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High-performance liquid chromatographic method for the determination of RGH-5002 in human, rabbit and rat plasma[1]

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Abstract

A sensitive, accurate and reproducible high performance liquid chromatography (HPLC) procedure for the analysis of RGH-5002 in biological fluids was developed. After a single step liquid–liquid extraction at pH 9 using *n*-hexane, RGH-5002 and internal standard were eluted from a Hypersil Si column with 5 mM ammonium acetate–methanol–acetonitrile (0.5:45:50 v/v/v) at 28°C. The method could accurately detect 5 ng ml^{-1} of RGH-5002

Keywords: Assay validation; Muscle relaxant; Normal-phase HPLC; Plasma; RGH-5002

1. Introduction

RGH-5002 is a new centrally acting muscle relaxant [1], which is currently undergoing preclinical studies (Fig. 1). To support preclinical studies and clinical trials a quick, specific and sensitive method was developed and validated for the analysis of RGH-5002 in rat, rabbit and human plasma.

The structural relatives of the compounds tolperisone and epirisone, which are widely used in therapy as antispastic agents, are measured in biological fluids mostly by gas chromatography (GC) techniques [2–5]. GC with nitrogen–phosphorus-flame ionisation detector (NP-FID) detection was also attempted for RGH-5002 in this laboratory but the method requiring program heating to avoid interference from late peaks and proved to be time-consuming and tedious for the analysis of a large number of samples. As an alternative analysis method a high performance liquid chromatography (HPLC) method was developed. Both normal and reversed phase chromatography were tested. Based on the pilot experiments the normal phase seemed more promising for the separation of the compound from the endogenous substances.

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2. Experimental

2.1. Materials and reagents

RGH-5002 (1-dimethyl-4-fluorobenzylsilyl methylpiperidine hydrochloride, and the internal standard benzyldimethyl-pyrrolidinomethylsilane were synthesised by Chemical Works of Gedeon Richter Ltd.

Human plasma was supplied by the National Institute of Haematology (Hungary). Rabbit and rat plasma were produced from drug-free blood drawn from the central artery of rabbit ear or from the femoral vein of rats respectively. Plasma samples of individual rabbits or rats were pooled and kept at −20°C until use.

Hexane was Uvasol grade for spectroscopy, methanol and acetonitrile were Lichrosorb gradient grade (Merck, Darmstadt, Germany), 25% ammonia solution and ammonium acetate obtained from Reanal (Hungary) were analytical grade.

2.2. Calibration standards and quality control (QC) samples

Two stock solutions of RGH-5002 (one for the calibration curve and the other for the control samples) were prepared in methanol at a target concentrtion of 1 mg ml^{-1}. The stock solutions were further diluted with mehtanol to obtain working solutions of appropriate concentrations.

Calibration standards used to generate the calibration curve were prepared on the day of analysis at six concentrations (range 5–500 ng ml^{-1}). To make the desired concentrations of calibration standards for rabbit and rat plasma the respective working solutions were pipetted to 300 μl of methanol, whereas for human plasma aliquots of appropriate working solutions were added to 1.0 ml of blank plasma.

The reason for taking different approaches to the calibration procedure was as follows. The best and most common way is to process the calibration standards just like the experimental samples, i.e. using spiked plasma. However, there are cases where the availability of the blank biological matrix is limited (e.g. liquor, rat plasma) and so animals can be saved by using a calibration procedure without biological matrix. The appropriateness of the approach is checked and proved by the analysis of QC samples.

For the validation of the method QC samples were prepared in bulk at concentrations of 5, 10, 50, 200, 400, 1000 and 4000 ng ml^{-1} by diluting aliquots of the respective working solutions with pretested blank plasma. The samples were divided into 1.0 ml aliquots and stored at −20°C.

2.3. Sample preparation

RGH-5002 was extracted from plasma at a basic pH (pH 8.5–9) with *n*-hexane. Prior to sample processing QC samples at concentrations 1000 and 4000 ng ml^{-1} were diluted with blank plasma (1:10). 100 ng (20 μl) of the internal standard was pipetted into each 1.0 ml aliquot of QC samples and spiked calibration standards. After addition of 150 μl of 5% NH_4OH solution and 8 ml of hexane the samples were shaken mechanically, centrifuged, and the organic phase, transferred to a conical tube, was evaporated to dryness under a nitrogen stream at 40°C. In the case of the calibration standards prepared without plasma, 100 ng of the internal standard was added and the organic solvent was evaporated under a stream of nitrogen at 40°C. In order to minimise sample loss by incomplete recovery in a very small volume all dry residues were first redissolved in 250 μl of methanol and, after evaporation, 55 μl of methanol was added to the sample residues. 40 μl was injected into the chromatographic column.

