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Note

Determination of a disulfide-containing octapeptide antagonist of vasopressin in human plasma utilizing high-performance liquid chromatography with fluorescence detection

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The current interest in developing pharmacological agents based upon synthetic analogues of naturally occurring peptides requires the development of sensitive and specific analytical methodology for such species in biological matrices. SK&F 101 926 (Fig. 1, I), a synthetic octapeptide analogue of vasopressin (AVP), (1-pentamethylene propionic acid-2-(D)Tyr(Et)-4-Val-9-des-Gly)-AVP, is a potent antagonist of vasopressin-induced antidiuresis [1] currently undergoing clinical trials. This peptide antagonist is highly potent and is administered in low doses (10–20 μg/kg). The anticipated low limit of detection in plasma, 1 pmol/

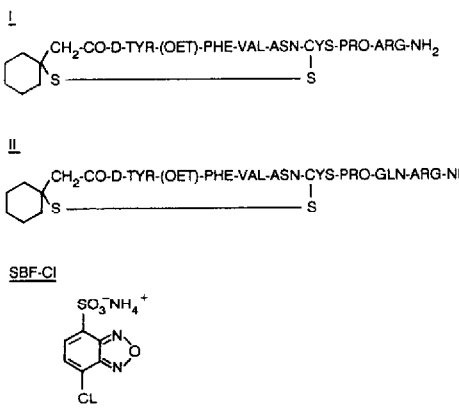

Fig 1. Chemical structures of SK&F 101 926 (I), SK&F 103 784 (II) and ammonium 4-chloro-7-sulfobenzofurazan (SBF-Cl).

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ml, necessitated enhancing the native detectability of the compound, via chemical derivatization, in order to develop sensitive and specific assay methodology. Compound I, however, has a blocked carboxy and amino terminus and generally non-reactive amino acid residues, which limit the possibilities for chemical modification. A potential site of modification, however, is the disulfide bond. Several approaches are available for the analysis of such species, including liquid chromatography with electrochemical detection [2–4] and pre- and post-column reduction and derivatization [5–7]. The methods reported to date for disulfide-containing peptides suffer either from a lack of sensitivity or routine applicability for the clinical analysis of human plasma samples.

Here we report on novel assay methodology for the detection of the disulfide-containing peptide drug I in human plasma samples utilizing high-performance liquid chromatography (HPLC). Following isolation of the drug from plasma samples by solid-phase extraction, the disulfide bond was reduced with dithiothreitol (DTT). The reduced peptide was then derivatized with ammonium 4-chloro-7-sulfobenzofurazan (SBF-Cl, Fig. 1), a highly selective fluorescent reagent specific for thiol groups in primarily aqueous media [8]. Following reaction, removal of excess derivatized DTT was performed by solid-phase extraction. Quantitative analysis of I was then performed by gradient HPLC on columns of reduced internal diameter (2 mm I.D.) with fluorescence detection. The methodology developed has general applicability in the analysis of disulfide-containing peptides and peptide drugs.

EXPERIMENTAL

Materials

SK&F 103 784 (Fig. 1, II) was used as the internal standard. Compounds I and II (purity > 98%) were obtained from Peptide Chemistry, Smith Kline and French Laboratories (Swedeland, PA, U.S.A.). Methanol and hexane were HPLC grade obtained from J.T. Baker (Philipsburg, NJ, U.S.A.). HPLC-grade water (Ultrapure Water Systems, Durham, NC, U.S.A.) was used in the preparation of solutions, buffers and in the mobile phase. Tetrabutylammonium phosphate (PIC A ion-pairing reagent) was obtained from Waters Assoc. (Milford, MA, U.S.A.). Trifluoroacetic acid (TFA, 99%) and ammonium 4-chloro-7-sulfobenzofurazan (SBF-Cl) were obtained from Pierce (Rockford, IL, U.S.A.). Dithiothreitol (99.8%) was purchased from Behring Diagnostics (La Jolla, CA, U.S.A.). Sodium tetraborate (99.9%) was obtained from Sigma (St. Louis, MO, U.S.A.). Phosphoric acid (HPLC grade) and ethylenediaminetetraacetic acid (EDTA, 99.5%) were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). All other chemicals were reagent grade and obtained from local sources.

