

The most difficult aspect of the development of the analytical procedure for the determination of I-a at low ng/ml levels was the isolation of I-a from the plasma matrix. The plasma apparently contained relatively large amounts of components chemically similar to I-a. Numerous SPE procedures were investigated ranging from adsorption onto non-polar C_{18} phases to adsorption based on both cation- and anion-exchange mechanism. In addition, a number of combination cartridges were studied such as, for example, C_{18} followed by ion exchange. The results showed that while many of these approaches provided good recovery of I-a from plasma, unac-

ceptable levels of endogenous components were always present. The choice of the cyclohexyl SPE as an initial clean-up was made because this cartridge was found to provide good and reproducible retention of I-a and easy elution with a small volume of eluent compatible with the subsequent derivatization and chromatographic steps.

Column switching

The inability of SPE cartridges alone to provide the necessary clean-up led us to the application of HPLC with column switching. The choice of linking two PLRP-S polymeric columns rather than two columns with different packings resulted from the findings of superior retention behavior with he PLPR-S columns. The primary observation was that small changes in the amount of acetonitrile in the mobile phase resulted in large changes in retention of the derivatized I-a. The 25% acetonitrile in mobile phase 1, for example, resulted in a retention time of nearly 30 min, yet, 32.5% acetonitrile in mobile phase 2 was strong enough to effect peak compression and elution in just 7 min. The pH of the mobile phase was found to effect both the stability of the derivative and the retention on the PLPR-S column; higher pH resulted in better stability and shorter retention. Consequently, a pH of 7.5 was chosen for mobile phase 1 to ensure stability during the 30 min that the derivative would be on column 1. A lower pH 5.1 was chosen for mobile phase 2 to further improve specificity. Despite the fact that both columns contained the same packing, a multi-dimensional chromatographic effect was achieved by lowering the pH and increasing the acetonitrile content in the second mobile phase. The stability of the derivative on column 2 was not seriously effected by the lower pH since the retention on this column was relatively short. Essentially, we have "traded" some sensitivity for the needed resolution and specificity.

Automated chemical derivatization

The utilization of the NDA–NAP reagent system has been developed from our previous experiences with the NDA–CN^- reagent [12–14] and pre-column derivatization for the determination of an amino bisphosphonate in urine [15]. These and other studies [11] have demonstrated that compounds containing primary amine functionality form isoindole and benzo[*f*]isoindole analogues after derivatization with OPA–thiol or NDA–CN^- reagents. By analogy with these reactions it is assumed that the benzo[*f*]isoindole derivative II-a is formed during reaction of I-a with NDA–thiol (Fig. 1). This is supported by the similarity in the absorption and fluorescence spectra of the derivative generated *in situ* (which exhibited the absorption bands in the region of 420–450 nm and an emission band with the maximum at 500 nm), with the characteristic spectra of the benzo[*f*]isoindole derivatives of other primary amines and amino acids which were synthesized and fully spectrally characterized earlier [12,15]. By analogy, the formation of highly fluorescent mono-derivatized II-b from I-b (Fig. 1) is invoked based on the previous observations that di-derivatized amino acids containing two primary amino groups (as in I-b) are practically non-fluorescent due to intramolecular fluorescence quenching [14]. However, the unequivocal confirmation of the structure of derivatives II-a and II-b have to await the synthesis of the derivatives which was not attempted.

The substitution of NAP for cyanide in the analysis of I-a was desirable from a laboratory safety point of view. However, the use of a thiol instead of cyanide could have resulted in some additional derivative instability. The potential stability problems were addressed through the application of an automated chemical derivatization procedure. The automation eliminated the need for careful control of the reaction kinetics and eliminated the problems associated with the potential chemical and photochemical instability of II-a. Instead of a typical pre-column derivatization of many samples and subsequent analyses of these samples over an extended period of time, each sample was derivatized at precisely the same time prior to the analysis. This was accomplished utilizing the Varian 9090 autosampler in the "automix" mode. The samples were processed manually to the point of placing aliquots of the eluent from the cyclohexyl SPE cartridge into

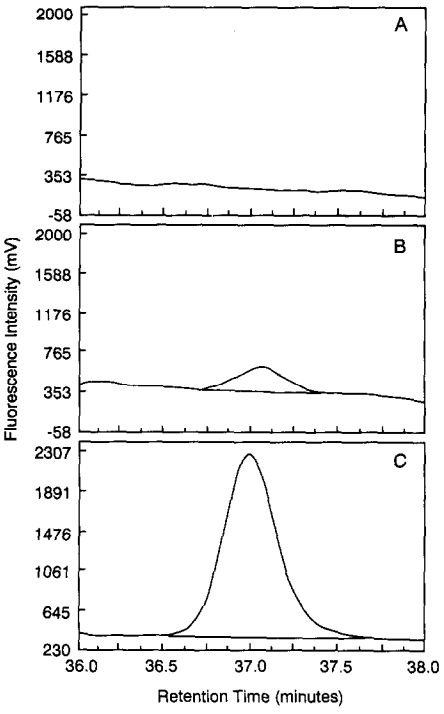

Fig. 4. Representive chromatograms of human plasma spiked with I-a. (A) Blank control plasma; (B) control plasma spiked with 1.0 ng/ml I-a; (C) control plasma spiked with 10 ng/ml I-a.

sample vials. The autosampler was then programmed to add appropriate volumes of solutions containing NAP and NDA. The sample solution was allowed to react for 1 min and then an aliquot was injected onto the first HPLC column.

TABLE I

INTRA-DAY VARIABILITY AND ACCURACY OF THE ASSAY OF I-a IN PLASMA

Concentration (ng/ml)	Coefficient of variation (n = 5) (%)	Accuracy[a] (%)
0.5	12.8	93
1.0	7.7	100
2.5	7.8	105
5.0	5.8	101
10.0	3.3	102
Mean		100 ± 5

[a] Calculated as (mean observed concentration/nominal concentration) multiplied by 100.

Assay validation

Fig. 4 illustrates typical chromatograms of II-a after isolation of I-a from plasma, pre-column derivatization and HPLC with column switching. The assay for I-a was linear in the concentration range 0.5–10.0 ng/ml of plasma. The typical equation of the linear regression line was y = 1 086 900x + 131 470 (using peak area in mV for calculations), with a correlation coefficient of 0.9986. The intra-day precision of the assay was less than 10% for all concentrations within the standard curve range except for the 0.5 ng/ml concentration which resulted in a coefficient of variation of 12.8% (Table I). Based on our criteria for validation (which demands coefficients of variation < 10%), this procedure was validated to 1 ng/ml. The accuracy of the assay was 92–106% (Table I).

CONCLUSION

A sensitive assay in human plasma for the determination of I-a based on SPE and automated pre-column derivatization with NDA–NAP reagent has been developed. The highly fluorescent derivative was quantified utilizing HPLC with column switching and fluorescence detection. The lowest limit of quantification was 1 ng/ml.

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