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Development and validation of a chiral liquid chromatographic method, based on Chiralpak® to quantify enantiomers of (±)-DRF 2725 in rat plasma: lack of inversion of ragaglitazar (*S*(−)-DRF 2725) to its antipode in plasma

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Abstract

A selective, accurate and reproducible high-performance liquid chromatographic (HPLC) method for the separation of individual enantiomers of DRF 2725 [*R*(+)-DRF 2725 and *S*(−)-DRF 2725 or ragaglitazar] was obtained on a chiral HPLC column (Chiralpak®). During method optimization, the separation of enantiomers of DRF 2725 was investigated to determine whether mobile phase composition, flow-rate and column temperature could be varied to yield the base line separation of the enantiomers. Following liquid–liquid extraction, separation of enantiomers of DRF 2725 and internal standard (I.S., desmethyl diazepam) was achieved using an amylose based chiral column (Chiralpak® AD™) with the mobile phase, *n*-hexane-propanol-ethanol-trifluoro acetic acid (TFA) in the ratio of 89.5:4:6:0.5 (v/v). Baseline separation of DRF 2725 enantiomers and I.S., free from endogenous interferences, was achieved in less than 25 min. The eluate was monitored using an UV detector set at 240 nm. Ratio of peak area of each enantiomer to I.S. was used for quantification of plasma samples. Nominal retention times of *R*(+)-DRF 2725, *S*(−)-DRF 2725 and I.S. were 15.8, 17.7 and 22.4 min, respectively. The standard curves for DRF 2725 enantiomers were linear ($R^2 > 0.999$) in the concentration range 0.3–50 μg/ml for each enantiomer. Absolute recovery, when compared to neat standards, was 70–85% for DRF 2725 enantiomers and 96% for I.S. from rat plasma. The lower limit of quantification (LLOQ) for each enantiomers of DRF 2725 was 0.3 μg/ml. The inter-day precisions were in the range of 1.71–4.60% and 3.77–5.91% for *R*(+)-DRF 2725, *S*(−)-DRF 2725, respectively. The intra-day precisions were in the range of 1.06–11.5% and 0.58–12.7% for *R*(+)-DRF 2725, *S*(−)-DRF 2725, respectively. Accuracy in the measurement of quality control (QC) samples was in the range 83.4–113% and 83.3–113% for *R*(+)-DRF 2725, *S*(−)-DRF 2725, respectively. Both enantiomers and I.S. were stable in the battery of stability studies viz., bench-top (up to 6 h), auto-sampler (up to 12 h) and freeze/thaw cycles ($n = 3$). Stability of DRF 2725 enantiomers was established for 15 days at −20 °C. The application of the assay to a pharmacokinetic study of ragaglitazar [*S*(−)-DRF 2725] in rats is described. It was unequivocally demonstrated that ragaglitazar does not undergo chiral inversion to its antipode in vivo in rat plasma.

Keywords: Enantiomer separation; Ragaglitazar

1. Introduction

(±)-DRF 2725 [*R*(+)-DRF 2725, *S*(−)-DRF 2725 (Fig. 1)] is a novel phenoxazine analogue of phenylpropanoic acid derivative. Pharmacologically (±)-DRF 2725 is related to thiazolidinedione (TZD) class (troglitazone, rosiglitazone, pioglitazone) and fibrate class (fenofibric acid, bezafibrate), in that it exhibits properties that are common to both classes. TZD class of compounds control the blood glucose level in type 2 diabetes by activation of peroxisome proliferator–activator receptor gamma (PPARγ) and fibrates class of drugs reduce triglyceride and increase HDL level through activation of peroxisome proliferator–activator receptor alpha (PPARα). Our earlier studies revealed that *R*(+)-DRF 2725 has showed a weak transactivation for both PPARα and γ receptors than *S*(−)-DRF 2725, which

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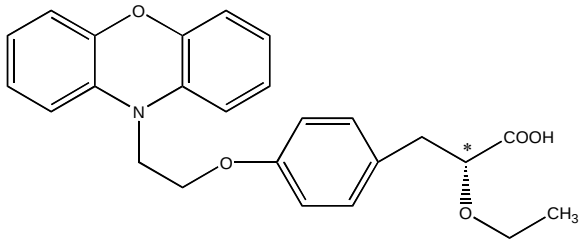
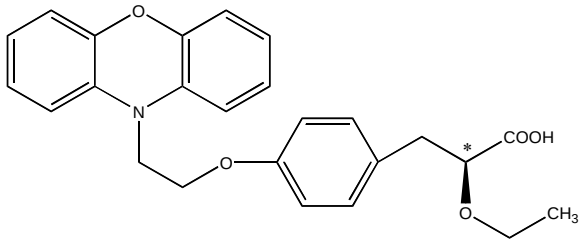


R(+)-DRF 2725



S(-)-DRF 2725

Fig. 1. Chiral structural representation of DRF 2725 enantiomers.

was the most potent form of the two enatiomers [1]. Hence, *S*(−)-DRF 2725 (ragaglitazar or NNC 61-0029) was profiled further, and because it showed an impressive pre-clinical profile in several models of diabetes and dyslipidemia, it was considered for further clinical development [2,3].