Weak cation-exchange (carboxymethyl–hydrogen form, CBA) solid-phase extraction cartridges (1 ml) were purchased from Analytichem International (Harbor City, CA, U.S.A.). All glassware was purchased from Thomas Scientific (Philadelphia, PA, U.S.A.) and was silylated before use with a 5% solution of dichlorodimethylsilane in toluene (v/v), rinsed and air-dried.

Extraction and derivatization procedures

An aliquot of plasma (1 ml), containing I as standard or as an unknown, was placed in a 10-ml screw-cap centrifuge tube and diluted with 1 ml of water, followed by the addition of 50 μl of an ethanol internal standard solution (II, 0.5 μg/ml). The resulting solution was mixed and allowed to stand for 0.5 h. A CBA cartridge was conditioned by successive washings with 1 ml of methanol and 1 ml of water. The plasma sample was applied to this cartridge using vacuum, followed by successive washes with 1 ml of 1% TFA in methanol, 1 ml of water, 1 ml of borate buffer (100 m*M*, containing 1 m*M* EDTA), 1 ml of water and 500 μl of methanol. The sample was eluted from the column with 400 μl of 1% TFA in methanol, and collected in a 2-ml centrifuge tube. The sample was evaporated with nitrogen at 40°C. The dried residue was then dissolved in 90 μl of borate buffer, mixed and allowed to stand for 15 min. To the sample were added 5 μl of 0.2 *M* DTT, and the sample was transferred to a 300-μl borosilicate vial, which was sealed with a PTFE-lined septum. The reduction proceeded for 0.5 h at 40°C. Subsequently, 20 μl of 0.2 *M* SBF-Cl were added to the reduced peptide and the reaction was allowed to proceed for 2 h at 60°C. Following reaction, the derivatization mixture was applied to a CBA cartridge, which had been conditioned with 1 ml of methanol and 1 ml of water. Following sample application, the cartridge was washed with 1 ml of water, 1 ml of borate buffer, 1 ml of water and 200 μl of hexane, and then was dried. The sample was eluted with two 150-μl aliquots of 0.5 *M* ammonium acetate in methanol and collected in a 1.5-ml polypropylene centrifuge tube. The sample was evaporated with nitrogen at 40°C, reconstituted with 100 μl of water and was allowed to stand for 0.5 h. A 20-μl aliquot was injected for analysis by HPLC.

High-performance liquid chromatography

The HPLC system consisted of a gradient microbore solvent-delivery system (Brownlee Labs., Santa Clara, CA, U.S.A.) and a Hitachi F-1000 fluorescence detector (EM Science, Cherry Hill, NJ, U.S.A.). The column used was a 22 cm×2.1 mm I.D. RP-8 5-μm octylsilica column (Brownlee Labs.). A 3 cm×2.1 mm I.D. 10-μm RP-8 guard column was coupled directly in-line. The eluents were 20 m*M* tetrabutylammonium phosphate–methanol (50:50, v/v, pH adjusted to 5.2 with phosphoric acid, solvent A) and methanol (solvent B). The gradient was 10–55% B in 22 min with a flow-rate of 300 μl/min. The column temperature was maintained at 50°C. Eluents were prepared daily and filtered through a 0.2-μm Nylon-66 filter. Fluorescence detection was accomplished by excitation at 380 nm while monitoring the fluorescence emission with a 470-nm cut-off filter. Samples were injected with an HPLC autosampler (WISP 710B, Waters Assoc.). A laboratory automation system (Beckman/Computer Inquiry Systems, Berkeley, CA, U.S.A.) was used for data acquisition and processing.

Standard curves

To establish calibration curves, a series of standard solutions of I were prepared in ethanol, from a stock solution of 1 mg/ml, containing 0, 0.5, 1, 2.5, 5, 10, 20, 50, 100 and 200 ng per 50 μl. A 50-μl volume of these solutions was added to 1 ml

of drug-free human plasma and the samples were processed by the extraction procedure above to generate a nine-point standard curve. The peak-height ratios of I and the internal standard were weighted 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. Calibration curve standard solutions were made fresh weekly and stored at 4 °C.

