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High-performance liquid chromatographic determination of the N-ethyl tricarbamate ester pro-drug of fenoldopam utilizing simultaneous post-column hydrolysis and fluorescence derivatization

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Abstract

A high-performance liquid chromatographic (HPLC) method was developed for the determination of SK&F 105058 (I), the N-ethyl tricarbamate ester pro-drug of fenoldopam, in dog plasma. Fenoldopam is a selective agonist of peripheral dopaminergic (DA-1) receptors and has been shown to improve blood flow and lower blood pressure. The method involves isolation of I and the internal standard (I.S.) from plasma by solid-phase extraction prior to chromatographic separation on an octyl silica column. Following chromatographic separation, the carbamate esters of I and I.S. were simultaneously hydrolyzed and the liberated alkylamines were derivatized, by mixing the column effluent with an alkaline solution of *o*-phthaldialdehyde and a thiol, to generate a highly fluorescent isoindole product which was subsequently detected with a fluorometer. Optimization of chromatographic and post-column reaction conditions resulted in an on-column detection limit of 0.4 ng. The recoveries for I and I.S. from plasma were 80.0 ± 5.0% and 62.0 ± 4.0%, respectively. The limit of quantification for I using 0.2 ml of plasma was 5 ng/ml. Linear response was observed for concentrations of I ranging from 5 to 2000 ng/ml plasma. The method was suitably specific and sensitive for pharmacokinetic and metabolic studies of I in dogs. The methodology developed should be generally applicable to the determination of carbamate-type pro-drugs in biological media.

1. Introduction

Fenoldopam [SK&F 82526; 6-chloro-2,3,4,5-tetrahydro - 1 - (4 - hydroxy - phenyl) - 1*H* - 3 - benzazepine-7,8-diol; Fig. 1], a potent agonist of peripheral dopamine-1 (DA-1) receptors, has been shown to improve blood flow resulting in reduction in blood pressure following its intravenous and oral administration [1–3]. It undergoes extensive first-pass metabolism following oral administration and consequently pro-drugs of fenoldopam were investigated in order to circumvent the low bioavailability observed. Of the several carbamate esters of fenoldopam that were examined, the N-ethyl tricarbamate ester (I, SK&F 105058) was chosen for further evaluation as a potential pro-drug candidate [4].

In order to assess the overall pharmacokinetic

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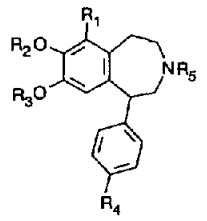

	R_1	R_2	R_3	R_4	R_5
Fenoldopam	Cl	H	H	OH	H
SK&F 105058 (I)	Cl	$CONHC_2H_5$	$CONHC_2H_5$	$OCONHC_2H_5$	H
SK&F 101167 (II)	H	SO_3H	H	H	H
IS	H	SO_3H	$CONHC_3H_7$	H	$CONHC_3H_7$

Fig. 1. Structures of fenoldopam, I and I.S.

and pharmacodynamic relationship between I and fenoldopam, sensitive analytical methods were required to quantitate both I and fenoldopam in plasma. Although HPLC methods [5–9] with electrochemical detection were described for fenoldopam and its metabolites, these methods could not be adopted for the quantitation of I since the catechol group required for electrochemical detection was blocked by carbamate moieties. However, the carbamate moieties provided an alternate method of detection for I, due to ease of generation of primary alkylamine groups in a post-column mode, which could be subsequently reacted with an amine specific derivatization reagent [10].

This report describes a specific and sensitive HPLC method for the detection of I in biological fluids. The approach involves isolation of I and the internal standard from plasma, by solid-phase extraction, prior to quantitative analysis by HPLC with a selective post-column reaction, followed by on-line fluorescence detection. The selective detection of I is achieved by post-column base hydrolysis of its carbamate esters to liberate ethyl amine, followed by reaction with *o*-phthaldialdehyde and 3-mercaptopropionic acid to form a highly fluorescent substituted isoindole moiety, which can be measured at high sensitivity. This method, although developed for the quantitative measurement of I in plasma, should prove to be a highly general and useful tool for the measurement of carbamate ester pro-drugs in biologic media.

