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An HPLC/UV method for the determination of RGH-1756 in dog and rat plasma

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Abstract

RGH-1756 (1-(2-methoxy-phenyl)-4-{4-[4-(6-imidazo[2,1-b]-thiazolyl)-phenoxy]-butyl}-piperazine dimethansulphonate) is a novel atypical antipsychotic candidate of Gedeon Richter Ltd. A new HPLC method has been developed and validated for the quantitative determination of RGH-1756 in dog and rat plasma. The compound and the internal standard are extracted from the biological samples by a simple and fast liquid–liquid extraction method, using 1-chlorobutane. The recovery for RGH-1756 is about 90%. The extracts are analyzed by reversed phase HPLC (column: Supelcosil-LC-18-DB 250*4.6 mm, 5 μm, eluent:acetonitrile:methanol:0.2 molar ammonium-acetate 40:25:35, $\lambda = 254$ nm). The assay is specific for RGH-1756. The standard curves are linear in the range between 10 and 2000 ng ml^{-1}. The overall precision (expressed as CV%) and accuracy (expressed as bias%) of quality controls and calibration standards are within 15%. The validated lower limit of quantification is 10 ng/ml. No indications have been found for possible instabilities of RGH-1756 in plasma, in the extraction solvent, or after repeated thawing-freezing cycles. The method has been succesfully applied for the bioavailability studies of RGH-1756 in the two animal species. In these studies results of the inprocess method validation have shown the reliability of the method, too. CV% of quality controls in the rat study has been found between 7.4 and 10.0%, in the dog study between 4.1 and 12.5%. The bias has ranged from 0.4 to 3.8% and from −4.5 to 1.2% in the rat and dog study, respectively.

Keywords: RGH-1756; Rat; Dog; Method validation; HPLC

1. Introduction

RGH-1756 (1-(2-methoxy-phenyl)-4-{4-[4-(6-imidazo[2,1-b]-thiazolyl)-phenoxy]-butyl}-piperazine dimethansulphonate) is a novel atypical antipsychotic candidate developed in Gedeon Richter Ltd. Preclinical profile of the drug suggests that this agent could be a new atypical antipsychotic to treat not only positive and negative symptoms but cognitive disturbances of schizofrenia, too [1–7]. In vitro RGH-1756 has a strong and selective antagonist activity on human D_3, and somewhat less pronounced activity on human D_{2L} and $5HT_{1A}$ receptors [5].

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To conduct the pharmacokinetic, bioavailability and toxicokinetic studies necessary in drug development a new HPLC method with UV detection has been developed and validated for the quantitative determination of RGH-1756 in rat and dog plasma. 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 RGH-1756 in rats and dogs.

2. Materials and methods

2.1. Chemicals

RGH-1756 free base (Fig. 1.) and dimethansulphonate salt as well as the internal standard (IS) (6-{4-[2-(4-〈2-methoxy-phenyl〉-piperazine-1-il)-etoxy]-phenyl}-2,3-dihydro-imidazo[2,1-b]thyazol-trihydrochloride) (Fig. 2.) were synthesized at Gedeon Richter Ltd. Chemical purity of RGH-1756 was more than 98%. Acetonitrile, methanol, chlorobutane and tetrahydrofuran were of analytical grade and obtained from Merck (Darmstadt, Germany). Ammonium acetate was MicroSelect and supplied by Fluka (Buchs, Switzerland). Heparinised blank plasma was obtained from Wistar rats and Beagle dogs.

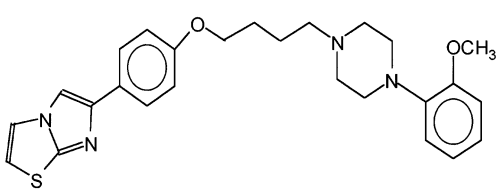

Fig. 1. Chemical structure of RGH-1756.

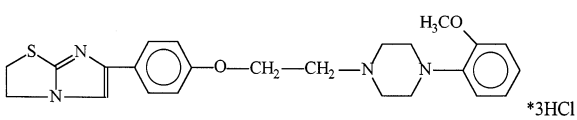

Fig. 2. Chemical structure of the internal standard.

