High-performance liquid chromatographic determination of the insulin sensitizing agent DRF-2189 in rat plasma[1]

H.K. Jajoo[a],[*], N.V.S. Rao Mamidi[a], Katneni Kasiram[a], A. Surya Prakash[a], V.V.S. Swaroop Kumar[a], P. Bheema Rao[b], Vidya Bhushan[b], S. Subramaniam[a]

[a]*Drug Metabolism and Pharmacokinetics Laboratory, Department of Clinical Research, Dr. Reddy's Research Foundation, 7-1-27, Ameerpet, Hyderabad 500 016, India*
[b]*Department of Medicinal Chemistry, Dr. Reddy's Research Foundation, 7-1-27, Ameerpet, Hyderabad 500 016, India*

Abstract

A high-performance liquid chromatographic method for the determination of DRF-2189, using troglitazone as internal standard, is described. A dichloromethane–ethyl acetate solvent mixture (6:4, v/v) was used as the extraction solvent. A Kromasil C_{18} column with a mobile phase consisting of 0.05 M phosphate buffer–acetonitrile–methanol (22.5:37.5:40) (pH 5.0) was used at a flow-rate of 1.0 ml/min. The eluate was monitored by using fluorescence detection with excitation and emission wavelengths at 292 nm and 325 nm, respectively. Ratio of peak area of analyte to internal standard was used for quantification of plasma samples. Using this method, the absolute recovery of DRF-2189 from rat plasma was >95% and the limit of quantitation was 50 ng/ml. The intra-day relative standard deviation (R.S.D.) ranged from 1.74 to 7.24% at 1 μg/ml and 1.86 to 3.83% at 10 μg/ml. The inter-day R.S.D.s were 8.34 and 4.91% at 1 and 10 μg/ml, respectively. The method was applied to measure plasma concentrations of DRF-2189 in pharmacokinetic studies in Wistar rats. © 1998 Elsevier Science B.V.

1. Introduction

Type 2 diabetes mellitus (DM), also known as non-insulin-dependent diabetes mellitus (NIDDM), is the most common metabolic disease and is characterized by sub-normal tissue response to insulin, resulting in hyperglycemia [1]. Thiazolidinedione analogues are known to possess significant insulin sensitizing activity in vivo [2,3]. One of these thiazolidinedione analogues, troglitazone [4] is already approved for human use in the USA, Japan and the UK while BRL-49653 [5], which is more potent as compared to troglitazone is currently in Phase-III clinical trials. Other compounds such as α-alkylthio and α-alkoxy carboxylic acid derivatives are also reported to exhibit insulin sensitizing effect at much lower dose and are under development as euglycemic agents [6]. DRF-2189 (Fig. 1a) is a new thiazolidinedione that has been shown to possess potent euglycemic and hypolipidaemic effects in animal models of type 2 DM and is now in preclini-

*Corresponding author.
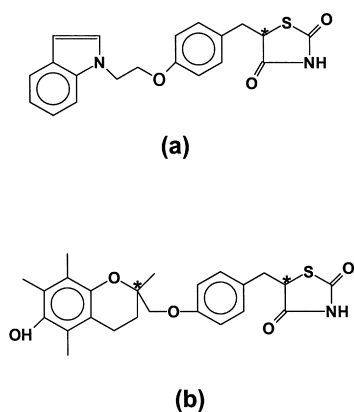[1]This is publication number 25 from Dr. Reddy's Research Foundation.

Fig. 1. Structures of (a) DRF-2189 {5-[4-[2-(1-indolyl)ethoxy]-phenylmethyl]-thiazolidine-2,4-dione} and (b) troglitazone. Asterisks indicate the presence of chiral centers in the molecule.

cal development [7]. DRF-2189 reduces blood sugar levels by 55% in db/db mice at 3 mg/kg p.o. and is thus comparable to BRL-49653 [8].

To support the pharmacokinetic and bioavailability evaluations necessary for drug development, a simple and sensitive high-performance liquid chromatographic (HPLC) assay method with fluorescence detection has been developed for the quantitative determination of DRF-2189 in rat plasma using troglitazone (Fig. 1b) as an internal standard. The method offers the advantage of simplicity with adequate sensitivity, selectivity, precision and accuracy. The analytical method has been used to evaluate the pharmacokinetics of DRF-2189 in male Wistar rats.