2.4. HPLC system

A Hewlett-Packard 1050 liquid chromatograph module system consisted of a quaternary pump,

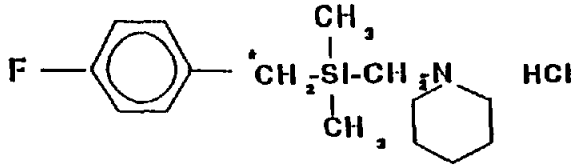

Fig. 1. Structure of RGH-5002.

Table 1
Parameters of calibration curve[a]

	Slope m	Intercept b	$\frac{\text{Intercept} \times 100}{\text{slope} \times X_1}$	Correlation coefficient
Human plasma $n = 5$				
Mean	0.007944	−0.00162	4.2	0.9992
SD	0.000154	0.000634	1.60	0.00104
% RSD	1.9	37.5	38.2	0.1
Rat plasma $n = 4$				
Mean	0.00743	0.00131	3.2	0.9988
SD	0.000120	0.00029	0.97	0.00125
% RSD	1.6	22.3	30.6	0.125
Rabbit plasma $n = 4$[b]				
Mean	0.6878	0.00148	4.9	0.9998
SD	0.0214	0.00228	6.6	0.0002
% RSD	3.11	153.0	137.5	0.02

[a] Calibration curve was plotted by fitting weighted least-squares regression line: $Y = mX + b$ (weighting factor: $W = 1/Y^2$); Y = peak area ratios of RGH-5002 to internal standard; X = concentration of RGH-5002.
[b] X = concentration ratio of RGH-5002 to internal standard, $X_1 = X$ at the lowest calibration point

an auto sampler, a variable-wavelength UV detector operating at 220 nm, and a HPLC^{2D} ChemStation were used. Chromatographic separation was achieved on a normal-phase column (200 × 4 mm^2, 5 μm i.d.) packed with Hypersil, supplied by Bio-Separation-Technology, Hungary. The analysis was carried out at 28°C. The mobile phase was methanol–acetonitrile (1:1) containing ammonium acetate at a concentration of 5 mM. The flow rate was 1.5 ml min^{-1}.

3. Results

3.1. Calibration curve

Quantitation was based on the peak area ratio of analyte to internal standard. On the basis of calibration analysis, calibration curve data were fitted to the linear regression model ($Y = mX + B$) with a weighting factor $W = 1/Y^2$. All calibration lines obtained during method validation showed a small intercept ($\leqslant 5\%$ of mX at the lowest calibration point) and the coefficient of correlation was 0.998 or better in every case. The relative standard deviations (RSDs) of the slopes for rat, rabbit and human plasma were 1.6, 3.1 and 1.9% respectively (Table 1).

3.2. Selectivity

Individual blank plasma samples of each species were processed and chromatographed. There was no interference of endogenous constituents from plasma and from reagents at retention times of either RGH-5002 or the internal standard. Typical chromatograms obtained from extracted plasma samples are shown in Fig. 2. Retention time was approximately 4.6 min for RGH-5002 and 5.3 min for the internal standard.

3.3. Limit of quantification

The limit of quantification of the method was defined as the lowest nominal concentration of the control sample for which the RSD and relative error were $< 20\%$ in the inter-assay analysis. The limit of quantification of the assay was 5.0 ng ml^{-1}, with a precision of RSD = 2.0, 7.3 and 3.0% for human, rat and rabbit plasma. The mean accuracy, expressed as relative error, was 2.7, 2.1 and 4.7% respectively.

