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Determination of a novel hematoregulatory peptide in dog plasma by reversed-phase high-performance liquid chromatography and an amine-selective *o*-phthaldialdehyde–thiol post-column reaction with fluorescence detection

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Abstract

A sensitive and selective high-performance liquid chromatographic method was developed for the determination of SB 107647 (**I**), a novel synthetic hematoregulatory peptide, in plasma samples of dog and rat. The method involves isolation of **I** and the internal standard (SB 203285, IS) from plasma by a solid-phase anion-exchange extraction column prior to reversed-phase ion-pair chromatographic separation on an octyl silica column. Following separation, a selective post-column reaction of the ϵ-amino groups of the lysine moieties of the peptide with *o*-phthaldialdehyde and a thiol under basic conditions was used to generate a highly fluorescent isoindole product, which was subsequently detected on-line with a fluorometer. Optimization of chromatographic conditions resulted in an on-column detection limit of 1 ng. The recovery of **I** from dog plasma at 20 and 4000 ng/ml was 50.0 ± 5.94 and 56.6 ± 1.45% (Mean ± S.D.), respectively. The limit of quantification for **I**, for 0.25-ml plasma samples, was 20 ng/ml. Linear response was observed for concentrations of **I** ranging from 20 to 4000 ng/ml of plasma. The assay was sufficiently sensitive, accurate and precise to support toxicokinetic studies in animal species.

1. Introduction

SB 107647 {**I**, Fig. 1; (S)-5-oxo-L-prolyl-L-α-glutamyl-L-α-aspartyl-N8-(5-amino-1-carboxypentyl)-8-oxo-N7-[N-[N-(5-oxo-L-prolyl)-L-α-glutamyl]-L-α-aspartyl]-L-*threo*-2,7,8-triaminooctanoyl-L-lysine} is a novel synthetic hematoregulatory peptide [1–3]. It selectively stimulates cytokinin production by stromal cells, particularly fibroblasts, at an early stage of hematopoiesis relative to the colony stimulating factors (CSFs).

Since **I** lacks appreciable absorbance in the ultraviolet region, development of a sensitive and specific HPLC method to support toxicokinetic studies was impractical using conventional HPLC detectors. However, the ϵ-amino groups of the lysine moieties of the peptide provided potential sites for chemical modification in order to improve detectability of **I** for development of a selective HPLC methodology with fluorescence detection. This report describes a rapid, selective and sensitive HPLC method for quantification of **I** in dog and rat plasma samples. The method involves isolation of **I** and the internal standard (IS) from plasma by solid-phase extraction prior to reversed-phase ion-pair chromatographic separation on an octyl

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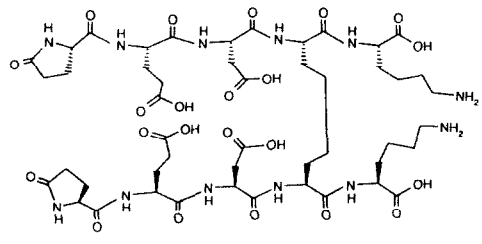
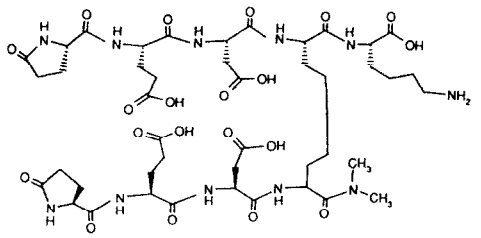


I



IS

Fig. 1. Structures of **I** and internal standard (IS).

silica column. Following separation, a selective post-column reaction of the ϵ-amino groups of the lysine moieties of the peptide with *o*-phthaldialdehyde and a thiol under basic conditions was used to generate a highly fluorescent isoindole product which was subsequently detected on-line with a fluorometer.

2. Materials and methods

2.1. Chemicals

SB 107647 (I) and the internal standard (SB 203285, IS) were supplied by Central Supply and Investigational Materials, SmithKline Beecham Pharmaceuticals (Swedeland, PA, USA). Glacial acetic acid and monochloroacetic acid were obtained from Mallinckrodt Chemical Company (Paris, KY, USA). Citric acid monohydrate, sodium hydroxide, HPLC-grade methanol and acetonitrile were obtained from J.T. Baker Chemical Company (Phillipsburg, NJ, USA). Disodium EDTA was obtained from E.M. Science (Cherry Hill, NJ, USA). Sodium salt of 1-octanesulfonate was purchased from Regis Chemical Company (Morton Grove, IL, USA). Thiofluor (N,N-dimethyl-2-mercapto ethylamine hydrochloride) was purchased from Pickering Laboratories (Mountain View, CA, USA). *o*-Phthaldialdehyde was purchased from Fluka Chemical Corporation (Ronkonkoma, NY, USA). Strong anion exchange (SAX) solid-phase extraction cartridges (100 mg, 1 ml) and the Vac-Elut manifold were purchased from Analytichem International (Harbor City, CA, USA).