2. Experimental

2.1. Chemicals and reagents

DRF-2189 and troglitazone (used as an internal standard) were synthesized and characterized by spectral methods [7]. Methanol, acetonitrile, dichloromethane and ethyl acetate were of HPLC grade (Qualigens, Mumbai, India) and potassium dihydrogenorthophosphate (analytical-reagent grade) was obtained from Glaxo (Mumbai, India).

2.2. Preparation of standards

Stock solutions of 1.0 mg/ml of DRF-2189 and internal standard (troglitazone) were prepared in methanol and stored at 4°C. Appropriate dilutions of DRF-2189 were made with methanol to produce working standard solutions containing 100 µg/ml, 10 µg/ml and 1 µg/ml. These dilutions were used to spike plasma to make calibration standards. Internal standard was used directly from the stock solution. Calibration samples were prepared by spiking 100 µl of blank plasma with appropriate amount of the drug on the day of analysis. Recovery, precision and accuracy samples were prepared in bulk at appropriate concentrations in blank rat plasma and stored at 4°C.

2.3. Extraction procedure

To 100 µl of plasma sample, 3.0 µg of troglitazone (internal standard) was added. The plasma was then vortexed for 20 s. A 2.0 ml volume of dichloromethane–ethyl acetate (6:4) solvent mixture was added and the tubes were vortexed for 3 min and centrifuged at 3000 rpm for 10 min on a table top centrifuge (Remi Instruments, Mumbai, India). The supernatant organic layer (1.5 ml) was separated and evaporated to dryness under nitrogen (N-Evap, Organomation, MA, USA) at 50°C. The residue was reconstituted with 200 µl methanol–water (1:1) mixture and 100 µl was injected onto the HPLC system. Calibration standards were prepared in the range 0.05–50 µg/ml.

2.4. Chromatography

A Shimadzu HPLC system equipped with a solvent-delivery system (Model LC-10A), fluorescence detection system (Perkin-Elmer LC-240) and a data processor (Millennium software, Waters, USA) was used. A Kromasil C_{18} ODS column (250 mm×4.6 mm I.D., 5 µm) was used. A solvent mixture consisting of 0.05 M sodium dihydrogenorthophosphate–acetonitrile–methanol (22.5:37.5:40, v/v), pH 5.0 was used as the mobile phase at a flow-rate of 1.0 ml/min. The fluorescence detection system, set at excitation and emission wavelengths of 292 nm and 325 nm, respectively, was used to monitor the

eluate. The attenuation and response factors for the detector were set at 128 and 2, respectively.

2.5. Pharmacokinetic study

The pharmacokinetic study was carried out in male Wistar rats, obtained from the National Institute of Nutrition (Hyderabad, India). The animals (175–200 g) were fasted for 12 h before starting the experiment and they had free access to water throughout the experiment period. Animals were fed 3 h after drug administration. DRF-2189 was administered to the animals through gavage at a dose of 10 mg/kg orally (p.o.) as a 0.5% carboxymethylcellulose suspension. Blood samples (0.4 ml) were collected from orbital sinus into heparinised microfuge tubes at different time points (0, 0.5, 1, 2, 3, 5, 8, 12 and 24 h) and centrifuged (Biofuge, Hereaus, Germany) at 9000 rpm for 9 min to separate plasma. Plasma samples (100 μl) were spiked with internal standard and processed as described above. Pharmacokinetic parameters were calculated employing non-compartmental model analysis. The area under the concentration–time curve (AUC) was calculated by trapezoidal rule, t_{max} is the time at which the maximum plasma concentration (C_{max}) 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 by formula $0.693/K_{el}$.

3. Results and discussion

3.1. Chromatography

Typical chromatograms corresponding to blank plasma and plasma sample obtained after oral administration of DRF-2189 at a dose of 10.0 mg/kg to Wistar rats, are shown in Fig. 2. No endogenous interfering peaks were visible in blank plasma at the retention times of DRF-2189 and the internal standard (Fig. 2a) thereby confirming specificity of the analytical method. Both analyte and the internal standards were well separated with retention times of 10.6 and 13.9 min, respectively (Fig. 2b). System suitability parameters for the method were as follows: theoretical plates for DRF-2189>3000,

asymmetry factor <1.5 and resolution between the DRF-2189 and troglitazone peaks was greater than four.