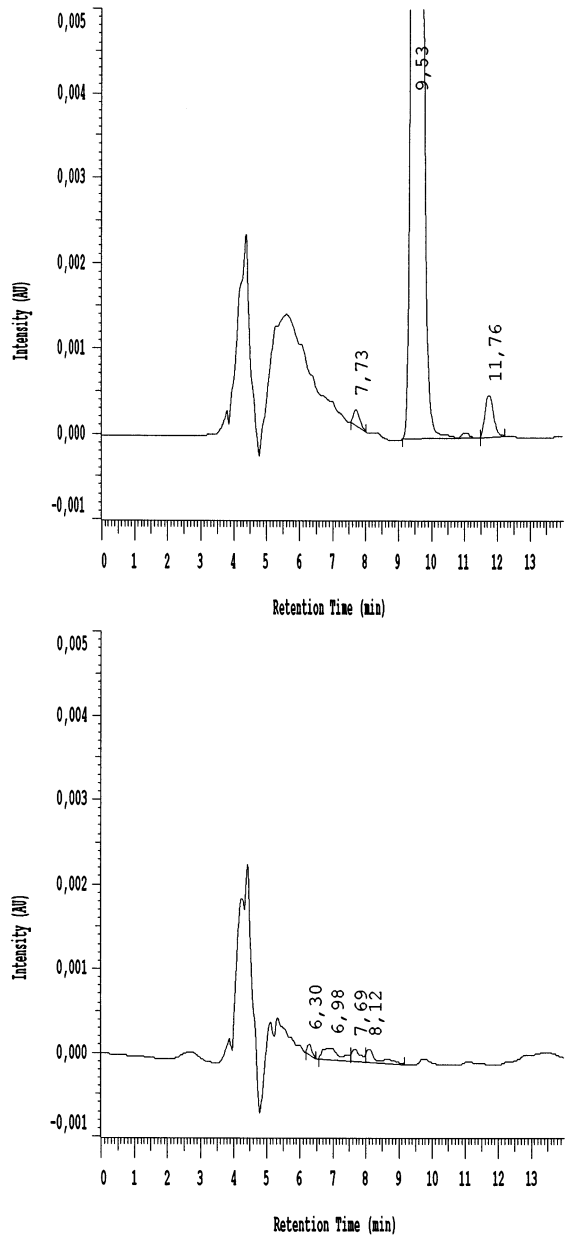

Fig. 3. Typical chromatogram of a calibration sample of 10 ng/ml (up) and a blank dog plasma (down).

2.2. Standard solutions

Calibration and quality control (QC) stock solutions of RGH-1756 base were prepared in tetrahydrofuran. Working solutions were made by

appropriate dilutions of the stock solutions with methanol. IS stock solution of 1 mg/ml and its dilution of 50 μg ml^{-1} were prepared in methanol. All solutions were stored at +4°C for maximum 12 weeks.