Examination of literature reveals several methods for chiral separation and quantitation, which are based on direct chiral separation, via chiral stationary phases, and indirect chiral separation, via conversion of enantiomers into diastereomers prior to chromatography [4]. Therefore, enantiomeric analysis of racemic chiral substrates including monitoring for chiral inversion has become routine for pharmacokinetic disposition. In this context, we were interested to develop a simple, specific and accurate HPLC method for quantitative determination of *R*,*S* (±)-DRF 2725 enantiomers in rat plasma, and the application of the method to study pharmacokinetics of *S*(−)-DRF 2725 and particularly evaluate the potential of chiral inversion of *S*(−)-DRF 2725 to *R*(+)-DRF 2725 in rats. In order to optimize the enantiomeric separation, different chromatographic conditions were also studied and are described in this paper.

2. Experimental

2.1. Materials

(±)-DRF 2725, *R*(+)-DRF 2725, *S*(−)-DRF 2725 and I.S. (for bioanalytical purpose) were synthesized by Discovery Chemistry Group, Dr. Reddy's Laboratories Ltd. (DRL), Hyderabad, India and were characterized using chromatographic and spectral techniques by Analytical Research Group, DRL. Purity was found to be more than 99% for all of these compounds. *n*-Hexane, propanol (Merck Limited, Mumbai, India) and ethyl alcohol (Sigma, St. Louis, USA) were of HPLC grade. All other reagents purchased from Qualigens (Mumbai, India) were of analytical reagent grade. Control rat plasma was obtained from Department of Pre-Clinical and Safety Evaluation, Discovery Research, DRL, Hyderabad.

2.2. Instrumentation

The HPLC system was an Agilent (Agilent Technologies, Waldbronn, Germany) 1100 series LC system equipped with degasser (G1322A), isopump (G1310A), column heater (G1316A), auto-injector with sample cooler (G1330A) and ultraviolet detector (G1314A). The data were acquired and processed with LC^{3D} Chemstation software (version A.08.01). Chromatographic separations were achieved using a 4.6 mm × 250 mm, 10 μm particle size chiral column (Chiralpak AD^{TM}, Daicel Chemical Industries Ltd., Japan) equipped with a 4.6 mm × 50 mm, 10 μm particle size guard column (Chiralpak AD^{TM}, Daicel Chemical Industries Ltd., Japan) maintained at 15 °C (±2 °C) in a column oven. Separation characteristics such as capacity factor (K'), selectivity or separation factor (α) and resolution factor (Rs) were determined by using standard formulas.

2.3. Assay conditions

The chromatograms were monitored by UV detector and the wavelength of 240 nm was selected. Samples were dissolved in mobile phase. The injection volume was fixed at 75 μl. The mobile phase was *n*-hexane-propanol-ethanol-trifluoro acetic acid in the ratio of 89.5:4:6:0.5 (v/v). Before the analysis the mobile phase was filtered through a 0.45-μm hydrophilic PVDF filter (Cat No: HVLP 04700, Millipore, USA), and then degassed ultrasonically for 15 min. The optimized column temperature and flow rate were 15 °C and 0.7 ml/min, respectively.

2.4. Standard solutions

Standards and QC stock solutions of each enantiomers of DRF 2725 and desmethyl diazepam (I.S.) were prepared in methanol. Appropriate dilutions of each enantiomers of DRF 2725 were made in methanol to produce working stock solutions of 5000, 1000, 500, 200, 100, 50, 20, 10, 5, 2 and 1 μg/ml. Stock solutions were stored at approximately 5 °C. Working stocks were used to prepare plasma calibration standards. A working I.S. solution (1 mg/ml) was prepared in methanol. Calibration samples were prepared by spiking 100 μl of control pooled rat plasma with the appropriate amount of the analyte (10 μl) and I.S. (10 μl) 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 (0.3, 0.9, 15.0 and 30.0 μg/ml) and 100 μl aliquots were distributed into different tubes. All the samples were stored at −20 °C.

2.5. Calibration curves

Calibration curves were acquired by plotting the peak area ratio of each enantiomer of DRF 2725:I.S. against the nominal concentration of calibration standards. The concentrations used were 0.3, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 μg/ml. The results were fitted to linear regression analysis without the use of a weighting factor.