3.2. Quantification

Peak-area ratios of DRF-2189 to the internal standard were measured. A representative calibration graph of DRF-2189 to troglitazone peak-area ratio versus DRF-2189 concentration in the range 0.05–50 μg/ml resulted in the regression equation $y = 0.268x - 0.0104$ ($r^2 = 0.999$). The lower limit of quantitation (LOQ) was established by determining concentration of spiked calibration standard, which had a signal-to-noise ratio>3. The LOQ for DRF-2189 in plasma was 0.05 μg/ml. The analyte to internal standard peak-area ratios were determined for plasma samples and were used for calculation of plasma concentrations.

3.3. Precision

Precision of the assay was determined by assaying plasma samples containing DRF-2189 at two concentrations (1 and 10 μg/ml). Samples for precision study were obtained by spiking blank plasma with analyte solution at each concentration in bulk, and 100-μl aliquots were distributed into screw-capped centrifuge tubes and stored at −20°C. The intra-day precision was determined by analyzing four spiked plasma samples at each concentration on the same day. For determination of inter-day precision, fortified plasma samples were analyzed on four different days. The intra-day relative standard deviation (R.S.D.) ranged from 1.74 to 7.24% at 1 μg/ml and 1.86 to 3.83% at 10 μg/ml. The inter-day R.S.D.s were 8.34 and 4.91% at 1 and 10 μg/ml, respectively (Table 1). These values are <15% which is the limit specified on inter- and intra-day precision [9,10].

3.4. Recovery and accuracy

The extraction recovery of DRF-2189 was assessed at concentrations of 1 and 10 μg/ml. Plasma samples (in quadruplicate) containing DRF-2189 and internal standard were extracted and injected. Four samples of the same amount of compound in mobile
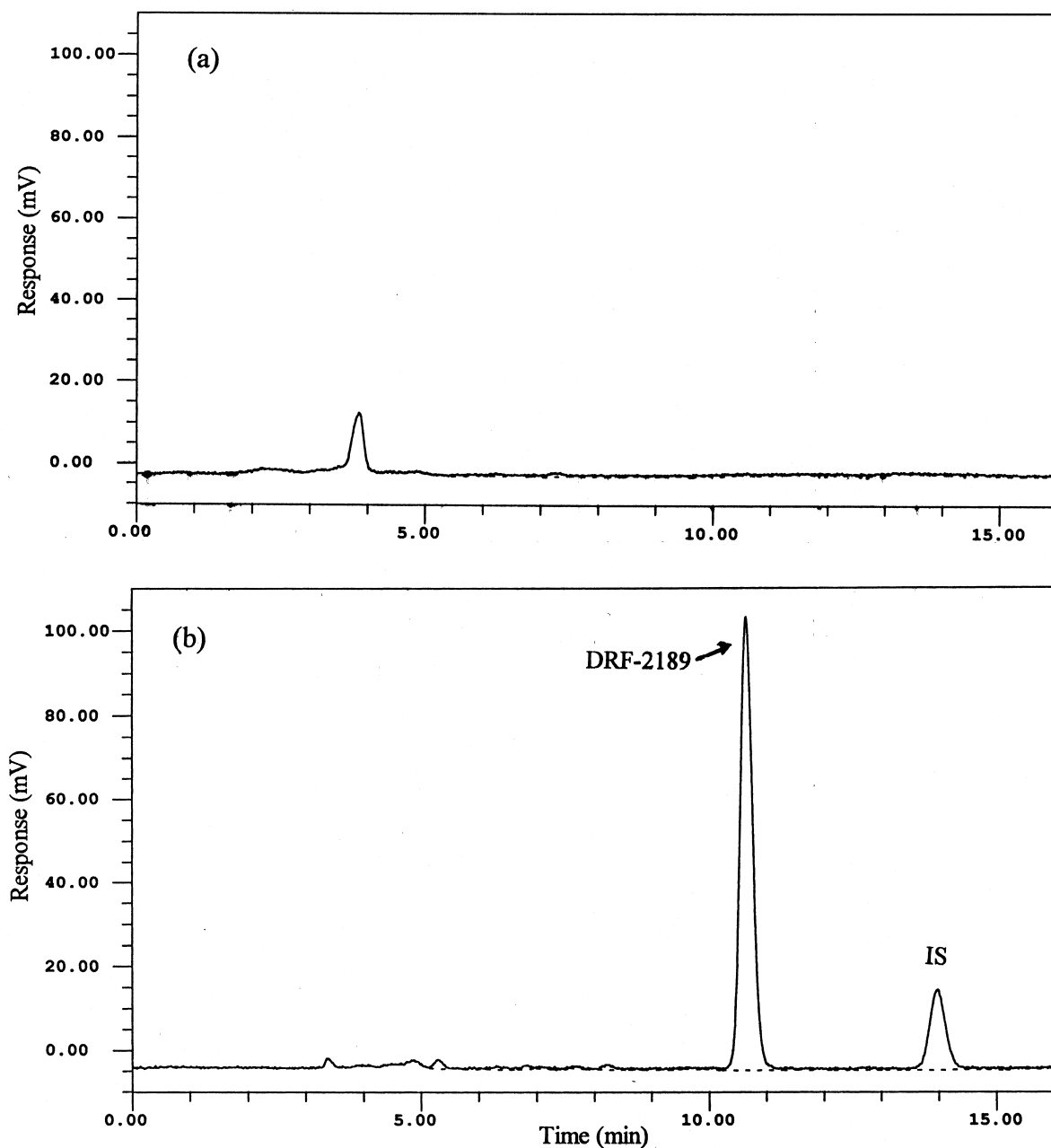
Fig. 2. Typical HPLC chromatograms for analysis of DRF-2189 in (a) blank rat plasma and (b) 5-h plasma sample from rat dosed with DRF-2189 at 10 mg/kg p.o. The concentration for this sample was found to be 14.3 μg/ml.