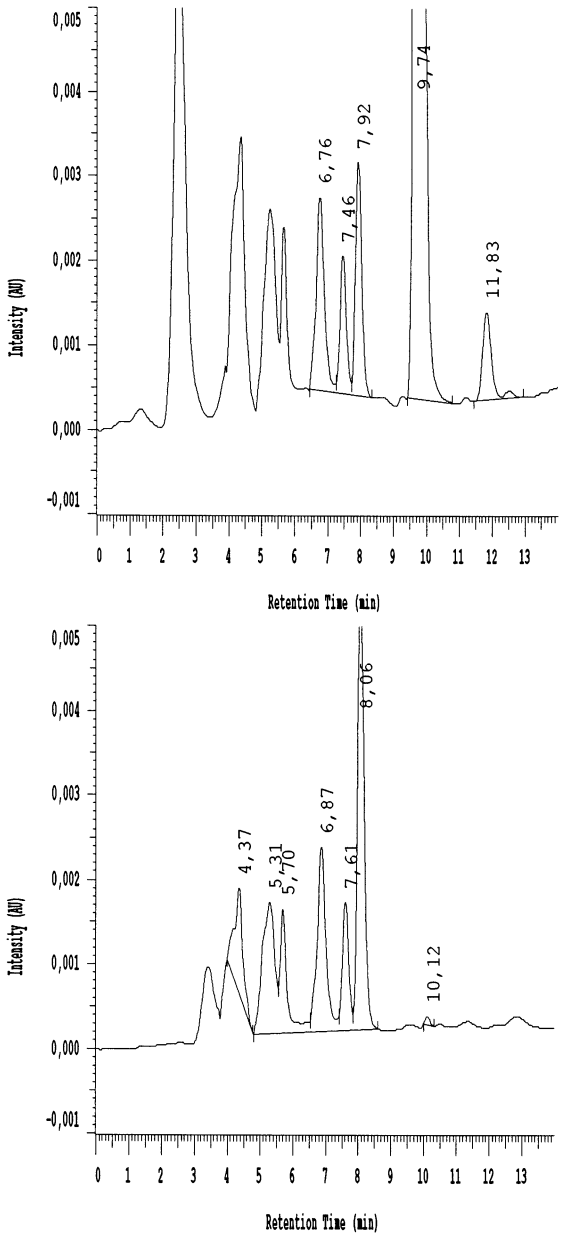

Fig. 4. Typical chromatogram of a calibration sample of 20 ng/ml (up) and a blank rat plasma (down).

2.3. Calibration standards and quality control samples

Calibration standards were prepared by spiking 1 ml of blank plasma with 20 μl of the appropriate calibration working solution in the range from 10 to 2000 ng ml^{-1}. QC samples were prepared in bulk at the concentrations of 10, 20, 100 and 1000 ng ml^{-1} and stored below −18°C.

2.4. Extraction procedure

One milliliter of plasma was spiked with 20 μl IS working solution (1 μg IS) 6 ml of 1-chlorobutane was added and after shaking for 10 min at 210 min^{-1} frequency on a horizontal shaker (HS250 basic, IKA) and centrifugation for 10 min at 2000 rpm (CR422, Jouan), 5 ml of the organic phase was transferred into a glass tube and evaporated to dryness under nitrogen stream in a 40°C water bath. The dry residue of samples was redissolved in 100 μl of methanol by shaking on a vortex mixer and transferred into a conical crimp-cap vial. Fifteen microliter was injected onto the column.

2.5. Chromatographic conditions

The HPLC system LaChrom (Hitachi, Tokyo, Japan and Merck, Darmstadt, Germany) consisted of a D 7000 interface, an L7400 UV detector, an L7360 column oven, an L7200 autosampler and an L7100 pump. D7000 HSM software was used for data processing. Chromatographic separations were accomplished using a Supelcosil LC-18-DB, 5 μm, 4.6 × 250 mm column (Supelco, Bellefonte, PA, USA). The mobile phase consisting of acetonitrile:methanol:0.2 molar ammonium acetate mixture (40:25:35 v/v/v) was pumped isocratically at a flow rate of 0.6 ml min^{-1} at a temperature of 40°C. Eluate was monitored using UV detector set at 254 nm. In the method development the eluent composition was optimized to separate the analytes from the extracted endogenous materials within a reasonable analysis time: the organic component was increased gradually and various acetonitrile:methanol ratios were tested as well. Ammo-

Table 1
Calibration curve parameters in prestudy validation

	Correlation coefficient	Slope $\times 10^{-3}$	Intercept $\times 10^{-3}$
Validation for dog plasma ($n = 5$)			
Mean	0.9998	1.61	−0.41
SD	0.0001	0.05	0.47
CV%	0.01	2.90	
Validation for rat plasma ($n = 10$)			
Mean	0.9996	1.54	1.18
SD	0.0009	0.08	1.11
CV%	0.09	4.95	

Table 2
Precision of the method in prestudy validation for dog and rat plasma

	Nominal concentration of QC samples (ng/ml)			
	10	20	100	1000
Validation for dog plasma (*CV%*)				
Inter-day ($n = 5$)	4.50	2.80	3.72	3.25
Intra-day ($n = 5$)	3.55	3.78	4.08	3.40
Validation for rat plasma (*CV%*)				
Inter-day ($n = 10$)	11.66	6.20	4.72	4.18
Intra-day ($n = 5$)	5.73	6.87	2.55	4.20

