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Journal of Pharmaceutical and Biomedical Analysis
22 (2000) 251–255

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Validated HPLC method for determination of PAT-5A, an insulin sensitizing agent, in rat plasma☆

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Received 13 May 1999; received in revised form 28 September 1999; accepted 16 October 1999

Abstract

A high performance liquid chromatographic method for the determination of PAT-5A (a potent insulin sensitizer) using DRF-2095 (a thiazolidinedione) as internal standard (I.S.) is described. A 1:1 v/v ethylacetate and dichloromethane solvent mixture was used for extraction of PAT-5A from plasma. A Kromasil KR100-5C18-250A, 5 μm, 4.6 × 250 mm SS column was used for the analysis. Mobile phase consisting of sodium dihydrogen phosphate (pH 4.0, 0.05 M) and methanol mixture (25:75, v/v) was used at a flow rate of 1.0 ml/min. The eluate was monitored using a UV detector set at 345 nm. Ratio of peak area of analyte to I.S. was used for quantification of plasma samples. Using this method the absolute recovery of PAT-5A from rat plasma was > 90% and the limit of quantification was 0.05 μg/ml. The intra-day relative standard deviation (RSD) ranged from 2.19 to 4.98% at 1.0 μg/ml, 1.05 to 3.68% at 10.0 μg/ml and 3.14 to 5.08% at 50 μg/ml. The inter-day RSD were 1.6, 2.24 and 1.54% at 1, 10 and 50 μg/ml, respectively. The method was applied to measure the plasma concentrations of PAT-5A in pharmacokinetic and bioavailability studies in male Wistar rats. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: PAT-5A-HPLC-method validation

1. Introduction

Type 2 diabetes, also known as non insulin dependent diabetes mellitus (NIDDM) is the most prevalent among the metabolic disorders and is characterized by sub-optimal tissue response to insulin [1]. The thiazolidinedione class of compounds are known to possess significant insulin sensitizing activity and offer a novel approach to treat type 2 diabetes since they normalize insulin action on the target tissues without stimulating secretion of insulin from pancreatic β-cells [2–4]. Troglitazone, a thiazolidinedione launched in USA as Rezulin®, has been found to cause hep-

☆ This is publication number 98 from Dr Reddy's Research Foundation.

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PII: S0731-7085(99)00262-9

atic dysfunction [5] and increase in lipoprotein-a levels [6]. This indicates the necessity for developing compounds with a better safety profile. Recently, phase III clinical trials have been completed with BRL 49653 (Rosiglitazone, Avandia®), which is a far more potent insulin sensitizer compared to troglitazone [6]. Our efforts to develop antidiabetic drugs on these lines have shown PAT-5A (Fig. 1), a maleic acid salt of a new thiazolidinedione, to possess potent euglycemic and hypolipidemic effects in animal models of type 2 diabetes [7,8]. The compound is presently in preclinical development.

To conduct the pharmacokinetic and bioavailability evaluation necessary for drug development, a simple and sensitive HPLC assay method with UV detection has been developed for the quantitative determination of PAT-5A in rat plasma using DRF-2095 as an internal standard. The method offers the advantage of simplicity with adequate sensitivity, selectivity, precision and accuracy. This analytical method has been used to evaluate the pharmacokinetics of PAT-5A in male Wistar rats.

2. Experimental

2.1. Materials

PAT-5A and DRF-2095 (I.S.) were synthesized and characterized by the Synthetic and Medicinal Chemistry Laboratory, Dr Reddy's Research Foundation, Hyderabad, India. Methanol, ethylacetate, dichloromethane (HPLC grade) and sodium dihydrogen orthophosphate (Analytical Reagent Grade) were obtained from Qualigens, Glaxo (India), Mumbai, India. MilliQ (Millipore, USA) grade water was used. Heparinised rat control plasma was obtained from Wistar rats (National Institute of Nutrition, Hyderabad, India).

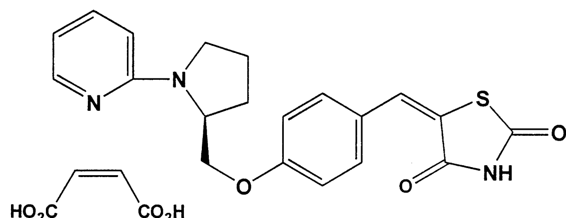

Fig. 1. Structure of PAT-5A (5-[4-[*N*-(20pyridyl)-(2s)-pyrrolidine - 2 - methoxy]phenylmethylene]thiazolidine - 2,4 - dione, maleic acid salt).