2. Experimental

2.1. Chemicals

Fenoldopam (SK&F 82526), SK&F 105058 (I) and SK&F 101167 (II, 1-phenyl-8-hydroxy-2,3,4,5-tetrahydro-1*H*-3-benzazepine-7-yl-hydrogen sulfate; Fig. 1) were prepared in the Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals (Swedeland, PA, USA). HPLC-grade water (Millipore Corporation, Bedford, MA, USA) was used in the preparation of reagent solutions, buffers and mobile phase. Propyl isocyanate (99%) was purchased from Aldrich (Milwaukee, WI, USA). Glacial acetic acid and monochloroacetic acid were purchased from Mallinckrodt (Paris, KY, USA). Disodium ethylenediamine tetraacetic acid (disodium EDTA, 99.5%) was purchased from EM Science (Cherry Hill, NJ, USA). *o*-Phthaldialdehyde (OPA) was obtained from Pickering Laboratories (Mountain View, CA, USA). 3-Mercaptopropionic acid was obtained from Sigma (St. Louis, MO, USA). Pyridine was obtained from Pierce (Rockford, IL, USA). Ascorbic acid was purchased from Calbiochem (LaJolla, CA, USA). Sodium hydroxide, sodium acetate and HPLC grade methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). C_{18} Solid-phase extraction columns (1 ml) and the Vac-Elut manifold were purchased from Varian Sample Preparation Products (Harbor City, CA, USA).

2.2. Reagents

o-Phthaldialdehyde (OPA) reagent solution was prepared by first dissolving sodium hydroxide (2 g) in 1 l of degassed HPLC-grade water and the solution was sonicated for approximately 10 min. To this alkaline solution, 2 ml of a freshly prepared methanolic solution of *o*-phthaldialdehyde (OPA, 2 mg/ml) and 80 μl of 3-mercaptopropionic acid were added and the contents were mixed by swirling. The solution was filtered through a 0.45-μm filter (type HA, Millipore) and sparged continuously with helium

during its use as a post-column reagent. The reagent was stable for 48 h at room temperature.

2.3. Synthesis of propyl carbamate ester of II

The propyl carbamate ester of II, used as the internal standard in this report was synthesized according to the method described earlier [10]. Briefly, II (10 mg) was dissolved in 1 ml of anhydrous pyridine and propyl isocyanate (1 ml) was added. The sample was vortex-mixed, sealed and placed in a heating block and maintained at 50°C for 1 h. The solvents were evaporated at 45°C under nitrogen. The residue was first dissolved in 100 μl of 50% aqueous methanol and then diluted with water (1 ml). A C_{18} extraction cartridge (100 mg, 1 ml) was conditioned by successive washings with 1 ml of methanol and 1 ml of water. The dilute methanolic solution, containing the crude carbamate derivative of II, was then applied onto the cartridge and vacuum was applied. The column was washed with 1 ml of water followed by 1 ml of 10% aqueous methanol. The carbamate ester of II was then eluted from the column with 2 ml of 30% aqueous methanol into a borosilicate tube. Glacial acetic acid (10 μl) was added to the eluate and the eluate stored at 4°C. To confirm the formation of the propyl carbamate ester of II, the derivative was subjected to continuous-flow fast-atom bombardment (CF-FAB) mass spectral analysis. The presence of molecular ion ($[M-H]^-$ at m/z 504) as the base peak in the negative ion CF-FAB mass spectrum indicated that both the hydroxy and secondary amine moieties were derivatized by propylisocyanate forming a propyl carbamate ester with the hydroxy group and an propyl urea group with the secondary amine group.