2.2. Reagents

0.3 M Monochloroacetate (MCA) buffer

28.3 g of monochloroacetic acid, 9.8 g of sodium hydroxide and 1.0 g of disodium EDTA were dissolved in 1 l of HPLC-grade water. The final pH of the solution was 3.2.

Mobile phase buffer

Sodium salt of 1-octane sulfonate (10 m*M*, 2.16 g) was dissolved in 100 ml of 0.3 *M* monochloroacetate buffer, pH 3.2 and 900 ml of HPLC-grade water. The solution was filtered through a 0.45-μm membrane filter (type HA, Millipore, USA).

o-Phthaldialdehyde (OPA) reagent solution

Sodium hydroxide (1.0 g) was first dissolved in 500 ml of HPLC-grade water and filtered through a 0.45-μm membrane filter (type HA, Millipore). The solution was sonicated for approximately 10 min and then sparged with helium for approximately 10 min. To this alkaline solution, 2 ml of freshly prepared methanolic solution of *o*-phthaldialdehyde (OPA, 2 mg/ml) and 80 mg of thiofluor were added and the contents were mixed by swirling. The solution was sparged continuously with helium during its use as post-column reagent. The solution was stable for 48 h.

Stock standard solutions

The stock standard solutions of **I** and IS were prepared by dissolving appropriate amounts of the peptide in 0.05 *M* acetic acid to give a final solution concentration of 1 mg/ml. These stock solutions were stable for 2 months when stored at 4°C. Appropriate dilutions of the stock solution of **I** were made fresh every day with 0.05 *M*

acetic acid solution to generate a series of working standard solutions (100, 10, 1 and 0.1 μg/ml). The stock solution of IS was diluted 1:200 with 0.05 *M* acetic acid to give a solution concentration of 5 μg/ml. The working internal standard solution was stable for 2 months when stored at 4°C.

2.3. Calibration

A set of plasma calibration standards (concentrations of **I**: 20, 40, 100, 200, 400, 1000, 2000, 4000 ng/ml) was analyzed with every determination of **I** in plasma samples of unknown concentration, by adding appropriate volumes of the working standards of **I** to 0.25 ml of plasma. A weighted $(1/y)$ linear regression was used to construct a calibration curve for the peak height ratio of analyte to internal standard *vs.* analyte concentration. The concentration of **I** in plasma samples was then calculated using the equation $y = bx + a$, where b = slope of regression line, a = y-intercept of regression line, x = concentration of **I** in ng/ml and y = peak height of **I** in millivolts (mV)/peak height of IS in millivolts (mV)

2.4. Extraction of I from plasma

An aliquot of heparinized plasma (0.25 ml) mixed with 200 μl of 0.05 *M* acetic acid (contains standards when preparing standard curve) and 50 μl of internal standard solution (5 μg/ml, IS) was added to 500 μl of acetonitrile in a 75 × 12 mm borosilicate tube in order to precipitate plasma proteins. The sample was then centrifuged at approximately 2000 *g* for 5 min. An SAX extraction column was conditioned by successive washings with 1 ml of methanol and 1 ml of water. The deproteinated plasma sample was then applied onto the SAX column under reduced pressure at 1–2 in. of Hg (1 in. Hg ≈ 3386 Pa). After passage of the sample through the column, the vacuum was increased to 10–15 in. of Hg to remove any traces of plasma from the extraction column. The column was then washed with 1 ml of water and the washing solvent was completely removed from the sorbent bed prior to elution of analytes. The analytes were then eluted from the column with 0.25 ml of citric acid (0.1 *M*, pH 2.2) under reduced pressure (1–2 in. of Hg) and the eluate was collected into a 1.5 ml polypropylene Eppendorf micro centrifuge tube (Brinkmann Instruments, Westbury, NY, USA). The sample was centrifuged in a micro-centrifuge (Model microfuge E, Beckman Instruments, Palo Alto, CA, USA) at approximately 2000 *g* for 5 min and the supernatant was transferred to an autosampler vial. A portion of the extract (25–130 μl) was injected into the HPLC system for analysis.