RESULTS AND DISCUSSION

Optimal conditions for the reduction of I with DTT were determined with the ^{3}H-labelled compound [3,3-$^3H_2$]D-Tyr using HPLC to separate the reduced and non-reduced peptide. At DTT concentrations above 5 m*M*, complete reduction of I was accomplished within 0.5 h. The DTT concentration utilized in the procedure reported here was 8.7 m*M*.

The use of fluorescence methodology in the analysis of suitably derivatized I offers advantages, most notably the very high sensitivity attainable with properly chosen conditions. Although other thiol-specific fluorescence probes are available, SBF-Cl offers several advantages [8] including water solubility, extremely high specificity in aqueous environments, a 100-nm wavelength shift in the emission maximum upon binding to a thiol and the formation of a single, stable derivative product with good fluorescence properties for reversed-phase HPLC.

Recovery and stability

The recovery of I from plasma was estimated with four determinations at each of two concentrations (15 and 75 ng/ml) utilizing the ^{3}H-labelled compound. A mean plasma recovery of 73.4 ± 5.1% was obtained for I. The recovery of the internal standard, II, from plasma at 25 ng/ml was 76 ± 4.3%.

Freshly prepared plasma standard solutions were compared with plasma standard solutions frozen at −20 °C for one month. The variations in peak-height ratios at each drug level examined were found to be insignificant.

In addition, derivatized I and internal standard were found to be stable in water at room temperature for at least 24 h. Derivatized 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, linearity and selectivity

Utilizing a 2.1 mm I.D. HPLC column, the on-column limit of detection (signal-to-noise ratio = 3) for derivatized I was 10 pg (9.3 fmol), a four-fold improvement over that obtained on a 4.6 mm I.D. column. 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 0.46 pmol (0.5 ng), which corresponded to an injected amount of I of ca. 70 fmol.

Calibration curves obtained were linear over the range 0.46–185 pmol/ml I. In this range, no interferences from endogenous substances were observed. In ad-