2.6. Precision and accuracy

The intra-assay precision and accuracy were estimated by analyzing six replicates containing DRF 2725 at four different QC levels i.e., 0.3, 0.9, 15 and 30 μg/ml. The inter-assay precision was determined by analyzing the four levels QC samples on four different runs. The criteria for acceptability of the data included accuracy within ±15% deviation (DEV) from the nominal values and a precision of within 15% relative standard deviation except for LLOQ, where it should not exceed ±20% of CV [5].

2.7. Extraction and recovery

Two sets of standards containing the analyte and I.S. at three different concentrations (0.9, 15 and 30 μg/ml) and at the lower limit of quantification (LLOQ) were prepared. One set was prepared in rat plasma and the other set was prepared in methanol. The recovery was determined by comparing peak areas of spiked plasma extracts with those of unextracted neat standards prepared in methanol. The recovery value was calculated at the various concentrations of each enantiomers of DRF 2725. The recovery of the I.S. was determined at a single concentration of 50 μg/ml.

2.8. Stability experiments

The stability of (±)-DRF 2725 and I.S. in the injection solvent was determined periodically by injecting replicate preparations of processed samples for up to 12 h (in the auto sampler at 5 °C) after the initial injection. The peak-areas of the analyte and I.S. obtained at initial cycle were used as the reference to determine the stability at subsequent points. Stability of each enantiomers of DRF 2725 in the biomatrix during 6 h (bench-top) was determined at ambient temperature (25 ± 3 °C) at four concentrations in quadruplicates. Freezer stability of ragaglitazar in rat plasma was assessed by analyzing the QC samples stored at −20 °C for at least 15 days. The stability of each enantiomers of DRF 2725 in rat plasma following repeated freeze–thaw cycles was assessed using QC samples spiked with each enantiomers of DRF 2725. The samples were stored at −20 °C between freeze/thaw cycles. The samples were thawed by allowing them to stand (unassisted) at room temperature for approximately 2 h. The samples were then returned to the freezer. The stability of each enantiomers of DRF 2725 was assessed after three freeze–thaw cycles. The samples were processed using the same procedure as described in the sample preparation section. Samples were considered stable if assay values were within the acceptable limits of accuracy (i.e., ±15% DEV) and precision (i.e., ±15% R.S.D.) except for LLOQ, where it should not exceed ±20%.

2.9. Sample preparation

Sample preparation was carried out by liquid–liquid extraction. A 100 μl aliquot of plasma sample was pipetted out into a 15-ml centrifuge tube, methanolic solution (10 μl) of desmethyl diazepam equivalent to 5 μg was added and mixed for 15 s on a cyclomixer (Remi Instruments, Mumbai, India). After the addition of 3 ml of dichloromethane:ethyl acetate (1:2), the mixture was vortexed for 2 min; followed by centrifugation for 10 min at 1760 × *g* on a tabletop centrifuge (Remi Instruments, Mumbai, India). The organic layer (2.7 ml) was separated and evaporated to dryness at 40 °C using a gentle stream of nitrogen (Zymark® Turbovap®, Kopkinton, MA, USA). The residue was reconstituted in 150 μl of the mobile phase and 75 μl aliquot was injected onto HPLC column.

2.10. Study in rats

Male Wistar rats, 12–14 weeks of age and weighing between 190 and 200 g, were fasted overnight (∼14 h) before the dosing day and they had free access to water throughout the experimental period. *S*(−)-DRF 2725 was administered orally at a dose of 30 mg/kg, as a suspension (in 0.25% carobxy methyl cellulose). Animals were provided with standard diet 3 h post dosing. The rats were anaesthetized in ether and blood samples (approximately 0.25 ml) were collected from the retro-orbital plexus into microfuge tubes (containing 10 μl of saturated EDTA) at 0.5, 1, 2, 3, 5, 8, 12 and 24 h post-dosing. Plasma was harvested by centrifuging in a micro centrifuge (Biofuge, Hereaus, Germany) at 7500 × *g* at 4 °C for 3 min and stored at −20 °C until bioanalysis. Plasma (100 μl) samples were spiked with I.S. and processed as described above.

2.11. Pharmacokinetic analysis

Pharmacokinetic parameters were calculated by employing a non-compartmental analysis [6]. The peak plasma concentration (C_{max}) and the corresponding time (T_{max}) were directly obtained from the raw data. The area under the plasma concentration versus time curve up to the last quantifiable time point, $AUC_{(0-t)}$ was obtained by the linear and log-linear trapezoidal summation. The $AUC_{(0-t)}$

extrapolated to infinity (i.e., AUC $_{(0-\infty)}$) by adding the quotient of C_{last}/K_{el}, where C_{last} represents the last measurable time concentration and K_{el} represents the apparent terminal rate constant. K_{el} was calculated by the linear regression of the log-transformed concentrations of the drug in the terminal phase. The half-life ($t_{1/2}$) of the terminal elimination phase was obtained using the relationship $t_{1/2} = 0.693/K_{el}$.