2.5. High-performance liquid chromatography

The HPLC system consisted of a 665A-12 high pressure gradient semi-micro solvent delivery system (Hitachi Instruments, Danbury, CT, USA), an autoinjector (WISP, Model 710B; Waters Assoc., Millford, MA, USA), a 1 ml post-column reaction coil (ABI Analytical, Ramsey, NJ, USA) and a FL-750B fluorescence detector (McPherson, Acton, MA, USA). Chromatographic separations were carried out on a 15 cm × 2.1 mm I.D. Zorbax Rx 5 μm octyl silica column (Mac Mod Analytical, Chadds Ford, PA, USA) which was preceded by a 3 cm × 2.1 mm I.D. C_8 guard column (ABI Analytical). The column was maintained at 40°C with a column heater (Model 725-1010, Rainin Instruments, Woburn, MA, USA). Standard HPLC tubing (0.007 in. I.D. × 1/8 in. O.D.) was used to connect the various components of the HPLC system. The isocratic mobile phase was composed of 30 m*M* MCA buffer with 10 m*M* octane sulfonate, pH 3.2, and methanol mixed on-line at a ratio of 80:20 (v/v) and pumped at 300 μl/min. An additional pump (Model 114, Beckman) was utilized to deliver the OPA reagent solution at a flow-rate of 100 μl/min to the post column reaction coil (1 ml), where it was mixed with the column effluent utilizing a low dead-volume 10 μl Visco Mixer (The Lee Company, Westbrook, CT, USA). The resulting fluorescent reaction product was detected with the fluorome-

ter. The fluorescence detector was equipped with a High Sensitivity Accessory (HSA) and utilized a 200 W xenon–mercury lamp. The xenon–mercury lamp allowed optimum signal-to-noise (S/N) ratio via use of the lamp emission maxima instead of compound extinction maxima. Since the mercury line emission maximum spans a relatively narrow range which is within the range where the fluorescence of the peptide derivative is excited, an increase in sensitivity for **I** was observed. The excitation wavelength was set approximately 336 nm (the mercury emission line may vary slightly depending on the lamp). A band pass filter (UT-1), which transmits the light between 250 and 400 nm, was also installed on the excitation side of the monochromator. The fluorescence emission was monitored at HSA utilizing an 400 nm cut-off filter. An automated laboratory system (PE/Nelson Access Chrom, Version 1.8, Cupertino, CA, USA) was used for data acquisition and processing. Chromatographic peak height data were collected and used for the generation of standard curves.

2.6. Validation procedures

Four pools of plasma precision samples containing 20, 40, 400 and 4000 ng/ml of **I** were prepared by adding appropriate volumes of standard solutions to drug-free dog plasma. These plasma samples were stored at − 80°C until analysis was performed. Six replicate samples from each pool were extracted and analyzed on three separate days. Concentrations were determined by comparison with a calibration curve prepared on the day of analysis. From the data obtained, intra-day precision (determined as the mean of the daily relative standard deviations, R.S.D.s), inter-day precision (determined as the R.S.D. of the daily means) and mean accuracy were calculated.

2.7. Absolute recovery

Known amounts of **I** and the internal standard were dissolved in drug-free plasma samples (n = 6) and processed according to the methods described earlier. In order to estimate recovery, the peak heights observed were compared with those obtained by direct injection in triplicate of known amounts of the two compounds equivalent to 100% recovery. Recovery of **I** and internal standard was expressed as a percent value relative to the peak height observed following direct injection.

3. Results and discussion

The need to measure endogenous bioactive peptides and their synthetic analogs in biological fluids requires a highly sensitive and specific analytical methodology. The application of HPLC to such a problem typically requires chemical derivatization in order to improve the native detectability of the peptide analyte. For many peptide analytes containing an α- or ϵ-amino group, derivatization with one of the many fluorescent reagents available for primary amines is an attractive approach to high sensitivity detection by HPLC. Of the several fluorescent reagents [4–7] available for the derivatization of primary amines, only *o*-phthaldialdehyde (OPA) reacted readily with ϵ-amino group of the lysine moiety of **I**, in presence of a thiol and base, and yielded a highly fluorescent substituted isoindole product. Since pre-column derivatization of **I** with OPA–thiol was precluded due to presence of multiple sites of reaction and limited stability of the peptide in base, the reaction was carried out in the post-column mode following chromatographic separation on a reversed-phase column.

