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Sensitive LC–MS/MS methods for the quantification of RGH-188 and its active metabolites, desmethyl- and didesmethyl-RGH-188 in human plasma and urine

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Abstract

Selective and sensitive LC–MS/MS methods have been developed and validated for simultaneous determination of RGH-188, a novel atypical antipsychotic, and its two active metabolites, desmethyl- and didesmethyl-RGH-188 in human plasma and urine. Deuterated analytes, [$^2H_6$]-RGH-188, [$^2H_3$]-desmethyl-RGH-188 and [$^2H_8$]-didesmethyl-RGH-188 were used as internal standards (IS). The compounds were isolated from the alkalized biological matrix using liquid–liquid extraction (LLE) and the extracts were analysed by reversed-phase HPLC with MS/MS detection. The chromatographic run time was 5.0 min per injection. The PE Sciex API 365 mass spectrometer was equipped with a TurboIonSpray® interface and operated in positive-ion, multiple reaction monitoring (MRM) mode. The mass transitions monitored were *m/z* 427.3 → 382.2, 413.2 → 382.2, 399.2 → 382.2, 433.3 → 382.2, 416.2 → 382.2 and 407.3 → 390.2 for RGH-188, desmethyl-RGH-188, didesmethyl-RGH-188, [$^2H_6$]-RGH-188, [$^2H_3$]-desmethyl-RGH-188 and [$^2H_8$]-didesmethyl-RGH-188, respectively. The lower limit of quantification (LLOQ) was 0.05 and 0.1 ng/ml for RGH-188 and its metabolites, respectively, using 1 ml of plasma. LLOQ in 1 ml of urine was 0.1 ng/ml for all three analytes. The methods were validated for selectivity, linearity, accuracy and precision. The lower limit of quantification, dilution integrity, matrix effect, stability of the analytes in the biological matrix during short- and long-term storage and after three freeze–thaw cycles were also tested. The assays were simple, specific and robust enough to support clinical development of RGH-188.

Keywords: RGH-188; Desmethyl-RGH-188; Didesmethyl-RGH-188; LC–MS/MS; Human plasma and urine

1. Introduction

RGH-188, trans-4-{2-[4-(2,3-dichlorophenyl)-piperazine-1-yl]-ethyl}-*N*,*N*-dimethylcarbamoyl-cyclohexyl-amine hydrochloride, is a novel atypical antipsychotic [1,2] with potent dopamine D_3/D_2 receptor antagonism/partial agonism, currently in Phase II development for the treatment of schizophrenia and bipolar mania. To support the preclinical development several HPLC and LC–MS/MS methods were developed and validated for quantitative determination of RGH-188 in animal (mouse, rat, dog) plasma. All these methods utilized liquid–liquid extraction (LLE) and could be used for monitoring only the parent compound. The *in vitro* and *in vivo* metabolite profiling studies showed that one of the main metabolic pathways of RGH-188 is dealkylation, yielding two pharmacologically active metabolites, desmethyl- and didesmethyl-RGH-188. Since exposure of the animals to these metabolites was comparable with that to the parent compound, analytical methods for the purpose of human clinical studies were required to provide simultaneous quantification of all the three compounds.

This paper describes two validated methods combining LLE and liquid chromatography coupled to tandem mass spectrometry for the simultaneous determination of RGH-188 and its active metabolites in human plasma and urine. In order to improve the assay ruggedness, deuterated analytes were used as internal standards (IS). The chemical structures of RGH-188, desmethyl- and didesmethyl-RGH-188 and their

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IS, [$^2H_6$]-RGH-188, [$^2H_3$]-desmethyl-RGH-188 and [$^2H_8$]-didesmethyl-RGH-188 are shown in Fig. 2. The LLE procedure has been optimized in order to obtain sufficiently high recoveries for all the three analytes and the MS/MS conditions were also investigated and adjusted in order to achieve quantification of as low concentrations as possible since the doses of RGH-188 to healthy volunteers in first in man studies were expected to be very low. The developed methods were validated for performance parameters such as selectivity, linearity, accuracy and precision.

2. Experimental

2.1. Materials

RGH-188 HCl (purity 99.16%), desmethyl-RGH-188 (purity 98.3%), didesmethyl-RGH-188 (purity 99.1%), [$^2H_6$]-RGH-188 (IS, purity 99.9%), [$^2H_3$]-desmethyl-RGH-188 (IS, purity 97.8%) and [$^2H_8$]-didesmethyl-RGH-188 (IS, purity 98.8%) were manufactured in-house at Gedeon Richter Plc. (Budapest, Hungary). Methanol, *tert*-buthyl-methyl-ether and 1-chlorobutane were of HPLC grade from Merck (Darmstadt, Germany). Ammonia solution 32% and buffer solution pH 11 (boric acid/potassium chloride/sodium hydroxide) were of reagent grade also from Merck. Water was produced at HPLC grade in-house by an Elga and PureLab ultra water purificator. Ammonium acetate was of reagent grade from Fluka (Buchs, Switzerland). Blank human plasma containing EDTA K3 as anticoagulant and human urine collected from healthy donors were stored below −20 °C until use.