Table 3
Accuracy of the method in prestudy validation for dog and rat plasma

	Nominal concentration of QC samples (ng/ml)			
	10	20	100	1000
Validation for dog plasma (*bias%*)				
Inter-day ($n = 5$)	0.20	2.74	−1.24	−1.69
Intra-day ($n = 5$)	−2.18	−1.15	0.90	2.66
Validation for rat plasma (*bias%*)				
Inter-day ($n = 10$)	3.31	−0.29	−0.06	1.03
Intra-day ($n = 5$)	7.54	0.04	−1.19	−1.56

nium acetate was applied to give better peak shape. The flow rate was optimized, too, 0.6 ml min^{-1} resulted in better performance than higher flow rates.

2.6. Prestudy validation

In prestudy validation of the method for dog and rat plasma the following parameters were investigated: selectivity, linearity, precision and accuracy, limit of quantitation (LOQ), stability, sample dilution and recovery.

Selectivity is generally defined as the lack of interfering peaks at the retention times of the assayed drug and the internal standard in the chromatograms. This was tested by the injection of the extracts of blank plasma samples of five individual animals.

Table 4
Stability of RGH-1756

	Validation for dog plasma		Validation for rat plasma	
	Nominal concentration (ng/ml)			
	20	1000	20	1000
Long-term stability (*mean relative error%*)				
2-week storage	8.0	4.6	0.9	−3.8
6-week storage	1.8	−0.8	−2.8	0.2
8-week storage	8.2	−1.8	4.7	5.8
9-week storage	3.8	9.0	Not measured	Not measured
Short-term room temperature stability (*mean relative error%*)				
4 h	9.3	7.8	−4.0	−0.7
Autosampler stability (*difference from reference%*)				
96 h (dog)	2.9	0.5	0.8	−0.8
72 h (rat)	2.9	0.5	0.8	−0.8
Freeze and thaw stability (*mean relative error%*)				
1 cycle	4.7	9.1	0.1	8.0
2 cycles	7.5	7.3	−0.2	2.4
3 cycles	4.5	5.0	−3.6	5.5

Calibration was performed with linear weighted least squares analysis with the weighting factor of $1/y^2$. It gave the best fit when examining the weighting factors of 1, $1/y$ and $1/y^2$. Linearity of the calibration was examined in inter-day assay.

Inter-day precision and accuracy data were obtained by measuring single set of QC samples against daily standard curves. The intra-day performance data (or repeatability) express precision and accuracy of the assay under the same conditions and within one day. To determine the repeatability, five sets of quality controls were analyzed with a standard curve. Accuracy (expressed as bias: percent deviation of the measured versus the nominal values) and precision (expressed as coefficient of variation of the measured values) of the QC samples were calculated.

Stability of RGH-1756 was investigated under different conditions by measuring QC samples of 20 and 1000 ng/ml, these conditions were: in frozen plasma (long-term stability), in plasma at room temperature (r.t.), after repeated freezing and thawing and in the extract at r.t. in the autosampler.

Sample dilution was investigated in three runs by QC samples of 20 000 ng/ml. On each day 100 μl aliquots ($n = 5$) were taken into extraction tubes and made up to 1 ml with 900 μl of blank plasma.

The recovery of the extraction for both RGH-1756 and the internal standard was calculated by comparison of peak height data of extracted calibration standards and those of working solutions in five runs. The examined concentrations were 10, 20, 100, 200, 1000 and 2000 ng ml^{-1} for RGH-1756 and 1000 ng ml^{-1} for the internal standard.

Table 5
Sample dilution

Validation for dog plasma	Run 1 ($n = 5$)	Run 2 ($n = 4$)	Run 3 ($n = 5$)
CV%	2.54	4.28	5.09
bias%	3.77	−1.64	−5.55
Validation for rat plasma	($n = 5$)	($n = 5$)	($n = 5$)
CV%	0.90	1.08	3.18
bias%	2.86	7.34	2.56