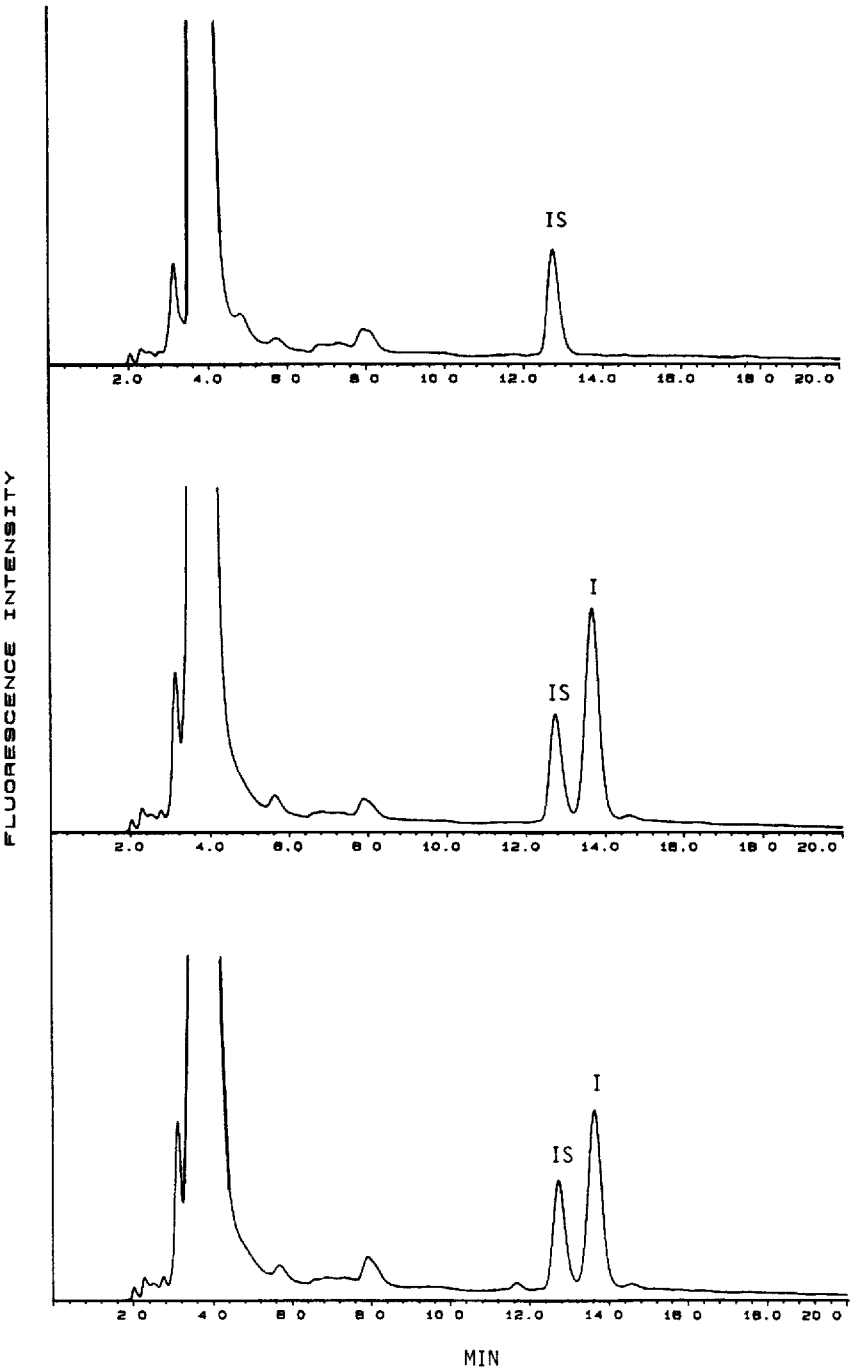

Fig. 2. Typical chromatograms from extracted 1-ml human plasma samples. (Top) drug-free plasma; (middle) drug-free plasma sample seeded with 46 pmol/ml I; (bottom) plasma sample taken following an intravenous dose of I; the concentration of I was calculated to be 39.6 pmol/ml. IS = internal standard.

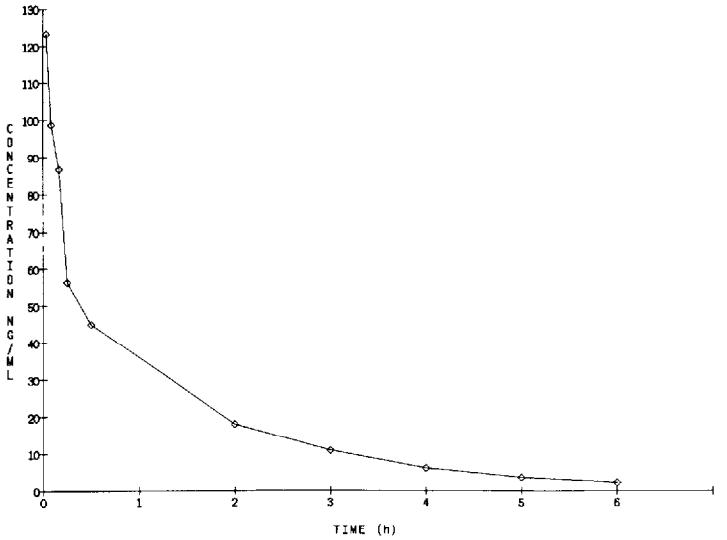

Fig. 3. Plasma concentration-time profile from a human subject following a short intravenous infusion (30 min) of 20 μg/kg I.

dition, derivatized I was chromatographically separable from its derivatized major metabolites in plasma. Weighted $(1/y)$ linear regression analysis of standard curves typically provided 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 nine seeded concentrations, was within 13% across the calibration range. The average concentration back-calculated from the composite curve was within 11% of the seeded value at each concentration.