2.2. Preparation of solutions, calibration standards and validation QC samples

Separate stock solutions of RGH-188, desmethyl- and didesmethyl-RGH-188 were prepared at concentration of 0.1 mg/ml (calculated for pure free base) in methanol. Combined working solutions were prepared from the stock solutions at concentrations of 25, 2.5, 2, 0.5, 0.1, 0.02, 0.01 and 0.005 μg/ml for all three analytes using methanol as solvent. When stored below +10 °C these stock- and working solutions were stable for at least 12 weeks. Internal standard stock- and working solutions were prepared in a similar way. IS working solution with concentration of 0.01 μg/ml for all three IS was freshly prepared on each day of analysis from an intermediate working solution of 1 μg/ml using water as solvent.

Calibration standards were freshly prepared on each day of analysis by adding 10 μl of the appropriate combined standard working solution to a 1 ml aliquot of blank biological fluid. Standards were prepared at concentrations of 0.05, 0.1, 0.2, 1, 5, 20 and 25 ng/ml for plasma and 0.1, 0.2, 1, 5, 20 and 25 ng/ml for urine.

Validation QC samples at levels of 0.05, 0.1, 0.25, 2.5, 20, 250 and 500 ng/ml were prepared in 5, 25 and 50 ml pools by spiking blank human plasma with appropriate amount of combined working solutions of RGH-188 and its metabolites. The amount of methanol in the QC samples was ≤2%. Validation QC samples for urine analysis were prepared in the same way at levels of 0.1, 0.25, 2.5 and 20 ng/ml for all three analytes. All QC samples were divided into aliquots and stored below −20 °C until use.

2.3. Plasma extraction procedure

1 ml of each sample except blank was spiked with 100 μl of combined internal standard working solution (0.01 μg/ml for all three IS) and vortex mixed. 1 ml of ammonia solution (ammonia 32%–water (1:4, v/v)) was added into all tubes. After vortexing, 6 ml of *tert*-butyl-methyl-ether was added. The sample was shaken for 20 min using a horizontal shaker at 240 strokes/min, and centrifuged for 5 min at 2000 *g* at approximately +4 °C. 5 ml of upper organic layer was transferred into a glass tube and evaporated to dryness under nitrogen stream in a 40 °C water bath. The residue was reconstituted in 100 μl of methanol–water (9:1, v/v) by vortexing for 60 s, and transferred into a chromatographic vial containing low-volume inserts. 40 μl of reconstituted sample was injected into the LC column for analysis.

2.4. Urine extraction procedure

1 ml of each sample except blank was spiked with 100 μl of combined internal standard working solution (0.01 μg/ml for all three IS) and vortex mixed. 1 ml of buffer solution (pH 11, boric acid/potassium chloride/sodium hydroxide) was added into all tubes. After vortexing, 6 ml of 1-chlorobutane was added. The sample was shaken for 20 min using a horizontal shaker at 240 strokes/min, and centrifuged for 5 min at 2000 *g* at approximately +4 °C. 5 ml of upper organic layer was transferred into a glass tube and evaporated to dryness under nitrogen stream in a 40 °C water bath. The residue was reconstituted in 100 μl of methanol–water (9:1, v/v) by vortexing for 40 s, and transferred into a chromatographic vial containing low-volume inserts. 40 μl of reconstituted sample was injected into the LC column for analysis.

2.5. Chromatographic conditions

The LC system was an Agilent 1100 system (Agilent Technologies, Waldbronn, Germany) consisted of degasser, binary pump, thermostatted autosampler and column oven. The analytes were separated on an XTerra RP_{18}, 150 mm × 4.6 mm, 5 μm (Waters, Ireland) column equipped with an XTerra RP_{18}, 20 mm × 3.9 mm, 5 μm (Waters, Ireland) precolumn, which were both maintained at 40 °C. The chromatographic analysis was performed under isocratic conditions. The mobile phase was methanol–ammonium acetate (10 mM) (90:10, v/v) at a flow rate of 1 ml/min, which was split so that approximately 250 μl/min was directed towards the mass spectrometry (MS) interface. The chromatographic run time was 5.0 min per injection. The samples were kept at approximately +10 °C in the autosampler.

