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Liquid chromatographic assay for a butenolide endothelin antagonist (PD 156707) in plasma

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Abstract

A sensitive and selective liquid chromatographic assay for determining the non-peptide endothelin A receptor antagonist PD 156707 (I) in rat plasma has been developed and validated. The analyte was isolated from matrix by solid-phase extraction. Liquid chromatographic separation was achieved isocratically on a 3.2 mm I.D., ODS column with a mobile phase of acetonitrile–ammonium phosphate (50 m*M*, pH 3.5) (44:56, v/v). Column effluent was monitored fluorometrically. Peak-height ratios (analyte/IS) were proportional to I concentrations in rat plasma from 25 to 1000 ng/ml. Assay precision and accuracy for I, based on quality controls, was 9.5% relative standard deviation, with relative error of ±6.5%. The quantitation limit was 25 ng/ml for a 200-μl sample aliquot.

Keywords: Butenolide; Endothelin antagonist

1. Introduction

Interest in the pathophysiology of endothelin, a potent vasoconstrictor, is increasing as an understanding of its involvement in several human disease states is gained [1]. Butenolides have recently attracted attention as a series of low molecular weight, non-peptide antagonists for the endothelin receptor. The synthesis, receptor binding and preliminary non-clinical pharmacokinetics for selected butenolide analogs have been described recently [2]. Analogs of this series are currently being evaluated as possible therapeutic agents in stroke, subarachnoid hemmorrhage (SAH) and other vasoconstrictor related ailments. Butenolides exhibit a characteristic, pH dependent tautomerization between keto-acid and hydroxy-lactone forms [2], which has a unique impact on the analytical approaches used to characterize and quantify them. Information regarding bioanalytical methodology for trace determination of butenolides in biological matrices has been previously unavailable.

This report describes the development and validation of extractions and liquid-chromatographic separations for the trace quantitative determination of two selected butenolides, PD 156707 (I) and PD 158312 internal standard (IS), in rat plasma. The structures for the keto-acid salt forms of these compounds are shown in Fig. 1.

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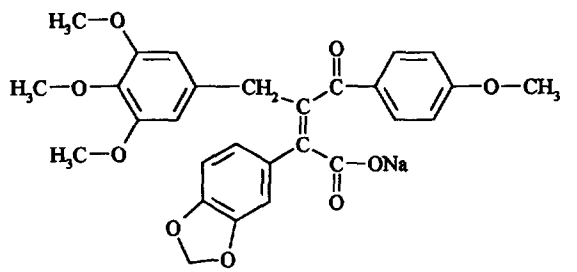
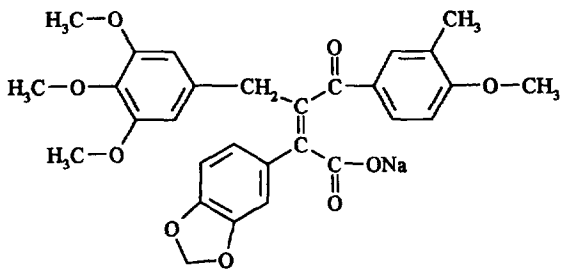


PD 156707



PD 158312

Fig. 1. Structures for drug (I) and internal standard (IS).

2. Experimental

2.1. Reagents and materials

All chemicals were of analytical-reagent grade, unless noted otherwise. Compound I and IS were obtained from Parke-Davis Pharmaceutical Research (Division of Warner-Lambert Company, Ann Arbor, MI USA). All chemical reagents were used without further purification.

Compound I (PD 156707, Lot 2Q, 93.3% purity, molecular mass of free acid is 520.93 g/mol) is chemically described as 2-benzo[1,3]dioxol-5-yl-4-(4-methoxy phenyl)-4-oxo-3-(3,4,5-trimethoxybenzyl)-but-2-enoic acid, sodium salt.