2.2. Standard solutions

Stock solutions of 1.0 mg/ml of PAT-5A and DRF-2095 were prepared in methanol and stored at 4°C. Appropriate dilutions of PAT-5A were made in methanol to produce working stock solutions of 100, 10 and 1.0 μg/ml. These dilutions were used to spike plasma in the preparation of calibration curves. I.S. was used directly from the stock solution. Calibration samples were prepared by spiking 100 μl of blank plasma with the appropriate amount of the drug on the day of analysis. Samples for the determination of recovery, precision and accuracy were prepared by spiking control rat plasma in bulk at appropriate concentrations (1, 10 and 50 μg/ml) and stored at −20°C.

2.3. Extraction Procedure

To 100 μl of plasma sample, methanolic solution of DRF-2095 equivalent to 2.5 μg was added and mixed for 10 s using a cyclomixer (Remi Instruments, Mumbai, India). A 2 ml volume of extraction solvent mixture (ethylacetate: dichloromethane; 1:1, v/v) was added. The contents were vortexed for 1 min and centrifuged at 2500 rpm for 10 min on a tabletop centrifuge (Remi Instruments). 1.5 ml of organic layer was separated and evaporated to dryness under nitrogen using an evaporating system (N-evap, Organomation, MA, USA) at 50°C. The residue was reconstituted to 200 μl with mobile phase and 100 μl was injected onto HPLC. Calibration standards were prepared in the range of 0.05 to 100 μg/ml.

2.4. Chromatographic conditions

The HPLC system consisted of a Waters™ LC Module-I, Millennium 2010 software (version 2.15) and a Kromasil KR100-5C18-250A, 5 μm,

4.6 mm × 250 mm column (Hichrom, UK). Mobile phase consisting of NaH_2PO_4 buffer (pH 4.0, 0.05 M) and methanol mixture (25:75, v/v) was pumped at a flow rate of 1.0 ml/min. Eluate was monitored using UV detector set at 345 nm.

2.5. Pharmacokinetic study

The pharmacokinetic study was carried out in male Wistar rats. The animals were fasted overnight (~14 h) and had access to water throughout the experimental period. Animals were given feed 3 h after drug administration. PAT-5A was administered through a gavage at a dose of 10 mg/kg p.o as a 0.25% carboxymethylcellulose suspension. About 0.25 ml of blood sample was collected into heparinised microfuge tubes at different time points (0, 0.5, 1, 2, 3, 5 and 8 h) from the orbital sinus and centrifuged using Biofuge (Hereaus, Germany) at 12 800 rpm for 5 min to separate plasma. Plasma (100 μl) samples were spiked with I.S. and processed as described above. Pharmacokinetic parameters were calculated employing non-compartmental model analysis. The AUC was calculated by trapezoidal rule and t_{max} noted is the time at which maximum concentration (C_{max}) in plasma was achieved. Elimination rate constant (K_{el}) was calculated from slope of the semi-logarithmic plot of plasma concentration versus time. Half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/K_{el}$.

3. Results and discussion

3.1. Chromatography

Typical chromatograms corresponding to blank plasma and plasma sample obtained after oral administration of PAT-5A at a dose of 10 mg/kg to Wistar rats are shown in Fig. 2. No endogenous interfering peaks were visible in blank plasma at the retention times of PAT-5A and the I.S. (Fig. 2(a)) thereby confirming the specificity of the analytical method. Both analyte and the internal standard were well separated with retention times of 5.75 and 8.8 min, respectively (Fig. 2(b)). System suitability parameters for the method were as follows: Theoretical plates for PAT-5A >4200 and for I.S. >3700, Asymmetry factor was <1.5 and resolution between PAT-5A and I.S. was >5.

3.2. Quantification

Peak-area ratios of PAT-5A to the I.S. were measured. A representative calibration graph of peak-area ratio (PAT-5A to I.S.) versus PAT-5A concentration in the range of 0.05–100 (μg/ml resulted in the regression equation $y = 0.4711x + 0.3576$ ($r^2 = 0.999$). The lowest concentration with the relative standard deviation (RSD) $<20\%$ was taken as lower limit of quantitation (LLOQ) [10,11] and was found to be 0.05 μg/ml. The RSD and S/N ratio at LLOQ were found to be 16% and 7, respectively.