In order to optimize the reaction conditions for the 2.0 mm I.D. reversed-phase columns used here, several post-column reaction parameters were examined using **I** as a substrate. The post-column reaction conditions were optimized for reagent flow-rate, base concentration, concentration of OPA and thiol, and reaction time by injecting 100 ng of **I** onto the column and monitoring the intensity of the fluorescence signal obtained. Results from these experiments led to the development of a post-column reagent that contains 8 mg of OPA and 160 mg of thiofluor in 1 l of 0.05 *M* sodium hydroxide

solution. Use of sodium hydroxide in place of the traditionally used borate buffer to prepare the OPA–thiol reagent solution not only improved the sensitivity of the method by reducing the volume of post-column reagent needed but also reduced the fluorescence background and noise. Optimum results were obtained when this OPA–thiol reagent was pumped at a flow-rate of 100 μl/min and the reaction was allowed to take place for 2.5 min in a 1 ml reaction coil at room temperature. Fig. 2 displays a chromatogram of a standard solution of **I** and the internal standard (IS). Application of this methodology provided a sensitive assay to determine **I** concentrations in dog and rat plasma samples. The assay involved anion-exchange solid-phase extraction of the peptide from deproteinated plasma, as a preliminary isolation step, followed by ion-pair chromatographic separation on a reversed-phase column and post-column reaction detection.

3.1. Recovery and stability

Due to presence of several carboxyl groups in the peptide chains, both **I** and the internal standard are well retained on a strong anion-exchange solid phase extraction column and provide a highly selective method of isolation of these analytes from the acidified and deproteinated plasma. Deproteination of plasma with acetonitrile is essential in order to retain the peptide analytes on SAX column. Use of other deproteinating agents such as trichloroacetic acid in place of acetonitrile significantly reduced the recovery of these peptides. Following the application of a deproteinated plasma sample onto the SAX column, the analytes are eluted with a minimum volume of dilute aqueous (0.1 *M*) citric acid. The recovery of **I** from dog plasma at 20 and 4000 ng/ml was 50.0 ± 5.94 and 56.6 ± 1.45% (mean ± S.D.), respectively. The recovery of the internal standard from plasma at a concentration of 250 ng/ml was 72.6 ± 3.28% (mean ± S.D.). Elution of the peptide analytes from the SAX column with excess 0.1 *M* methanolic citric acid (1 ml) followed by removal of methanol by evaporation, greatly decreased the recovery of these peptides. Although the recovery of **I** from the SAX column was improved through the use of higher molar concentrations of aqueous citric acid (0.5 to 1.0 *M*), the analytical column life was greatly reduced when these extracts were injected onto the column during routine use. Both **I** and the internal standard were stable in the final extract for at least 48 h.

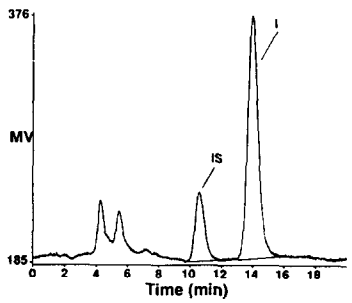


Fig. 2. Chromatogram of aqueous standard solution of **I** and IS. 100 ng of **I** and 25 ng of IS injected on column. Chromatographic conditions: column, 150 × 2.1 mm Zorbax Rx C_8 silica maintained at 40°C; mobile phase, 30 m*M* MCA buffer with 10 m*M* octane sulfonate, pH 3.2 and methanol (80:20, v/v); flow-rate, 300 μl/min; injection volume, 130 μl.

3.2. Sensitivity, linearity and selectivity

By utilizing a 2.1 mm I.D. HPLC column, the on-column limit of detection of **I** (signal-to-noise ratio 3) was 1 ng. The limit of detection and quantification for **I** in 0.25 ml plasma samples was 10 and 20 ng/ml, respectively. The calibration curves were linear over the range of 20–4000 ng/ml of **I**. Based on the analysis of drug-free plasma samples, endogenous plasma components did not interfere with the drug and the internal standard over the concentration range described here. Weighted ($1/y$) linear regression analysis of calibration curves provided the equation $y = 0.00093\,x + 0.001443$ and a correlation coefficient greater than 0.99. The calibration curves were highly reproducible. The precision, as measured by the relative standard deviations at each of the spiked concentrations, and accuracy, evaluated by the average concentration back-calculated from the respective standard curves, are shown in Table 1.