2.12. Statistical analysis of data

Statistical analysis (ANOVA) was performed using Sigma Stat (Scientific Software, Jandel Scientific, version 2.0, USA) and the significance level adopted for all statistical comparisons was $P < 0.05$.

3. Results and discussion

3.1. Effect of assay conditions on the separation

3.1.1. Effect of mobile phase composition

n-Hexane-propanol-ethanol-trifluoro acetic acid in the ratio of 89.5:4:6:0.5 (v/v) was chosen as mobile phase to obtain base line separation of individual enantiomers. Being as one of the component of the mobile phase, the percentage of propanol, a well-known organic modifier, had strong effect on the separation. Fig. 2 shows the chromatograms of the DRF 2725 enantiomers employing different propanol content. The reduction of propanol content from 10 to 4% resulted in a complete baseline separation of the two enantiomeric peaks of DRF 2725. Although it also increased the run times for separation, it was considered manageable given the objective of the experiment.

3.1.2. Column temperature

The examples cited in the literature demonstrate that a decrease in column temperature caused an increase in enantioselectivity. We found that a raise in temperature shortens the elution time while it hampered the resolution and therefore a right balance of column temperature and resolution had to be obtained. In the present work various value of temperatures were selected to evaluate the effects on separation. The effect of raising the column temperature in 10 °C increments was investigated on the DRF 2725 enantiomers separation. For DRF 2725 enantiomers the separation factor (α) remained constant at 1.12, resolution decreased from 2.23 to 1.45 across the tested temperature range (15–65 °C). Fig. 3 and Table 1 show that maximum resolution of DRF 2725 enantiomers is obtained at 15 °C. Therefore, the optimum column temperature for the separation of DRF 2725 enantiomers was set at 15 °C.

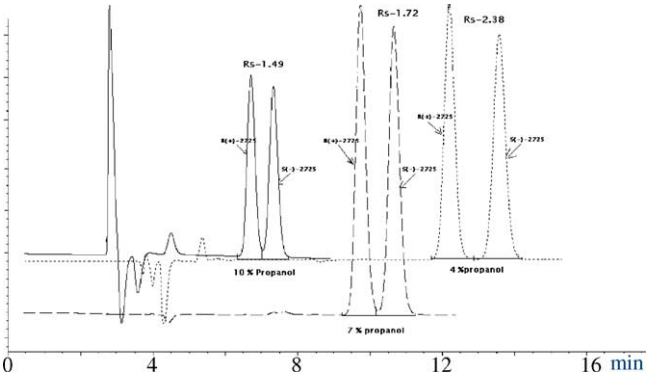


Fig. 2. Effect of propanol content upon the separation of the DRF 2725 enantiomers.

Table 1
Effect of temperature on the separation of DRF 2725 enantiomers

Temperature (°C)	Rs	K'_1	K'_2	α
15	2.23	2.49	2.82	1.14
25	1.93	2.31	2.60	1.13
35	1.74	1.97	2.21	1.12
45	1.80	2.14	2.39	1.13
55	1.65	2.01	2.23	1.11
65	1.45	1.87	2.06	1.10

K': capacity factor; Rs: resolution factor; α: separation factor.

3.1.3. Effect of flow-rate

The influence of flow-rate upon resolution was examined under the optimum mobile phase and temperature conditions, and this rate was increased from 0.5 to 1.5 ml/min (rates higher than 1.5 ml/min were not attempted because it has potential to damage the column). It was found that the flow-rate has little effect on α but significantly effects resolution. Although high flow rates will accelerate the speed of analysis, the resolution between enantiomers is compromised. The optimum flow-rate was established at 0.7 ml/min (Table 2). At the flow-rate the run time for the separation of all analytes of interest were within 30 min. As the assay was being developed to evaluate the interconversion potential of ragaglitazar to its antipode, emphasis was placed on an ideal separation as opposed to the speed of separation. It is quite likely that if the chiral assay needs to be employed on a routine basis, the method would need modification to cut short the run time with a minimal impact on resolution of the enantiomeric peaks.