Internal standard (PD 158312, Lot 1P, 99.6% purity, molecular mass of free acid is 550.96 g/mol) is chemically described as 2-benzo[1,3]dioxol-5-yl-4-(4-methoxy-3-methylphenyl)-4-oxo-3-(3,4,5-trimethoxybenzyl)-but-2-enoic acid, sodium salt.

Rat plasma was prepared in house by separating heparinized whole blood collected from male Wistar rats. Several pools of plasma were produced. All rat plasma was centrifuged (1000 *g*, 10 min) before use to remove any sediments.

2.2. Preparation of reference standards and controls

I. Fresh stock solutions were prepared for each batch run. Stock solution I, containing 0.5 mg/ml I free-acid equivalents, was prepared by dissolving the compound in acetonitrile. To prepare stock solution II, stock solution I was volumetrically diluted 10-fold with acetonitrile. These stock solutions were further diluted volumetrically with acetonitrile to prepare working standards for addition to blank plasma. A 20-μl aliquot of each working standard was added to 200 μl of blank plasma and 500 μl of buffer to prepare calibration standards for validation runs. Final calibration standard concentrations were 1000, 500, 250, 100, 50, 37.5, and 25 ng/ml.

IS. A 500 μg/ml (free-acid equivalents) stock solution was prepared for each batch run by dissolving IS in a small volume of methanol and diluting with acetonitrile. This solution was volumetrically diluted 100-fold with acetonitrile to prepare a working IS solution of 5000 ng/ml. Addition of 20-μl aliquots of the working solution to plasma samples gave an IS concentration of 500 ng/ml.

Quality controls

Plasma controls containing 40, 200, and 800 ng/ml I (free-acid equivalents) were prepared by diluting 1.00-ml aliquots of 1, 5, and 20 μg/ml aqueous stock solutions (prepared by a weighing of I which was independent of those for the standards) to 25.0 ml with centrifuged (1000 *g* for 10 min) blank rat plasma. These controls were subdivided into 500- or 700-μl aliquots and were stored in polypropylene tubes at −20°C.

2.3. Apparatus and conditions

Chromatography

The reversed-phase HPLC system consisted of a solvent delivery system (Model 410, Perkin-Elmer, Norwalk, CT, USA), autosampler (Model ISS 100, Perkin-Elmer), fluorescence detector (Model LC 240, Perkin-Elmer), data system (Model A/D 970 Series Interface, TurboChrom, PE Nelson, Norwalk, CT, USA, version 3.2), column (5 μm particle size, C_{18},

Spherex, 3.2 × 150 mm, Phenomenex, Torrence, CA, USA), and guard column (New Guard, 5 μm particle size, C_{18}, 3.2 × 10 mm, Brownlee, Santa Clara, CA, USA).

Mobile-phase composition was acetonitrile–ammonium phosphate buffer (50 m*M*, pH 3.5) (44:56, v/v) at a flow-rate of 0.5 ml/min. Fluorescence detection was at 312 nm (excitation) and 430 nm (emission, cut-off filter) with a response factor of 2.8 s and an attenuation of 256.

Solid-phase extraction (SPE) was performed using phenyl bonded phase (1 ml/100 mg, Bond Elut, Varian Sample Preparation, Harbor City, CA, USA) and a custom-made 144-port SPE Vacuum manifold (Parke-Davis Pharmaceutical Research, Ann Arbor, MI, USA). Extract evaporation was performed under nitrogen at 40°C (Turbovap, Zymark, Hopington, MA, USA).