2.6. Mass spectrometric conditions

A PE Sciex API 365 triple quadrupole mass spectrometer (Sciex, Toronto, Canada) equipped with a TurboIonSpray® interface was used for MS detection. The mass spectrometer was operated in positive-ion, multiple reaction monitoring (MRM) mode. The temperature and flow rate of the turbo gas was adjusted to 300 °C and 8 l/min, respectively. The ionization voltage was set to 4500 V. Nitrogen was used as curtain gas, nebulizing gas and collision gas, their flows were at instrument settings of 8, 10 and 3, respectively. Collision energy was 36 V for RGH-188 and its IS, 32 V for desmethyl-RGH-188 and its IS, and also 32 V for didesmethyl-RGH-188 and its IS. Singly charged precursor-product ion (MS–MS) transitions were monitored at *m/z* 427.3 → 382.2, 413.2 → 382.2, 399.2 → 382.2, 433.3 → 382.2, 416.2 → 382.2 and 407.3 → 390.2 for RGH-188, desmethyl-RGH-188, didesmethyl-RGH-188, [$^2H_6$]-RGH-188, [$^2H_3$]-desmethyl-RGH-188 and [$^2H_8$]-didesmethyl-RGH-188, respectively. The dwell time was 200 ms for all the six components. The Q1 and Q3 quadrupoles were maintained at unit resolution.

2.7. Data processing and quantification

The mass spectrometric data acquisition and the data analysis were done with the Analyst Version 1.2. software (Applied Biosystems/MDS SCIEX, Concord, Canada). A weighted $1/y^2$ linear regression was used to generate calibration curve from standards and calculate the concentrations of quality control samples. Equation of the standard curve: $y = mx + b$, where "*y*" is the peak area ratio of the analyte to IS, "*x*" is the theoretical concentration of the analyte divided by the theoretical concentration of IS, "*m*" is the slope and "*b*" is the intercept of the regression line.

2.8. Validation procedure

The methods were validated for selectivity, linearity, intra- and inter-batch accuracy and precision. The lower limit of quantification (LLOQ), sample dilution (only for plasma), matrix effect, stability of the analytes in the biological matrix during short- and long-term storage and after three freeze–thaw cycles as well as stability of the analytes in the reconstituted samples were also tested.

Selectivity of the methods was investigated by analysing individual blank plasma or urine samples of six donors for endogen interference with the analytes. Five calibration standards at LLOQ were used as reference samples.

For investigation of the calibration model calibration standards were prepared in five replicates and analysed in a single batch. Plasma standards were prepared at concentrations of 0.05, 0.1, 0.2, 1, 5, 20 and 25 ng/ml (standards at concentration of 0.05 ng/ml were evaluated only for RGH-188). Urine standards were prepared at concentrations of 0.1, 0.2, 1, 5, 20 and 25 ng/ml. A separate calibration line was constructed for RGH-188, desmethyl- and didesmethyl-RGH-188 by using linear weighted least squares analysis ($w = 1/y^2$).

Accuracy and precision were determined for each analyte by analysis of five replicates of combined validation QCs at defined concentration levels in three validation batches. The investigated concentration levels in plasma were 0.1, 0.25, 2.5, 20 ng/ml for RGH-188 and 0.25, 2.5, 20 ng/ml for the metabolites, while in urine they were 0.25, 2.5, 20 ng/ml for all three analytes.

The prior set quantification limit (0.05 ng/ml for RGH-188 and 0.1 ng/ml for the metabolites in plasma, 0.1 ng/ml for all three analytes in urine) was examined by analysis of five replicates of QC samples at LLOQ in three validation batches for assay of accuracy and precision.

The impact of dilution was tested only in plasma, for each analyte, by analysis of five replicates of QC samples at concentration levels of 250 and 500 ng/ml with 10- and 20-fold dilution, respectively, in three validation batches for assay of accuracy and precision.

Matrix effect was investigated on six different batches of blank plasma or urine. Spiked samples at a low concentration of 0.2 ng/ml were prepared in duplicates from each of the six matrix batches and then analysed as per method procedures with internal standards.

Stability tests were performed at concentration levels of 0.25 and 20 ng/ml using five replicates of QC samples subjected to different storage conditions and, as a reference, five replicates of freshly prepared QC samples.