Table 2
Effect of flow rate on the separation of DRF 2725 enantiomers

Flow rate (ml/min)	Rs	K'_1	K'_2	α
0.5	2.46	2.70	3.10	1.15
0.6	2.34	2.73	3.13	1.15
0.7	2.23	2.57	2.95	1.15
0.8	2.16	2.61	3.00	1.15
0.9	2.08	2.67	3.05	1.15
1.0	2.00	2.64	3.05	1.15
1.1	1.95	2.61	2.99	1.15
1.2	1.89	2.62	3.01	1.15
1.3	1.84	2.64	3.02	1.15
1.4	1.80	2.62	3.01	1.15
1.5	1.66	2.36	2.70	1.14

Rs: resolution factor; α: separation factor.

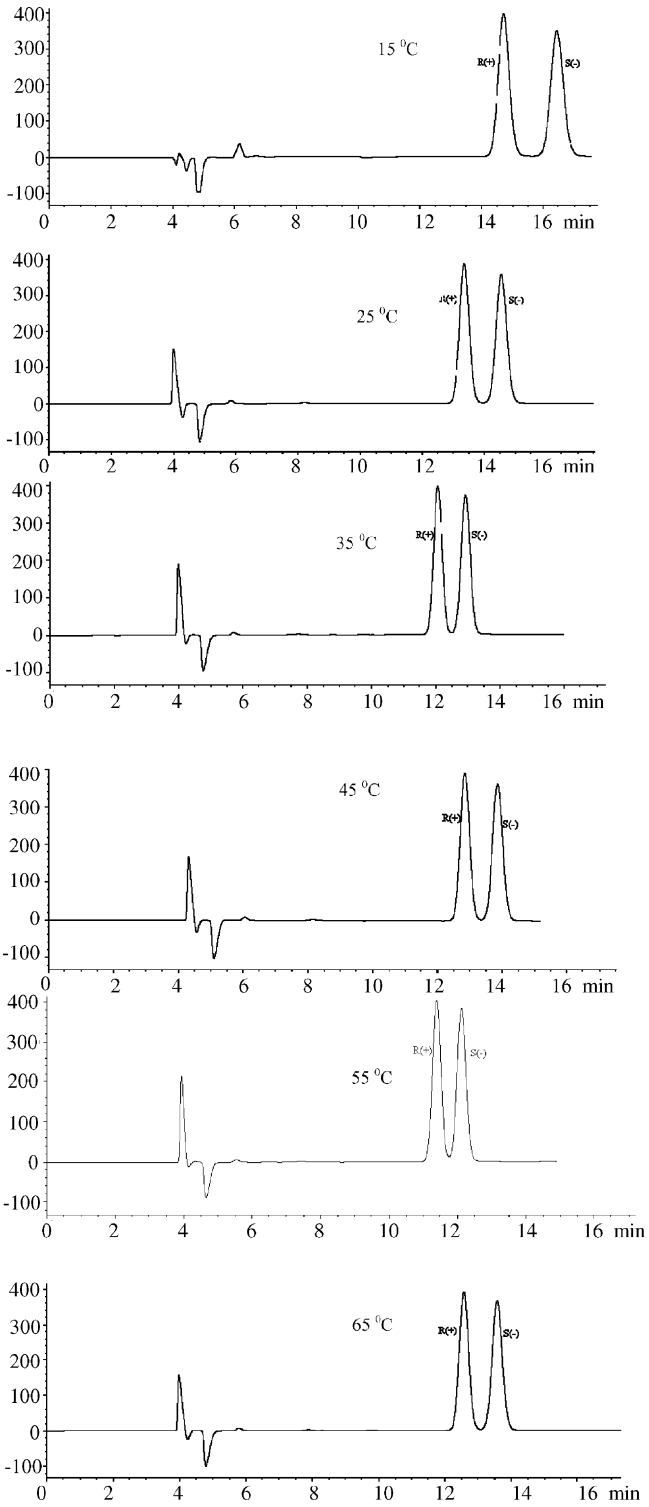


Fig. 3. Effect of temperature upon the separation of DRF 2725 enantiomers.

3.2. Specificity and chromatography

In the given chromatographic conditions, specificity of this method is indicated by the absence of any endogenous interference at retention times of peaks of interest as evaluated by chromatograms of blank rat plasma and plasma spiked with each enantiomers of DRF 2725 and I.S. Under the optimal conditions mentioned above, the experiment for checking the effect of interference at the retention times of individual enantiomers and an I.S. were performed. When single analytes were injected at the highest concentration in the chromatographic system, at the retention times of the other enanntiomer and/or I.S. no interference was observed (data not shown). Both enantiomers viz., *R*(+)-DRF 2725, *S*(−)-DRF 2725 and I.S. were well separated with retention time of 15.8, 17.7 and 22.4 min, respectively. System suitability parameters for the method were as follows: theoretical plates for *R*(+)-DRF 2725, *S*(−)-DRF 2725 and I.S. were 7632, 7273 and 2284, respectively. Asymmetry factor for *R*(+)-DRF 2725, *S*(−)-DRF 2725 was <1.03 and resolution was >2.38 between *R*(+)-DRF 2725 and *S*(−)-DRF 2725; between *S*(−)-DRF 2725 and I.S. was >3.18. Fig. 4 shows a typical overlaid chromatogram for the control rat plasma (free of analyte and I.S.), rat plasma spiked with DRF 2725 enantiomers at the LLOQ (0.3 μg/ml) and an in vivo plasma sample obtained after oral administration of *S*(−)-DRF 2725 (ragaglitazar) at 30 mg/kg.