2.4. Assay procedure

Into clean 12 × 75 mm glass test tubes, 200 μl of heparinized rat plasma (sample, control or standard), 500 μl of buffer (pH 3.0, 0.5 *M* potassium phosphate), 20 μl of IS (acetonitrile for blank), and 20 μl of standard (acetonitrile for blanks and controls) were added. All tubes were vortex-mixed for about 10 s. Solid-phase extraction columns were conditioned with 2 × 1 ml of acetonitrile and 2 × 1 ml of water under moderate (50–70 kPa) vacuum. Plasma samples were loaded with plastic transfer pipets and drawn through at 35–50 kPa. The solid-phase cartridges were washed with 2 × 1 ml of water (35 kPa), 2 × 0.75 ml of 40:60 acetonitrile–water (v/v) (35 kPa, then 70 kPa for 1 min) and 2 × 1 ml of hexane (35 kPa, then 70 kPa for 1 min). Analytes were eluted into clean 12 × 75 mm glass test tubes with 0.75 ml of 70:30 acetonitrile–water (v/v). Extracts were evaporated to dryness (~35 min at 40°C) with N_2. Residues were reconstituted with 200 μl of 40:60 acetonitrile–water, vortex-mixed for 10 s and transferred to autosampler vials containing glass inserts. Aliquots of 150 μl were injected into the chromatograph. The chromatographic column was cleaned after each large batch run by flushing with a solution of 80:20 acetonitrile–water (0.5 ml/min, 3–4 h). The guard column demonstrated minimal increase in back pressure after 300 injections.

2.5. Data reduction

The assay was validated from 25 to 1000 ng/ml of I, by assaying seven calibration standards and three quality control samples in triplicate in three separate batch runs. The best-fit line was determined by least-squares linear regression of peak-height ratio (I/IS) versus concentration from each batch run using a weighting factor of 1/concentration. PE-Nelson Turbochrom software (V 3.2) was used for data reduction. Concentrations of I in quality controls and samples were calculated using peak-height ratios and the regression parameters.

3. Results and discussion

3.1. Chromatographic performance and selectivity

Chromatograms, representing the separation of extracted analytes from matrix components, are shown in Fig. 2 for (a) blank rat plasma, (b) 25 ng/ml plasma standard, (c) 200 ng/ml standard and (d) I dosed rat plasma sample. No matrix components eluted at the retention times for I (11.1 min) or IS (15.4 min) in six independent matrix sources. A later eluting component (~30 min retention time) present in the plasma of some Wistar rats was effectively removed by inclusion of a 2 × 1 ml hexane wash prior to elution from the solid-phase cartridges.

The separation efficiency of the two analytes were 4700 and 5700 theoretical plates, for I and IS, respectively, on the 15 cm column. Capacity factors were 7.6 and 10.8, respectively. Asymmetry factors were less than 1.3, indicating only minor chromatographic peak tailing. System selectivity, gauged by R_s, the chromatographic resolution, in standard and sample chromatograms indicated that I was sufficiently well-resolved ($R_s > 3$) from all other sample components. The IS peak was baseline-resolved ($R_s = 1.8$) from small intermittent plasma peaks. Chromatographic performance parameters were calculated as described previously [3,4].

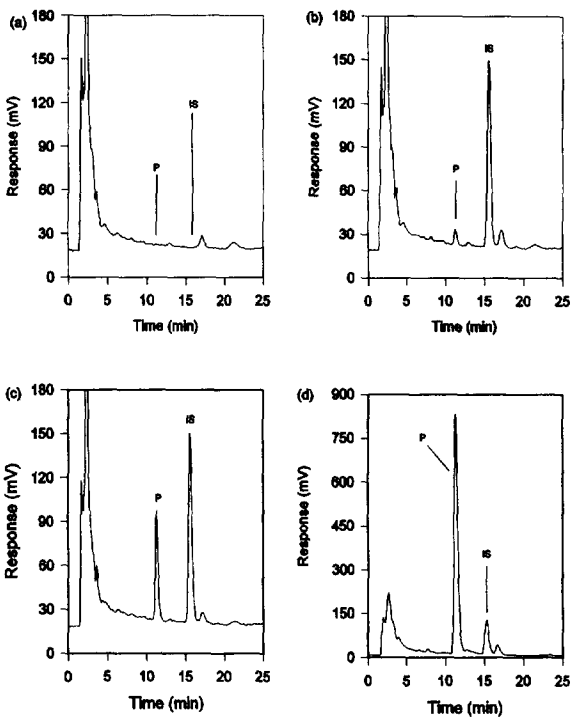


Fig. 2. Representative chromatograms. (a) Rat plasma without I (P) or internal standard (IS); (b) 25 ng/ml calibration standard plus IS in rat plasma; (c) rat plasma quality control, containing 200 ng/ml I plus IS; (d) plasma from a rat 0.50 h after receiving a single 10-mg/kg i.v. dose of I (concentration 9 μg/ml, dilution factor 8).