phase were directly injected and the peak areas were measured. Absolute recovery was calculated by comparing the peak areas for direct injection of pure DRF-2189 with those for plasma samples containing

the same amount of DRF-2189. The absolute recoveries of DRF-2189 ranged from ca. 101.27–103.27% (Table 2). The accuracy of the method was verified by comparing the concentrations measured

Table 1
Intra- and inter-day variation of DRF-2189 assay in rat plasma

Spiked concentration	Day	Measured concentrations[a]		
		Mean (μg/ml)	S.D	R.S.D. (%)
Intra-day variation				
At 1 μg/ml	0	0.97	0.06	5.71
	1	0.90	0.03	3.02
	3	1.10	0.02	1.74
	5	1.01	0.07	7.24
At 10 μg/ml	0	10.18	0.26	2.59
	1	11.29	0.29	2.59
	3	10.20	0.39	3.83
	5	10.60	0.20	1.86
Inter-day variation				
1 μg/ml		1.00	0.08	8.34
10 μg/ml		10.57	0.52	4.91

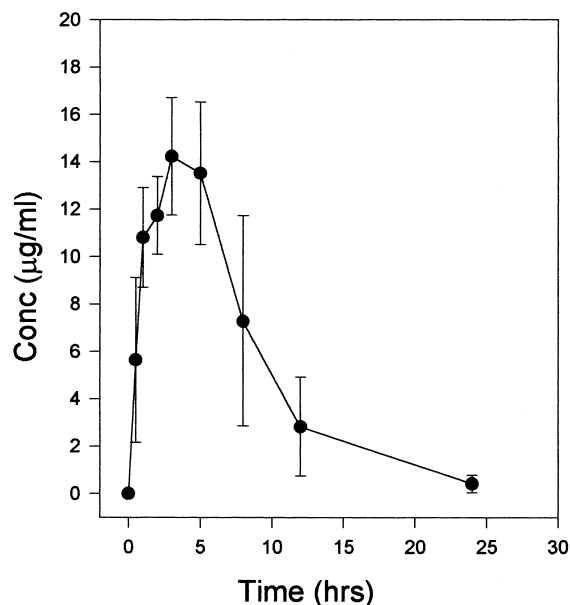[a] Values (mean and S.D.), are for $n=4$ observations.