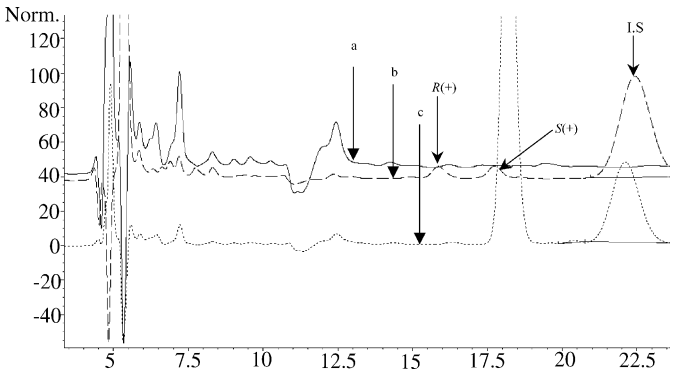


Fig. 4. HPLC chromatograms of a 75 μl injection of (a) rat blank plasma, (b) blank plasma spiked with *R*(+)-DRF 2725 and *S*(−)-DRF 2725 at LLOQ (0.3 μg/ml) and of internal standard (10 μg/ml), and (c) a 2.0 h in vivo plasma sample obtained from rat dosed with *S*(−)-DRF 2725 at 30 mg/kg, p.o.

3.3. Calibration curve and reproducibility

Peak area ratios of each enantiomer of DRF 2725 to the I.S. were measured and acted as a surrogate for quantitation. The results were fitted to linear regression analysis without the use of a weighting factor as well as weighting factor. The weighting options did not show any improvement in the performance of standard curve and quality control samples. Therefore, we have opted to the linear regression without weighting factor for validation of the analytical data. A representative calibration graph of peak-area ratio (individual enantiomer to I.S.) versus each enantiomer concentration in the range of 0.3–50 μg/ml was found to be linear. The average regression ($n = 4$) was 0.999 for both enantiomers

Table 3
Calibration curve of *R*(+)-DRF 2725/*S*(−)-DRF 2725 in rat plasma

Concentration (μg/ml)	Run-1	Run-2	Run-3	Run-4	Mean	% Accuracy
0.30	0.28/0.25	0.29/0.29	0.29/0.34	0.25/0.25	0.28/0.28	92.4/94.2
0.50	0.43/0.44	0.53/0.56	0.55/0.51	0.52/0.53	0.51/0.51	102/102
1.00	0.95/0.97	1.07/1.09	1.11/1.00	1.18/1.20	1.08/1.06	108/106
2.00	1.98/2.06	2.02/2.05	1.96/1.98	1.85/1.87	1.95/1.99	97.7/99.4
5.00	5.03/5.17	4.94/5.01	4.78/4.91	5.14/5.20	4.97/5.07	99.4/101
10.00	10.3/10.3	10.2/10.2	9.99/10.0	10.3/10.4	10.2/10.2	102/102
20.00	19.6/19.9	19.5/19.7	20.2/20.7	19.8/20.0	19.8/20.1	98.8/100
50.00	50.1/50.0	50.2/50.1	50.1/54.1	50.1/51.5	50.1/51.4	100/103
Intercept	0.006/0.012	0.005/0.006	0.007/0.006	0.008/0.009	–	–
Slope	0.051/0.065	0.049/0.061	0.052/0.060	0.051/0.061	–	–
R^2	0.999/0.999	0.999/0.999	0.999/0.999	0.999/0.999	–	–

of DRF 2725 (Table 3). The standard curve had a reliable reproducibility over the standard concentrations of the analyte across the calibration range. The lowest concentration with the R.S.D. <20% was taken as LLOQ [5] and was found to be 0.3 μg/ml for both DRF 2725 enantiomers. The R.S.D. and signal to noise ratio at LLOQ for *R*(+)-DRF 2725, *S*(−)-DRF 2725 were found to be 6.80% and 5.40, respectively. Therefore, on the basis of signal to noise ratio obtained in our experiment it is quite likely that limit of detection could be three-fold lower.