3.2. Limit of quantitation (LOQ) and limit of detection (LOD)

The LOQ was defined as the lowest concentration on the standard curve [5]. The LOQ in rat plasma, as determined by the intra-run precision of replicate standards, was 25 ng/ml for the parent drug. The inter-day variability at this level was 5.4% ($n = 9$), with relative error of +5.5%.

Other detection techniques were also evaluated. Absorbance detection at maxima 237 and 280 nm gave detection limits of 8 and 21 ng/ml ($S/N = 3$), respectively, from clean solution. Selectivities, however, in conjunction with a variety of solid- and liquid-phase extractions, were clearly unacceptable. Oxidative amperometry produced some response, but gave a detection limit of only 100 ng/ml ($S/N = 3$) at +1.05 V and pH 3.5, and was not pursued. Fluorescence intensity appeared to be insensitive to mobile phase pH. No difference in sensitivity was noted in changing from a mobile phase composition of acetonitrile–water (44:56) to acetonitrile–buffer (pH 3.5) (44:56).

The relative chromatographic response of a 250 ng/ml solution of I as a function of excitation and emission wavelengths was best at 312 nm (excitation) and 430 nm (emission filter). Excitation at 325 nm gave a response of 0.62 relative to excitation at 312 nm. This suggests that sensitivity could be further improved by the use of a laser-induced fluorescence detector, utilizing the 325 nm line of a He/Cd laser for excitation. These results are summarized in Table 1.

3.3. Recovery from rat plasma

The extraction efficiency of I from rat plasma, expressed as percent recovery, was determined by assaying six samples at each of three concentrations using the procedure described above and comparing the results with non-extracted standards run in triplicate. Mean recoveries of I (% RSD) from rat plasma were 109% (7.0), 91.6% (2.7), and 86.3% (2.9) at concentrations of 25.0, 250, and 1000 ng/ml.

Table 1
Relative chromatographic fluorescence response for various excitation and emission wavelengths

Excitation wavelength (nm)	Emission wavelength (nm)	Relative response
237	430[a]	0.82
280	430[a]	0.76
312	430[b]	0.07
312	430[a]	1.00
325	430[a]	0.62

[a]Emission wavelength was selected through use of a cut-off filter.
[b]Emission wavelength was selected through use of a grating.

Mean (% RSD) IS recovery was 75.5% (3.7) at 500 ng/ml. The lower recovery for IS was possibly due to slightly greater affinity for the solid-phase sorbent, relative to I.

Preliminary recovery studies of I with other solid-phase sorbents showed that, at sample pH 3.0, phenyl gave the best recovery (91%), followed by C8 (84%), C2 (82%), C18 (77%) and CH (65%). The relative selectivities of these sorbents were very similar. These extractions were performed without a wash step and elution was with 1.0 ml of acetonitrile.

The solid-phase extraction efficiencies for these butenolides are strongly influenced by the pH of the sample. For rat plasma samples buffered at pH 3.0 (typical assay conditions), recoveries of 92% and 76% were obtained for I and IS, respectively. Under the same extraction conditions, samples buffered at pH 5.0 gave recoveries of 88% and 90%, respectively. At pH 6.5 and at neutral pH, the recoveries of either compound were 0. It is likely that the higher pH causes formation of an open-ring structure containing an acid moiety, which is poorly retained on these sorbents. The closed-ring structure predominates at acidic pH [2] and is better retained by reversed-phase solid-phase extraction.