2.4. Stock standard solutions and reagents

The stock standard solution of I was prepared by dissolving an appropriate amount of I in methanol to give a final solution concentration of 1 mg/ml. The stock solution was stable for 2 months when stored at 4°C. Appropriate dilutions of the stock solution of I were made with methanol–0.1 *M* monochloroacetate (pH 4.0) (1:1, v/v) solution to generate a series of working standard solutions (10, 1 and 0.1 μg/ml). The working internal standard solution of I.S. was prepared by diluting the eluate containing the carbamate derivative of II, obtained from the above mentioned procedure, 1:100 with 50% methanolic solution of 0.05 *M* acetic acid (0.92 μg/ml). The working standard solutions of I and I.S. were stable for 1 month when stored at 4°C. Freshly prepared ascorbic acid solution in water was added to plasma to give a final concentration of 0.5% (w/v) in order to protect fenoldopam from decomposition. Samples were protected from light.

2.5. Calibration

A set of plasma calibration standards (concentrations of I: 5, 10, 20, 50, 100, 200, 500, 1000, 2000 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 0.2 ml of plasma. The peak-height ratios of I to the internal standard were weighted by $1/y$ (based on analysis of residual plots) and plotted against concentrations of I. Linear regression analysis gave calibration curves that were used to calculate the concentrations of I in spiked control or unknown plasma samples.

2.6. Extraction of I from plasma

An aliquot of heparinized plasma (0.2 ml, preserved with 20 μl of 5% ascorbic acid), containing I as standard or as an unknown, was mixed with 25 μl of internal standard solution (I.S., 10 μg/ml) and 1.0 ml of 0.1 *M* sodium acetate buffer (pH 5.0). A C_{18} column was conditioned by successive washings with 1 ml of methanol and 1 ml of water. The plasma sample was transferred to the column and then vacuum was applied. The column was washed with 2 ml of 0.01 *M* sodium acetate (pH 5.0). The analytes were then eluted from the cartridge with 1 ml of 0.3 *M* methanolic acetic acid solution into a 75 × 12 mm borosilicate tube. The eluate was

evaporated under a gentle stream of nitrogen at 35°C and the residue was reconstituted in 150 μl of methanol–0.05 *M* acetic acid (50:50, v/v) containing 0.5% ascorbic acid and transferred to an autosampler vial. The sample was centrifuged at 1300 *g* for 3 min and 5–50 μl was injected onto the HPLC system for analysis.

2.7. High-performance liquid chromatography

The HPLC system (Fig. 2) consisted of a Hitachi 665A-12 high-pressure gradient semi-micro solvent delivery system (Hitachi Instruments, Danbury, CT, USA), an autoinjector (WISP, Model 710B; Waters Assoc., Milford, MA, USA), a post column reactor module (PCRS Model 520, ABI Analytical, Ramsey, NJ, USA) and a McPherson fluorescence detector (Model FL-750B, McPherson, Acton, MA, USA). Chromatographic separation was carried out on a 25 cm × 2.0 mm I.D., 5 μm octyl silica column (Ultrasphere, Beckman Instruments, Palo Alto, CA, USA), maintained at 35°C (Rainin Instruments, Woburn, MA, USA). The isocratic mobile phase was 50 m*M* sodium acetate (pH 6.0)–methanol mixed on-line at a ratio of 53:47 (v/v) and pumped at a flow-rate of 300 μl/min. Mobile phase eluents were filtered through a 0.2 μm Nylon-66 filter and degassed before use. The post-column reactor module contained two independently heated zones which were used, in this case, as a column heating chamber and a reaction coil heating block. One additional pump (Model 114, Beckman Instruments) was utilized to deliver the OPA reagent solution at a flow-rate of 200 μl/min, to the post-column reactor (2 ml coil, 90°C) where it

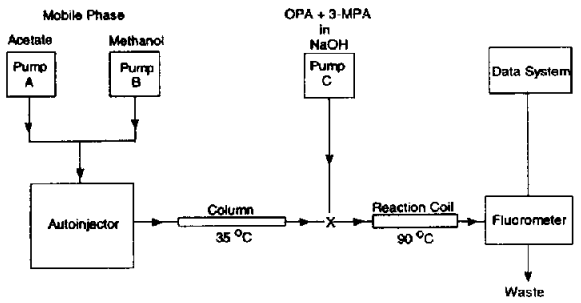

Fig. 2. Schematic diagram of the chromatographic system.