3.4. Precision and accuracy

Accuracy and precision data for intra- and inter-day plasma test samples are presented in Table 4.

3.5. Extraction recovery

The results of the comparison of neat standards versus plasma-extracted standards were estimated at 0.3, 0.9, 15 and 30 μg/ml concentrations for each enantiomer of DRF 2725. The absolute recoveries ranged from 67.2 to 70.2% for DRF 2725 enantiomers across the concentrations. The absolute recovery of internal standard at 50 μg/ml was about 96%.

3.6. Stability

3.6.1. Auto-sampler and bench top stability

Over a period of 12 h injection time in the auto-sampler at 5 °C and over the bench-top for 6 h period, the predicted

Table 4
Intra- and inter-day precision of determination of *R*(+)-DRF 2725/*S*(−)-DRF 2725 in rat plasma

Nominal concentration (μg/ml)	Run	Measured concentration (μg/ml)			
		Mean	S.D.	R.S.D.	Accuracy (%)
Intra-day variation (six replicates at each concentration)					
0.3	1	0.26/0.27	0.02/0.03	6.78/10.5	86.7/91.0
	2	0.27/0.27	0.02/0.03	6.49/12.7	90.3/90.8
	3	0.25/0.27	0.02/0.02	6.07/7.90	83.3/89.4
	4	0.26/0.25	0.01/0.01	2.88/3.58	87.2/83.3
0.9	1	0.93/0.97	0.02/0.02	2.58/1.70	103/108
	2	0.84/0.90	0.07/0.11	8.87/12.3	92.9/101
	3	0.89/0.89	0.01/0.06	1.35/6.56	99.0/98.9
	4	0.84/0.92	0.02/0.05	1.90/5.74	96.1/102
15	1	16.44/16.9	0.42/0.39	2.53/2.33	110/113
	2	15.8/16.0	1.83/1.46	11.5/9.16	106/107
	3	16.0/15.5	0.57/0.23	3.59/1.47	106/103
	4	16.3/16.6	0.31/0.24	1.93/1.43	109/111
30	1	32.0/32.4	0.37/0.34	1.17/1.07	107/108
	2	34.0/34.5	0.95/1.82	2.78/5.27	113/115
	3	30.5/29.8	0.32/0.17	1.06/0.58	102/99.4
	4	31.4/32.1	0.55/0.47	1.74/1.45	105/107
Inter-day variation (24 replicates at each concentration)					
0.3		0.26/0.27	0.01/0.07	1.71/3.77	86.7/90.0
0.9		0.88/0.92	0.04/0.04	4.28/3.87	97.8/102
15		16.1/16.2	0.28/0.62	1.71/3.83	108/108
30		32.0/32.2	1.47/1.90	4.60/5.91	107/108

R.S.D: relative standard deviation (S.D. × 100/mean).

Table 5
Summary of auto sampler stability of DRF 2725 enantiomers

Concentration (μg/ml)	*R*(+)-DRF 2725			*S*(−)-DRF 2725		
	Mean concentration (μg/ml)		% Deviation	Mean concentration (μg/ml)		% Deviation
	0 h	12 h		0 h	12 h	
0.3	0.29	0.29	0.00	0.26	0.25	3.85
0.9	0.98	0.96	2.04	0.97	0.96	1.03
15	16.9	16.9	0.30	17.1	17.0	1.05
30	32.6	32.8	0.52	32.5	32.7	0.62

% Deviation was calculated between 0 and 12 h.

Table 6
Summary of bench top stability of DRF 2725 enantiomers

Concentration (μg/ml)	*R*(+)-DRF 2725			*S*(−)-DRF 2725		
	Mean concentration (μg/ml)		% Deviation	Mean concentration (μg/ml)		% Deviation
	0 h	6 h		0 h	6 h	
0.3	0.26	0.25	1.25	0.24	0.26	8.09
0.9	1.01	1.01	0.20	0.94	1.01	7.00
15	14.6	15.7	7.80	16.5	16.8	1.81
30	31.4	34.2	9.02	30.0	32.1	6.97

% Deviation was calculated between 0 and 6 h.

Table 7
Summary of freeze–thaw stability of DRF 2725 enantiomers at −20 °C in rat plasma

Concentration (μg/ml)	*R*(+)-DRF 2725			*S*(−)-DRF 2725		
	Mean concentration (μg/ml)		% Deviation	Mean concentration (μg/ml)		% Deviation
	Cycle I	Cycle III		Cycle I	Cycle III	
0.3	0.25	0.26	3.84	0.27	0.28	3.70
0.9	0.93	0.98	5.37	0.97	0.96	1.04
15	16.4	15.7	4.56	16.9	16.7	0.88
30	30.0	31.6	5.29	32.4	33.6	3.70

% Deviation was calculated between cycles I and III.

concentrations for *R*(+)-DRF 2725 and *S*(−)-DRF 2725 at 0.3, 0.9, 15 and 30 μg/ml samples deviated within 10–15% of the nominal concentrations (Tables 5 and 6). Generally the results were found to be within the assay variability limits and showed chiral integrity of the individual enantiomers during the entire process.