Table 2 lists absolute recoveries of I and IS from rat plasma using solid-phase extraction on phenyl sorbents with various strengths of wash solvents. The minimum solvent strength required for adequate selectivity approached 40% acetonitrile. Stronger wash solvents gave greater selectivity with diminishing recovery. At 2.0 ml of 55% acetonitrile, negligible recovery was achieved for either analyte. Use of 30% tetrahydrofuran in place of acetonitrile gave acceptable recoveries but did not improve selectivity.

Table 2
Absolute recoveries of I and IS from rat plasma as a function of solid-phase extraction wash solvents

Wash volume (ml)	Acetonitrile[a] (%)	Recovery (%)	
		I	IS
1.5	40	92	76
3.0	40	83	76
2.0	45	55	67
2.0	50	15	32
2.0	55	0	2
3.0	30[b]	85	91

[a] ≤35% acetonitrile gave poor selectivity. Recovery could not be determined.
[b] Tetrahydrofuran was used instead of acetonitrile.

Liquid–liquid extractions of I and IS from acidified (pH 2) rat plasma were also evaluated for three different organic solvents. Using 5:1 ratios of solvent to plasma, recoveries of I and IS, respectively, were 91% and 98% (methyl *tert.*-butyl ether), 16% and 68% (hexane–2% ethanol) and 41% and 86% (cyclohexane–2% ethanol). The methyl *tert.*-butyl ether extraction gave the best selectivity and the best recoveries, but results were inferior to those obtained with solid-phase extraction.

3.4. Linearity, precision, and accuracy of calibration curves

Calibration curves were evaluated by the deviation of individual back-calculated standards from the regression line. RSDs for I calibration standards ($n = 9$) ranged from 1.1% through 9.2% over three batch runs, with REs of −7.6% through 8.0%. Based on the accuracy (% RE) and precision (% RSD) of the replicate calibration standards, the method demonstrated sufficient adherence to a linear model over 25 to 1000 ng/ml. For routine use, samples containing greater than 1000 ng/ml of I were diluted with blank rat plasma and reassayed.

3.5. Intra-run precision and accuracy

Intra-run (within-run) precision and accuracy were determined by assaying quality controls in triplicate, at each of three levels, in three separate batch runs. Intra-run precision estimates for I were less than 5.6%, 9.6%, and 13.8% RSDs at concentrations of 40.0, 200, and 800 ng/ml, respectively. Intra-run relative error for I was ±11.1% (Table 3).

3.6. Inter-run precision and accuracy

Inter-run (between-run) precision and accuracy were determined by pooling all individual assay results of triplicate quality controls over three separate batch runs. Inter-run precision estimates for I were 6.4%, 7.2%, and 9.5% at concentrations of 40.0, 200, and 800 ng/ml, respectively. Inter-run relative error for I was ±6.5% (Table 3).

Table 3
Rat plasma I concentrations in quality controls for three separate batch runs

	Concentration added (ng/ml) (free-acid equivalents)		
	40.0	200	800
Batch run 1			
Intra-run mean (ng/ml)	39.2	218	759
Intra-run SD (ng/ml)	2.20	11.2	83.5
Intra-run RSD (%)	5.6	5.1	11.0
Intra-run RE (%)	−2.0	9.2	−5.1
Batch run 2			
Intra-run mean (ng/ml)	44.4	205	711
Intra-run SD (ng/ml)	0.55	19.7	97.9
Intra-run RSD (%)	1.2	9.6	13.8
Intra-run RE (%)	11.0	2.5	−11.1
Batch run 3			
Intra-run mean (ng/ml)	40.6	199	774
Intra-run SD (ng/ml)	1.25	9.85	14.7
Intra-run RSD (%)	3.1	4.9	1.9
Intra-run RE (%)	1.5	−0.5	−3.3
N	9	9	9
Mean concentration found (ng/ml)	41.4	208	748
Inter-run SD (ng/ml)	2.7	15.0	70.9
Inter-run RSD (%)	6.4	7.2	9.5
Inter-run RE (%)	3.6	3.8	−6.5

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