was mixed with the column effluent utilizing a low dead volume (10 μl) mixer (The Lee Company, Westbrook, CT, USA). Following formation of the fluorescent reaction product, detection was accomplished utilizing a McPherson fluorescence detector equipped with a high sensitivity accessory (HSA) and 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 was within the range where the fluorescence of the OPA/thiol derivative of I was excited, an increase in sensitivity for I was observed. The excitation wavelength was set at approximately 336 nm (the mercury emission line may vary slightly depending on the lamp). The fluorescence emission was monitored in the HSA utilizing a 400 nm cut-off filter. An automated laboratory system (PE/Nelson Access Chrom, Version 1.6, Cupertino, CA, USA) was used for data acquisition and processing. Chromatographic peak-height data were collected and used for the generation of calibration curves.

3. Results and discussion

In order to optimize the reaction conditions for the 2.0-mm I.D. reversed-phase column 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, reaction temperature and reaction time, by repeated injections of 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 an optimized post-column reagent that contained 4 mg of OPA and 80 μl of thiol in 1 l of 0.05 *M* sodium hydroxide solution. Use of a single-stage reaction system [11,12], in which OPA/thiol was added to dilute sodium hydroxide solution for simultaneous hydrolysis and derivatization, not only improved the sensitivity of the method, by reducing the volume of post-

column reagent, but also reduced the fluorescence background and noise. Optimum results were obtained when the OPA/thiol reagent was pumped at a flow-rate of 200 μl/min with a reaction time of 4.0 min (2 ml reaction coil) at 90°C. Application of this methodology provided a sensitive assay to determine concentrations of I in dog plasma samples.

3.1. Recovery and stability

Known amounts of I and I.S. were dissolved in drug-free plasma samples ($n = 8$) 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 of known amounts of the two compounds equivalent to 100% recovery. Recoveries of I and I.S. were expressed as a percent value relative to the peak height observed following direct injection. Across the linear range, a mean plasma recovery of 80.0 ± 4.8% (mean ± S.D.) was obtained for the pro-drug. The recovery of I.S. from plasma, determined at an approximate concentration of 115 ng/ml, was 62.0 ± 3.9% (mean ± S.D.). In addition, the pro-drug 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 could be performed on the next day without observable quantitative changes.

3.2. Sensitivity, linearity and selectivity

By utilizing a 2.0-mm I.D. HPLC column, the on-column limit of detection of I (signal-to-noise ratio 3) was 0.4 ng. The limits of detection and quantification for I in 0.2 ml plasma samples were 2 and 5 ng/ml, respectively. Calibration curves were linear over the range of 5–2000 ng/ml of I. Based on the analysis of drug-free plasma samples, endogenous plasma components did not interfere with the drug or internal standard over the concentration range described here. Weighted ($1/y$) linear regression analysis of calibration curves provided a correlation coefficient greater than 0.999. The precision, as measured by the relative standard deviations (R.S.D.) at each of the spiked concentrations is shown in Table 1.

3.3. Accuracy and precision

Three pools of plasma precision samples containing 10, 100 and 1000 ng/ml of I were prepared by adding appropriate volumes of standard solutions to drug-free dog plasma preserved with 0.5% ascorbic acid. These plasma samples were stored at −80°C until analysis was performed. Five replicate samples from each pool were extracted and analyzed on four separate days.

Table 1
Back-calculated standard curve concentrations for I

Standard (ng/ml)	Day 1	Day 2	Day 3	Day 4	Mean	S.D.	R.S.D.
5.0	5.78	6.55	4.83	5.33	5.72	0.86	15.1
10.0	10.07	11.46	10.75	11.22	10.87	0.61	5.6
20.0	20.41	20.04	22.30	20.09	20.71	1.07	5.2
50.0	48.87	48.65	53.87	50.35	50.43	2.41	4.8
100.0	89.37	103.03	100.47	92.05	96.23	6.55	6.8
200.0	186.93	199.53	213.79	173.96	193.55	17.06	8.8
500.0	502.90	551.28	606.25	499.42	539.96	50.13	9.3
1000.0	1023.58	1004.78	1195.93	1141.75	1091.51	92.31	8.5
2000.0	NS	1957.84	1832.15	2038.11	1942.70	103.81	5.3

NS = no sample.