Table 1
Back calculated standard curve concentrations for **I**

	Nominal concentrations of **I** in plasma (ng/ml)							
	20	40	100	200	400	1000	2000	4000
Day 1	17.31	53.72	93.38	195.75	391.89	947.02	2079.28	4199.90
Day 2	22.75	42.67	94.07	192.37	394.20	967.95	1952.82	4099.56
Day 3	23.38	38.37	105.13	189.91	370.07	876.97	2037.38	4146.24
Mean	21.15	44.92	97.53	192.68	385.39	930.65	2023.16	4148.57
S.D.	3.34	7.92	6.59	2.93	13.32	47.65	64.42	50.21
R.S.D. (%)	15.79	17.60	6.76	1.52	3.46	5.12	3.18	1.21
Accuracy	105.75	112.30	97.53	96.34	96.35	93.07	101.16	103.71

3.3. Accuracy and precision

Table 2 summarizes the results obtained from a three-day dog plasma validation study in which six replicate spiked standards at four concentrations, 20, 40, 400 and 4000 ng/ml, were analyzed by this methodology. The mean accuracy of the assay at these concentrations ranged from 91.61 to 106.95%, whereas the intra-day precision, indicated by the mean of the daily R.S.D.s, ranged from 4.46 to 17.72%. The inter-day precision, indicated by the R.S.D.s of the daily means, ranged from 5.3 to 14.44%. The inter-day R.S.D.s of the method were also calculated by analyzing three pools of quality control dog plasma samples spiked with 40, 400 and 4000 ng/ml of **I** over period of 10 days. The inter-day R.S.D.s from the analysis of these samples (n = 19) were found to be 8.04, 12.26 and 10.14%, respectively. A one-day validation of the method was also carried out in rat plasma in order to check the suitability of this methodology for the analysis of rat plasma samples. At **I** concentrations of 20, 40, 400 and 4000 ng/ml of plasma,

Table 2
Accuracy and precision data for **I** in dog plasma

Parameter	Nominal concentrations of **I** in plasma (ng/ml)			
	20	40	400	4000
R.S.D				
Day 1	17.25	18.19	3.92	3.02
Day 2	15.45	7.43	1.98	3.25
Day 3	20.47	6.19	13.05	7.09
Error (%) [a]				
Day 1	+2.30	+2.41	−18.87	−6.27
Day 2	+1.27	+5.03	+4.07	+18.51
Day 3	−7.24	+13.42	−10.36	−7.49
Inter-day R.S.D. [b]	5.30	5.38	12.66	14.44
Intra-day R.S.D. [c]	17.72	10.60	6.32	4.46
Mean accuracy (%)	98.78	106.95	91.61	101.58

[a] (Calculated concentration − actual concentration)/actual concentration × 100.
[b] Coefficients of variation of daily means.
[c] Mean of the daily R.S.D.s.

the intra-day R.S.D.s were found to be 13.75, 8.11, 4.35 and 6.01%, respectively. The accuracy of the method in this study was found to be 101.3, 109.2, 97.3 and 94.3%, respectively.

3.4. Application of the procedure to plasma samples

The quantitative HPLC methodology described here provides a selective and sensitive detection of **I** in dog and rat plasma samples. A typical chromatogram of a plasma extract obtained from drug free dog plasma and a plasma sample spiked with 100 ng/ml of **I** is shown in Fig. 3. The chromatography was highly reproducible and provided a retention time for **I** and IS of 11.5 and 14.5 min, respectively. To date the method has been used successfully in the analysis of plasma samples from pre-clinical studies in dogs and rats. In conclusion, a sensitive and selective high-performance liquid chromatographic method has been developed for the determination of **I** in dog and rat plasma samples. The assay performed acceptably in a three-day validation over a concentration range of 20 to 4000 ng/ml, sufficiently accurate and precise to support toxicokinetic studies for **I** in dogs and rats.

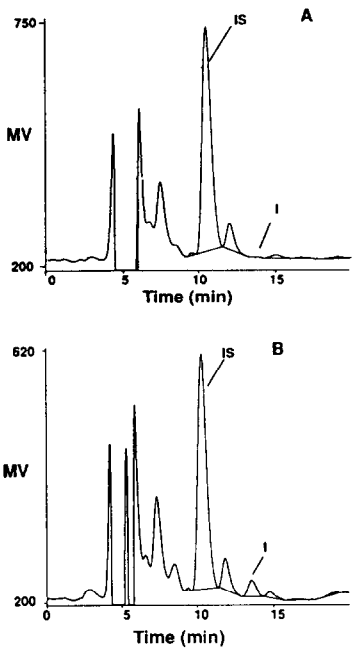


Fig. 3. Chromatograms of plasma extracts from dog plasma spiked with 250 ng/ml of IS (A) and plasma sample spiked with 250 ng/ml of IS and 100 ng/ml of **I** (B). See Fig. 2 for chromatographic conditions. Injection volume, 130 μl.

4. References

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