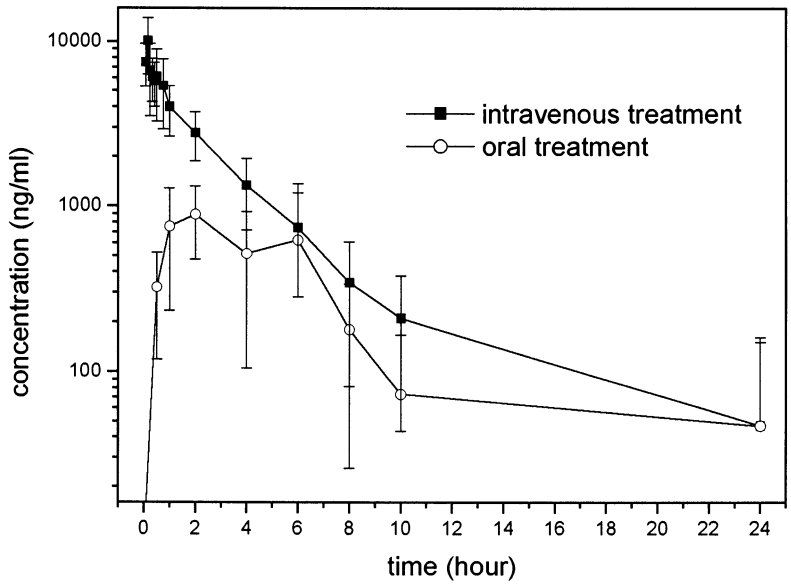

Fig. 5. RGH-1756 plasma concentrations in male Beagle dogs (mean ± SD, $n = 6$).

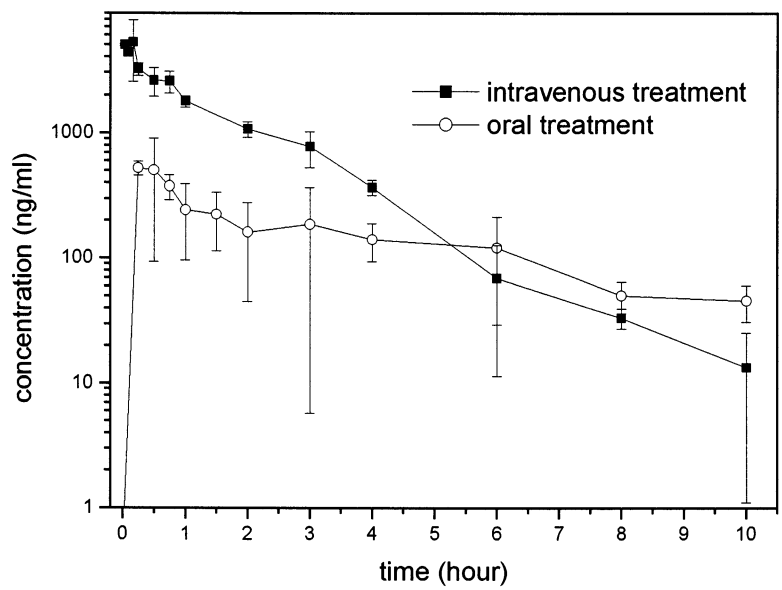

Fig. 6. RGH-1756 plasma concentrations in female Wistar rats (mean ± SD, $n = 3$/time points).

2.7. Application of the method for the bioavailability study of RGH-1756 in dogs and rats

Six male Beagle dogs were treated orally and intravenously at a dose of 14.15 mg kg^{-1} with RGH-1756 dimethansulphonate salt. Intravenous dose was administered as a 15 min-infusion in 5.5% glucose solution. Oral dose was administered in gelatine capsule.

Male and female Wistar rats were treated orally and intravenously at a dose of 10 mg kg^{-1} with

RGH-1756 dimethansulphonate salt. Three male and three female animals were exsanguinated at each of the predetermined sampling timepoints. The plasma samples obtained from these studies were stored below −18°C until the day of analysis. One milliliter of the plasma samples was analysed for RGH-1756 using the method described above. The concentration of the analyte in the samples was calculated in free base and then recalculated for dimethansulphonate salt. Reliability of the method was proven by inprocess validation. Duplicate QC samples were used at 3 concentration levels (20, 100 and 1000 ng ml^{-1}). Chromatographic runs were accepted, if the measured concentration of at least four out of the six QC samples was within nominal ±20% and at least one QC sample of each concentration level met this criteria.