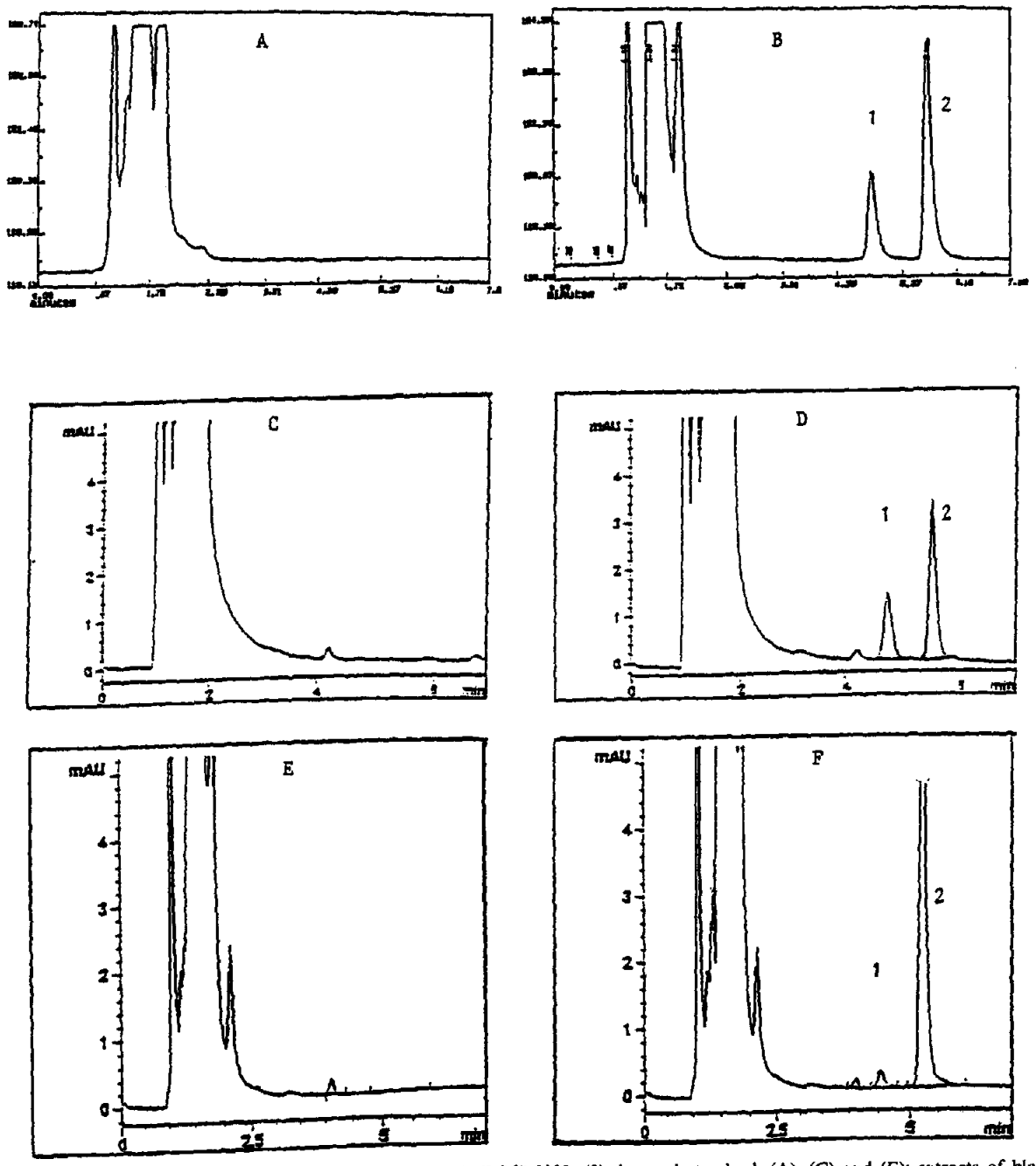

Fig. 2. Chromatograms of blank and control samples. (1), RGH-5002; (2), internal standard. (A), (C) and (E): extracts of blank human, rat and rabbit plasma respectively. (B): extract of human QC sample with 50 ng ml^{-1} RGH-5002; (D) extract of rat QC sample with 50 ng ml^{-1} RGH-5002; (F) extract of rabbit QC sample with 5 ng ml^{-1} RGH-5002.

3.4. Extraction recovery

To simplify and reduce the time of sample preparation the extraction was carried out in one step with a hexane : plasma ration of 8:1. This was the lowest ratio which ensured an aceptable-extraction recovery of at least 70%. The extraction recovery of the analyte was determined by

using radioactive RGH-5002 (the label is shown by the asterisk in Fig. 1). After extraction of the freshly prepared spiked samples the radioactivity of the organic phase was measured by liquid scintillation counting. The extraction recovery was calculated by comparing the amount added and was expressed as a percentage. The recovery was consistent over the concentration range showing slight differences between species (Table 2).

3.5. Precision and accuracy

Accuracy was defined as the difference between determined and nominal concentrations expressed as a percentage of the nominal value (relative error). Precision was given as % RSD of the determined concentration of QC samples.

Intra-assay precision and accuracy of the analytical method for rat and human plasma were determined by measuring replicate sets of QC samples. Inter-assay validation was carried out on different days by analysing a single set of QC samples for each matrix. Comparison of the results of the runs gave the between-batch accuracy and precision. The % RSD of intra-assay analysis of human and rat plasma at different concentrtions ranged from 2.25% to 6.60% and 1.87% to 5.60% respectively (Table 3). The inter-assay accuracy expressed as relative error ranged from −1.10% to 3.12%, −3.88 to 1.43% and −1.76% to 4.65% for human, rat and rabbit plasma respectively. RSD was 8.6% at the most (Table 4).