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 (Table 2). The mean accuracy of the assay at these concentrations ranged from 83.6 to 99.2%, whereas the intra-day precision ranged from 5.70 to 8.25%. The inter-day precision ranged from 2.82 to 4.60%.

3.4. Application of the procedure to plasma samples

The quantitative HPLC methodology described here provided selective and sensitive detection of I in dog plasma samples. Typical chromatograms of a plasma extract obtained from drug-free dog plasma and plasma samples spiked with 5 ng/ml and 100 ng/ml of I are shown in Fig. 3. Chromatography was highly reproducible and gave retention times for I and I.S. of 13.2 and 23.2 min, respectively.

In conclusion, a sensitive and selective high-performance liquid chromatographic method has been developed for the determination of the

Table 2
Accuracy and precision data for I in dog plasma

Parameter	Nominal concentrations of I in plasma (ng/ml)		
	10	100	1000
R.S.D			
Day 1	8.0	4.6	8.5
Day 2	2.1	5.9	12.2
Day 3	7.3	8.3	3.8
Day 4	9.3	4.0	8.5
Error (%)[a]			
Day 1	−5.7	−14.4	+4.2
Day 2	−4.9	−16.3	−6.2
Day 3	−13.5	−15.1	+1.5
Day 4	−9.9	−19.7	−2.8
Inter-day R.S.D.[b]	4.36	2.82	4.6
Intra-day R.S.D.[c]	6.68	5.70	8.25
Mean accuracy (%)	91.5	83.6	99.2

[a] [(Calculated concentration − actual concentration)/actual concentration] · 100.
[b] Relative standard deviation of daily means.
[c] Mean of the daily R.S.D.s.

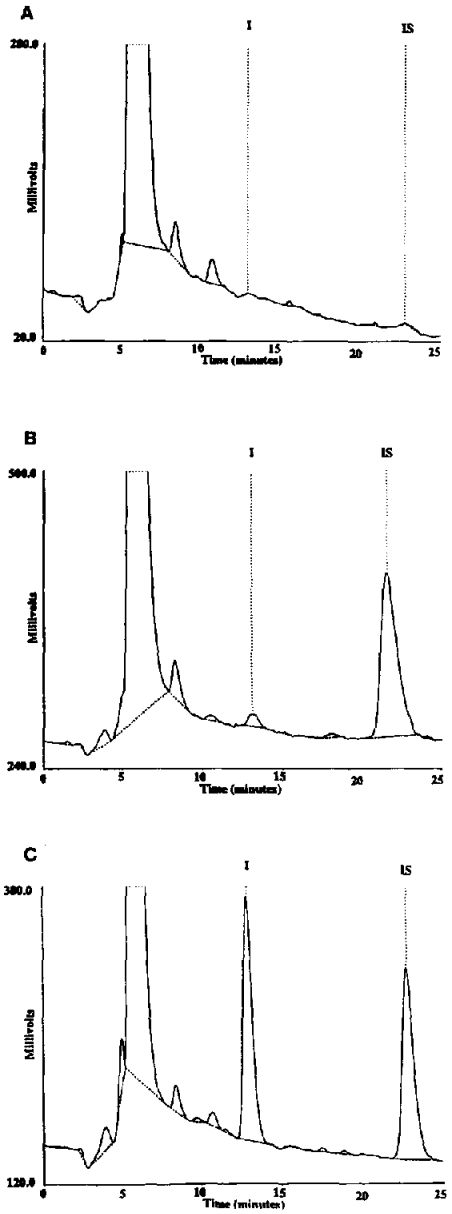

Fig. 3. HPLC chromatograms of extracts of drug-free plasma (A), plasma spiked with 5 ng /ml plasma of I (B), and plasma spiked with 100 ng /ml plasma of I (C).

carbamate pro-drug of fenoldopam in dog plasma samples. The assay performed acceptably over a concentration range of 5 to 2000 ng/ml and was sufficiently accurate and precise to support pharmacokinetic studies for I in dogs. The methodology developed should be generally applicable to the determination of carbamate-type pro-drugs in biological fluids.

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