3. Results and discussion

3.1. Specificity

Typical chromatograms of blank and spiked plasma samples are shown on Figs. 3 and 4. No endogenous interfering peaks were visible in blank plasma at the retention times of RGH-1756 ($\approx$9.6 min) and the internal standard ($\approx$11.8 min) thereby confirming the specificity of the analytical method.

3.2. Linearity

Calibration curves obtained from rat or dog plasma were linear in the range of 10–2000 ng ml^{-1}. Calibration curve parameters are given in Table 1. The correlation coefficients ($r = 0.9998 \pm 0.0001$, $r = 9996 \pm 0.0009$ for rat and dog plasma, respectively) indicate linearity over the whole calibration range. The slope of the standard curves showed low variance ($CV_{dog\ plasma} = 2.9\%$, $CV_{rat\ plasma} = 4.95\%$), which proved good inter-day reproducibility of the measurements. Mean intercept was just 2.5 (dog plasma) and 7.7% (rat plasma) of the response at the lowest calibration point.

3.3. Precision

Inter- and intra-day precision was well acceptable (<7%) for both dog and rat plasma. (Table 2.)

3.4. Accuracy

The method was found to be fairly accurate, as bias was within ±3% in inter- and intra-day assays. Relative errors of the mean values are given in Table 3.

3.5. Limit of quantification

The limit of quantitation was set at 10 ng ml^{-1}. Dog plasma samples could be measured with the precision of 4.50% in inter-day and 3.55% in intra-day study and with an accuracy of 0.20% and −2.18% in inter- and intra-day studies, respectively. The corresponding values for rat plasma were: 11.66 and 5.73% (precision) and 3.31 and 7.54% (accuracy) (Tables 2 and 3).

Table 6
Results of the inprocess method validation in bioavailability studies

	Nominal concentration of QC samples (ng/ml)		
	20	100	1000
Inprocess validation in dog study ($n = 8$, in 4 runs)			
Accuracy (bias%)	−4.46	1.20	1.09
Precision (CV%)	12.45	5.29	4.08
Inprocess validation in rat study ($n = 10$, in 5 runs)			
Accuracy (bias%)	3.8	0.4	3.2
Precision (CV%)	7.4	7.7	10.0

3.6. Stability

RGH-1756 was found to be stable in frozen dog plasma for at least 9 weeks, and in rat plasma for 8 weeks. There was no sign of RGH-1756 degradation in the plasma kept at room temperature for at least 4 h and in the reconstituted extracts for at least 72 h. Repeated freezing and thawing had no effect on the stability of RGH-1756 either in rat or in dog plasma (Table 4).

3.7. Sample dilution

If concentration of a trial sample exceeds 2000 ng ml^{-1}, the highest concentration of the calibration curve, a tenfold dilution can be rightly applied. Concentration of the diluted samples could be measured with acceptable error (Bias $< \pm 6\%$ in dog and $< \pm 8\%$ in rat plasma, Coefficient of variation $< 6\%$ in dog and $< 4\%$ in rat plasma) (Table 5).

3.8. Recovery

Average extraction recovery of RGH-1756 and the internal standard from dog plasma was 90.2 and 98.6%, respectively. The same values from rat plasma were: 91.2 and 98.4%.

3.9. Applicability of the method in bioavailability studies

The method described above could be successfully applied for bioavailability studies. Plasma concentration–time curves are shown on Figs. 5 and 6. The reliability of the method was confirmed by the results of the inprocess validation as shown in Table 6. Accuracy was within $\pm 5\%$, CV% characterizing the precision was less than 5% except for QC sample 20 ng ml^{-1} in the dog study, for which it was 12.5%.

4. Conclusion

These experiments confirm that the described method for the determination of RGH-1756 in dog and rat plasma is specific, accurate and precise. The calibration curve is linear and hence the method is suitable for analysis of plasma samples in the concentration range of 10–2000 ng ml^{-1} which can be extended to 20 μg ml^{-1} by the tenfold dilution of the trial samples. There are no stability problems during the usual storage and sample processing procedures. In conclusion, the HPLC method presented here fulfils the criteria generally required from the bioanalytical assays [8] and is suitable for the analysis of RGH-1756 during preclinical drug development.

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