Fig. 3. Plasma concentration versus time profile for DRF-2189 after a dose of 10 mg/kg p.o. in male Wistar rats. The data points are mean and error bars are standard deviation of three observations.

for DRF-2189 spiked plasma with the actual added concentrations. The results (Table 2) indicate that accuracy of the method is 97–102%.

These experiments confirm that the present method for determination of DRF-2189 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 obtained during a pharmacokinetic study in which a 10 mg/kg dose was administered orally to male Wistar rats ($n=3$) as a 0.5% carboxymethylcellulose suspension. A typical plasma concentration versus time profile is shown in Fig. 3. A HPLC chromatogram of plasma sample without internal standard showed no peaks at the retention time of the internal standard indicating that there was no interference from the metabolites. Peak purity index of the drug peak (determined using UV photodiode array detection) from the plasma sample was 0.99, thereby indicating that there was no

interference from metabolites. The pharmacokinetic parameters were calculated using non-compartmental model analysis. DRF-2189 was absorbed slowly, maximum concentration in the plasma (C_{max} 14.54±2.4 μg/ml; mean±S.D.) being reached at 3.67±1.1 h (t_{max}; mean±S.D.). The elimination half-life ($t_{1/2}$) of DRF-2189 was 3.28±1.4 h (mean±S.D.). In conclusion, the HPLC method presented here is suitable for analysis of DRF-2189 plasma samples during preclinical drug development.

References

[1] R.A. DeFronzo, E. Ferrannini, V. Koivisto, Am. J. Med. 74 (1983) 52.

Table 2
Absolute recovery and accuracy of determination DRF-2189 in rat plasma

Concentration (μg/ml)	Absolute recovery (mean±S.D., $n=4$)	Accuracy (%) (mean±S.D., $n=4$)	Range (μg/ml)
1.0	103.27±5.17	97.39±5.56	0.90–1.02
10.0	101.97±2.73	101.82±2.64	9.88–10.38

[2] N.C. Turner, Drug Discovery Today 1 (1996) 109.

[3] B. Hulin, P.A. McCarthy, E.M. Gibbs, Curr. Pharm. Design 2 (1996) 85.

[4] H. Horikoshi, T. Yoshioka, T. Kawasaki, K. Nakamura, N. Matsunuma, K. Yamaguchi, K. Sasahara, Annu. Rep. Sankyo Res. Lab. 46 (1994) 1.

[5] B.B.C. Cantello, M.A. Cawthrone, G.P. Coltam, P.T. Duff, D. Haigh, R.M. Hindley, C.A. Lister, S.A. Smith, L. Thurlby, J. Med. Chem. 37 (1994) 3977.

[6] B. Hulin, L.S. Newton, D.M. Lewis, P.E. Genereux, E.M. Gibbs, D.A. Clark, J. Med. Chem. 39 (1996) 3897.

[7] B.B. Lohray, V. Bhushan, P. Bheema Rao, G.R. Madhavan, N. Murali, K. Narasimha Rao, K. Anantha Reddy, B.M. Rajesh, P. Ganpathy Reddy, R. Chakraborti, R. Rajagopalan, Bioorg. Med. Chem. Lett. 7 (1997) 785.

[8] B.B. Lohray, V. Bhushan, P. Bheema Rao, G.R. Madhavan, N. Murali, K. Narasimha Rao, K. Anantha Reddy, B.M. Rajesh, P. Ganapathi Reddy, R.K. Vikramadithyan, R. Chakraborti, R. Rajagopalan, N.V.S. Rao Mamidi, H.K. Jajoo and S. Subramaniam, J. Med. Chem. (1997) submitted for publication.

[9] F. Bressolle, M. Bromet-Petit, M. Audran, J. Chromatogr. B 686 (1996) 3–10.

[10] V.P. Shah, K.K. Midha, S. Dighe, I.J. Mcgilveray, J.P. Skelly, A. Jacobi, T. Layoff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, J. Pharm. Sci. 81 (1992) 309.