3.3. Precision

Precision of the assay was determined by analysing plasma samples containing PAT-5A at three different concentrations. Samples for precision study were obtained by spiking blank plasma with the analyte solution at each concentration in bulk, and 100 μl aliquots were distributed into screw-capped tubes and stored at −20°C. The

Table 1
Intra and inter-day variation of pat-5a in rat plasma

Spiked concentration	Day	Measured concentrations[a]		
		Mean (μg/ml)	S.D.	RSD
Intra-day variation				
1.0 μg/ml	0	0.99	0.03	3.06
	2	0.97	0.02	2.19
	4	0.99	0.05	4.98
10.0 μg/ml	0	10.45	0.11	1.05
	2	10.73	0.40	3.68
	4	10.94	0.30	2.73
50.0 μg/ml	0	49.32	1.55	3.14
	2	49.56	2.52	5.08
	4	50.78	2.03	4.00
Inter-day variation				
1.0 μg/ml		0.98	0.02	1.60
10.0 μg/ml		10.71	0.25	2.24
50.0 μg/ml		49.89	0.78	1.54

[a] Values (mean and S.D.) are for $n = 4$ observations

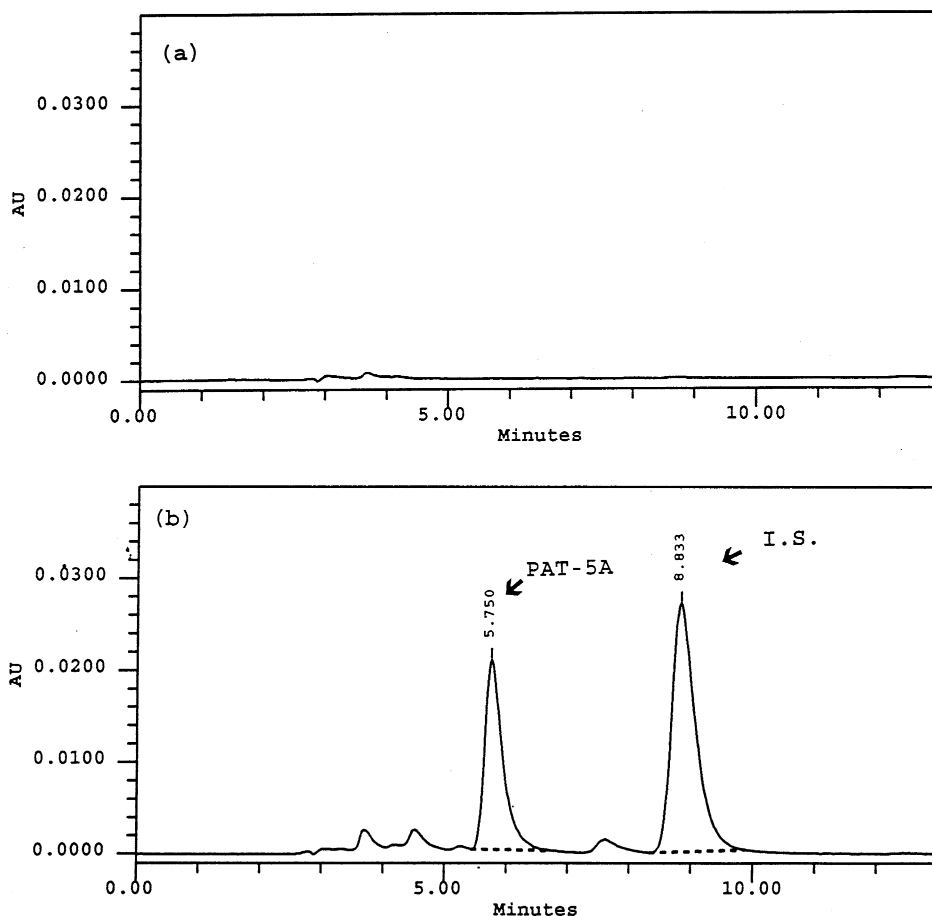

Fig. 2. Typical HPLC chromatograms for analysis of PAT-5A in (a) blank rat plasma and (b) 2 h plasma sample from rat dosed with PAT-5A at 10 mg/kg p.o. The concentration for this sample was found to be 5.52 μg/ml.

intra-day precision was determined by analyzing four spiked plasma samples at each concentration on the same day. For the determination of inter-day precision, fortified samples were analyzed on four different days. The intra-day relative standard deviation (RSD) ranged from 2.19 to 4.98 at 1 μg/ml, 1.05 to 3.68 at 10 μg/ml and 3.14 to 5.08 at 50 μg/ml. The inter-day RSD were 1.60, 2.24 and 1.54 for 1, 10 and 50 μg/ml, respectively (Table 1). These values are within the limits ($<15\%$) specified for inter and intra-day precision [9,10].