Typical results obtained from the analysis of human plasma samples are shown in Fig. 2, which displays HPLC profiles of processed samples of drug-free human plasma, a 1-ml drug-free plasma sample seeded with 46 pmol of I and a 1-ml 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 the internal standard of 13.8 and 12.8 min, respectively.

Accuracy and precision

The accuracy and precision of the assay were within 13% 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, 7, 40 and 125 ng/ml, were analyzed each day by this methodology. The mean accuracy of the assay at these concentrations ranged from 99 to 111%, whereas the within-day precision varied from 2.0 to 5.7%. The reproducibility of the assay was high with between-day precision ranging from 2.4 to 3.6%.

Application of the procedure to plasma samples

To date the procedure has been used successfully in the analysis of biological samples from clinical and pre-clinical studies. Fig. 3 shows the plasma concentration–time profile obtained from a patient following a short intravenous infusion (30 min) of 20 μg/kg I. Serial blood samples were drawn at selected time

TABLE I

SUMMARY OF THREE-DAY ASSAY VALIDATION STUDY

Five replicates at three concentrations analyzed on each of three successive days

Actual concentration (ng/ml)	Day	Concentration found (mean) (ng/ml)	Coefficient of variation (%)	Accuracy (%)	Precision (%)		Mean accuracy (%)
					Within-day[a]	Between-day[b]	
7	1	6.9	5.7	98.6	5.7	3.6	99.1
	2	7.2	9.4	102.9			
	3	6.7	1.9	95.7			
40	1	45.6	0.9	114.0	2.0	2.5	110.9
	2	44.1	3.8	110.3			
	3	43.4	1.4	108.5			
125	1	131.2	1.6	105.0	2.2	2.4	102.4
	2	125.0	3.9	100.0			
	3	127.7	1.1	102.2			

[a]Mean of the daily coefficients of variation.
[b]Coefficient of variation of the daily means

points up to 6 h following intravenous dosing. The assay methodology developed is sufficiently sensitive for use in the study of the plasma pharmacokinetics of I.

Additionally, the approach described here has general applicability in the detection of disulfide-containing peptides, especially those without other easily modifiable functional groups. Although optimal HPLC conditions vary slightly, we have successfully derivatized and analyzed several other synthetic and naturally occurring disulfide-containing peptides, such as vasopressin, atrial natriuretic factor and other synthetic analogues of vasopressin, with comparable results.

CONCLUSIONS

An HPLC–fluorometric assay for a disulfide-containing synthetic octapeptide antagonist of vasopressin (I) has been developed that is capable of measuring as low as 0.46 pmol of I in 1 ml of plasma. The assay has excellent linearity, accuracy and precision over the range 0.46–185 pmol/ml and has been shown to be suitable to study the human pharmacokinetics of I. In addition, the novel methodology reported here applies generally to the detection of disulfide-containing peptides in biological samples.

REFERENCES

1 W.F. Huffman, F.E. Ali, W.M. Bryan, J.F. Callahan, M.L. Moore, J.S. Silvestri, N.C.F. Yim, L.B. Kinter, J.E. McDonald, D. Ashton-Shue, F.L. Stassen, G.D. Heckman, D.B. Schmidt and L. Sulat, J. Med. Chem., 28 (1985) 1759.
2 L.A. Allison, J. Keddington and R.E. Shoup, L. Liq. Chromatogr., 6 (1983) 1785.
3 L.A. Allison and R.E. Shoup, Anal. Chem., 58 (1983) 8.
4 C.T. Garvie, K.M. Straub and R.K. Lynn, J. Chromatogr., 413 (1987) 43.
5 J.F. Studebaker, S.A. Slocum and E.L. Lewis, Anal. Chem., 50 (1978) 1500.
6 J.F. Studebaker, J. Chromatogr., 185 (1979) 497.
7 O.C. Ingebretsen and M. Farstad, J. Chromatogr., 210 (1981) 522.
8 J.L. Andrews, P. Ghosh, B. Ternai and M.W. Whitehouse, Arch. Biochem. Biophys., 214 (1982) 386.