3.6.2. Freeze/thaw stability

Table 7 shows the results of the analyses of the QC samples following repeated three freeze/thaw cycles. DRF 2725 enantiomers have shown to be stable in the frozen plasma at −20 °C for at least three freeze/thaw cycles. The data also reflect the chiral integrity and stability of the enantiomers during the freeze/thaw process.

3.6.3. Freezer stability

R(+)-DRF 2725 and *S*(−)-DRF 2725 were found to be stable when stored at −20 °C for at least for 15 days. Both accuracy and precision of QC samples in this evaluation were within the assay variability of ±15% reflecting the chiral integrity during a longer term storage.

3.7. Application of the method

After a single oral administration of 30 mg/kg *S*(−)-DRF 2725 (ragaglitazar) to male Wistar rats, the plasma concentrations of *S*(−)-DRF 2725 were determined by the described method. The mean plasma concentration versus time profiles for *S*(−)-DRF 2725 is depicted in Fig. 5. Inspection of Fig. 5 revealed that the newly developed analytical method had the required sensitivity to characterize the absorption, distribution and elimination phases of ragaglitazar or *S*(−)-DRF 2725 following oral dosing. None of the chromatograms showed the presence of the peak of *R*(+)-DRF 2725, indicating that following oral dosing of ragaglitazar there is no chiral inversion of *S*(−)-DRF 2725 to *R*(+)-DRF 2725 in vivo. The pharmacokinetic parameters of *S*(−)-DRF 2725 were calculated using a

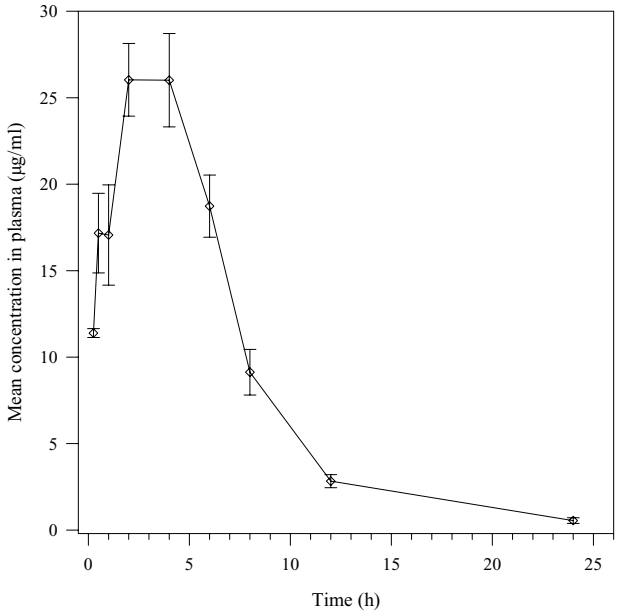


Fig. 5. Plasma concentration vs. time profiles of *S*(−)-DRF 2725 after single dose oral administration of 30 mg/kg in male Wistar rats. The data points are means and standard deviation bars of four observations.

non-compartmental analysis. Maximum concentration in plasma (C_{max} 30.0 ± 6.87 μg/ml) was achieved at 2.00 ± 0.00 h (T_{max}). The half-life ($t_{1/2}$) of ragaglitazar was 4.18 ± 0.37 h, while the $AUC_{(0-\infty)}$ was 206 ± 33.8. Ragaglitazar is primarily excreted via bile as unchanged drug in healthy humans and patients with type II diabetes and no formation of phase II metabolite(s) including acyl glucuronide and/or sulfate conjugate was reported [7]. On the basis of the data generated in this study, the in vivo formation of other antipode *R*(+)-DRF 2725 is unlikely following ragaglitazar oral administration and therefore, employment of a non-chiral method is justified for the quantification of ragaglitazar during the development process.

4. Conclusion

A high-performance liquid chromatography method for the determination of *R*(+)-DRF 2725 and *S*(−)-DRF 2725 in rat plasma was developed and optimized for various conditions and validated. The *R*- and *S*-enantiomers of DRF 2725 and I.S. were well-separated with Chiralpak® column within 25 min of total run time. The developed method was successfully applied to show lack of inversion of ragaglitazar to its antipode following oral administration of ragaglitazar.

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