3.4. Recovery and accuracy

The extraction recovery of PAT-5A was estimated at 1, 10 and 50 μg/ml concentrations. Plasma samples (in quadruplicate) containing PAT-5A and I.S. were extracted and analyzed. Four samples containing similar concentrations of the compound in mobile phase were directly injected and peak areas were measured. Absolute recovery was calculated by comparing the peak areas for direct injection of pure PAT-5A in methanol with those obtained by plasma samples containing same amount of PAT-5A. The absolute recoveries ranged from ca. 91.5 to 99.7 (Table 2). The accuracy of the method was verified by comparing the concentrations measured for PAT-5A spiked plasma with the actual added concentrations. The results (Table 2) indicate that accuracy of the method was 95.7 to 103.8%.

Table 2
Absolute recovery and accuracy of determination of pat-5a in rat plasma

Concentration (μg/ml)	Absolute recovery (mean ± S.D., $n = 4$)	Accuracy (%) (mean ± S.D., $n = 4$)	Range (μg/ml)
1.0	91.50 ± 1.72	96.60 ± 2.11	0.95–1.00
10.0	99.70 ± 1.05	103.87 ± 0.41	10.36–10.42
50.0	97.58 ± 3.07	95.76 ± 0.69	47.63–48.12

These experiments confirm that the present method for determination of PAT-5A in plasma samples is specific, accurate and precise. The calibration curve was linear and hence the method was suitable for analysis of plasma samples in the concentration range 0.05–50 μg/ml. This method was used for analysis of plasma samples collected during a pharmacokinetic study in which a 10 mg/kg dose was administered orally to male Wistar rats ($n = 4$) as a 0.25% carboxymethylcellulose suspension. A typical plasma concentration versus time profile was shown in Fig. 3. A HPLC chromatogram of plasma sample without I.S. showed no peaks at the RT of the I.S. indicating that there was no interference from metabolites or plasma endogenous peaks. The pharmacokinetic parameters were calculated using non-compartmental model analysis. PAT-5A was absorbed rapidly with maximum concentration in plasma (C_{max} 10.75 ± 2.97 μg/ml; mean ± S.D.) being reached at 0.75 ± 0.29 h (t_{max}: mean ± S.D.). The half-life ($t_{1/2}$) of PAT-5A was 1.31 ± 0.16 h (mean ± S.D.). In conclusion, the HPLC method presented here is suitable for the analysis of PAT-5A plasma samples during preclinical drug development.

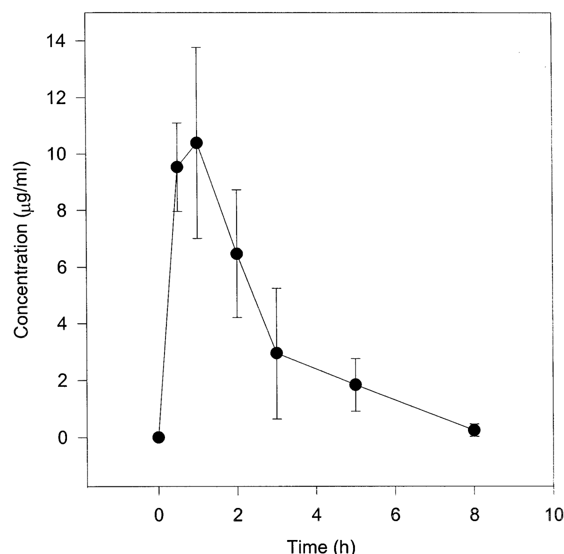

Fig. 3. Plasma concentration versus time profile for PAT-5A after single oral dose of 10 mg/kg in male Wistar rats. The data points are means and error bars are standard deviation of four observations.

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