Table 2
Recovery data for the extraction of RGH-5002 from human, rat and rabbit plasma

Nominal concentration (ng ml^{-1})	Number of samples	Recovery (%)	SD	% RSD
Human				
10	5	88.8	4.4	5.0
200	4	88.8	5.5	6.2
500	4	85.0	1.4	1.6
Rat				
12	5	70.6	6.4	9.0
233	5	77.4	2.6	3.4
583	5	72.7	5.5	7.6
Rabbit				
50	4	90.3	8.3	9.2
500	4	88.6	14.3	16.1

3.6. Stability in plasma stored at −20°C

For each concentration (50 and 400 ng ml^{-1}) five stored samples were analysed against five freshly spiked control samples without a calibration curve. The statistical method applied for the analysis of the data is described by Timm et al., [6]. RGH-5002 was stable at −20°C for at least 4 months in human plasma, and for 7 and 8 weeks in rat and rabbit plasma respectively. No significant or relevant decrease in concentration of RGH-5002 during these storage periods was detected. The lower limit of the 90% confidence interval for the difference between stored and freshly prepared samples did not reach −10% (Table 5).

3.7. The effect of freeze–thaw cycles

The study was done over three cycles at −20°C using samples of human and rat plasma at concentrations of 50 and 400 ng ml^{-1}. For each concentration and each cycle five aliquots of 1 ml were analysed by the HPLC method within one run. The samples of cycle one, which were thawed only once, were used as 100% control. The effect of freeze–thaw cycles was tested statistically as described above for stability. The frozen/thawed samples remained stable under the applied conditions: the 90% confidence intervals were within ±10% (Table 6).

Table 3
Intra-assay precision and accuracy of the method for human and rat plasma

	Nominal concentration (ng ml^{-1})						
	5	10	50	200	400	1000	4000
Human plasma							
Samples (*n*)	5	5	5	5	5	5	5
Mean relative error (%)	−11.55	−5.88	−7.00	−3.21	−2.27	−1.64	−4.72
% RSD	4.12	4.07	2.55	1.87	1.96	2.32	5.60
Rat plasma							
Samples (*n*)	5	5	5	5	5	–	–
Mean relative error (%)	5.27	2.24	3.94	1.95	−1.32	–	–
% RSD	2.53	2.25	3.15	6.60	3.96	–	–

Table 4
Inter-assay precision and accuracy of the method for human, rat and rabbit plasma

	Nominal concentration (ng ml^{-1})						
	5	10	50	200	400	1000	4000
Human plasma							
Samples (*n*)	5	5	5	5	5	5	5
Mean relative error (%)	2.70	3.12	−1.82	−1.14	−1.10	−1.15	0.78
% RSD	1.95	5.50	3.45	4.70	3.73	5.23	2.01
Rat plasma							
Samples (*n*)	3	3	3	3	3	3	3
Mean relative error (%)	−2.12	−3.38	1.43	−0.62	−3.24	−0.74	−2.16
% RSD	7.30	4.13	6.33	7.74	4.51	3.98	3.56
	Nominal concentration (ng ml^{-1})						
	5	10	50	200	400	500	–
Rabbit plasma							
Samples (*n*)	4	4	4	4	4	4	–
Mean relative error (%)	4.65	3.65	−1.76	1.66	2.35	0.70	–
% RSD	2.93	1.87	4.43	7.54	6.27	8.62	–

4. Conclusion

A sensitive, accurate and reproducible HPLC procedure for the analysis of RGH-5002 in human, rat and rabbit plasma was developed. The standard curve obtained by weighted least-squares linear regression was linear from 5.0–500.0 ng ml^{-1}. The method could accurately detect 5 ng ml^{-1} of RGH-5002 using a 1 ml plasma volume. The mean inter-assay accuracy and precision were within 5% and 9% respectively, for all matrixes and concentrations investigated.

The procedure has been successfully applied in toxicokinetic study to the analysis of plasma samples from rats and rabbits receiving a range of oral doses.

Table 5
Stability of RGH-5002 in plasma stored at −20°C

Matrix	Storage period (weeks)	Concentration (ng ml^{-1})	Lower limit (%)	Upper limit (%)
Human	16	50	−1.8	+8.9
	16	400	−6.9	+1.5
Rat	7	50	−5.47	−1.01
	11	50	−14.1	−0.84
	16	400	−14.9	−6.3
Rabbit	8	10	−5.5	+6.8
	8	400	−5.1	+0.4

Table 6
The effect of freeze–thaw cycles

Plasma	Cycle	Concentration (ng ml^{-1})	Lower limit (%)	Upper limit (%)
Human	2	50	−5.5	+4.0
Human	2	400	−4.8	+7.9
Human	3	50	−1.7	+5.5
Human	3	400	−6.7	+2.2
Rat	2	50	−1.7	+6.2
Rat	2	400	+3.0	+9.6
Rat	3	50	+1.6	+7.4
Rat	3	400	−5.7	+2.3

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