3. Results and discussion

3.1. Development of LLE procedure

The LLE procedure was optimized in order to obtain sufficiently high recoveries for all the three analytes. The recovery was assessed by comparing the analyte peak areas from extracted samples and following injection of standard solutions. The analytical methods available for quantification of RGH-188 in animal (mouse, rat, dog) plasma utilized liquid–liquid extraction with 1-chlorobutane (CBT). When using CBT for extraction of RGH-188 and its metabolites from alkalized human plasma, excellent recovery (>90%) was observed for RGH-188 and its desmethyl-metabolite, however the recovery for didesmethyl-RGH-188 was only 43%. In order to increase the recovery for didesmethyl-RGH-188 with keeping the high recovery for the parent compound and its desmethyl-metabolite, several organic solvents (CBT, *tert*-butyl-methyl-ether (TBME), hexane (HEX)) and pH conditions (neutral and alkaline) were investigated. Alkalization was done using ammonia solution (ammonia 32%–water (1:4, v/v)). As shown in Fig. 1 the maximum recovery reached for didesmethyl-RGH-188 was 54% using extraction with TBME from alkalized plasma. Although the recovery for RGH-188 and desmethyl-RGH-188 decreased significantly (56% vs. 98% and 51% vs. 96%, respectively), these extraction conditions were chosen as a compromise. Double extraction and SPE were also tried but even lower recoveries were obtained.

For extraction of the analytes from human urine CBT and TBME was tested at different pH values. As shown in Fig. 1 the recovery increased with increasing pH for all the three analytes.

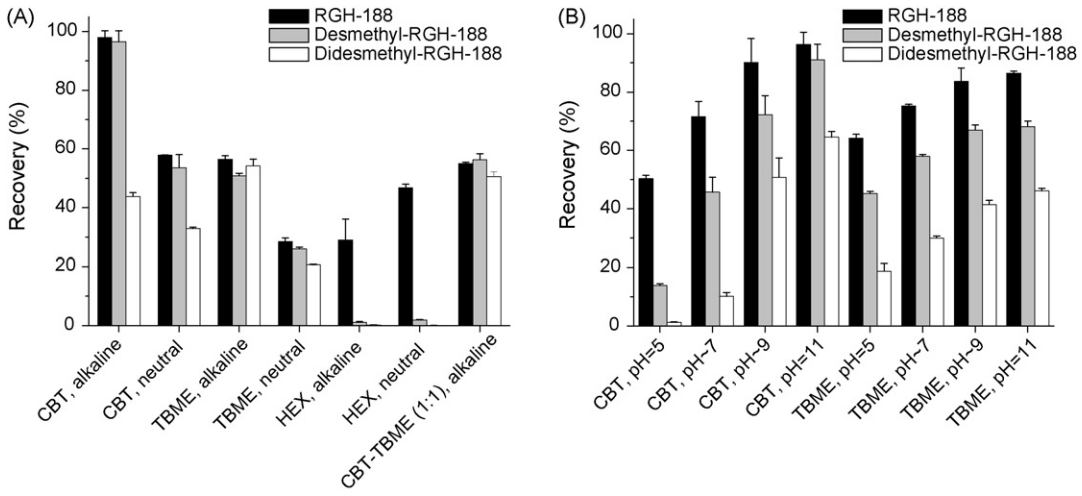

Fig. 1. Recovery for RGH-188 and its metabolites obtained with liquid–liquid extraction of plasma (A) and urine (B) samples containing the analytes at concentration of 1 ng/ml ($n = 2$).

The maximum recoveries (96%, 91% and 65% for RGH-188, desmethyl- and didesmethyl-RGH-188, respectively) were obtained using CBT at pH 11, which was provided using a commercially available buffer solution (pH 11, boric acid/potassium chloride/sodium hydroxide).

3.2. Optimization of MS conditions

For optimization of MS conditions, each compound (in 1 μg/ml methanol solution) was directly infused into the mass spectrometer at a flow rate of 0.6 ml/h using a Harward syringe pump (Harward Apparatus, Saint-Laurent, Canada) and parameters such as ionization voltage, focusing and declastering potential, flow of curtain and nebulizer gas were investigated in order to obtain the maximum intensity for the protonated pseudomolecular ions of the analytes and the internal standards. On the full scan mass spectra of RGH-188, desmethyl-RGH-188, didesmethyl-RGH-188, [$^2H_6$]-RGH-188, [$^2H_3$]-desmethyl-RGH-188 and [$^2H_8$]-didesmethyl-RGH-188 the pseudomolecular ions [MH^+] were observed at a mass to charge ratio (m/z) of 427.3, 413.2, 399.2, 433.3, 416.2 and 407.3, respectively.

The product ion scan resulted in a major fragment at m/z 382.2 for RGH-188, desmethyl-RGH-188, didesmethyl-RGH-188, [$^2H_6$]-RGH-188 and [$^2H_3$]-desmethyl-RGH-188 and m/z 390.2 for [$^2H_8$]-didesmethyl-RGH-188. Collision energy and collision cell exit potential were investigated in order to obtain the best product ion/precursor ion intensity ratio. As shown in Fig. 2, the maximum attainable ratio decreased in the order of RGH-188, desmethyl- and didesmethyl-RGH-188, therefore sensitivity for the metabolites was lower than that for the parent compound. Fig. 2 also shows the structures for the fragment ions.

3.3. Selectivity

No interfering peaks were detected in any extracts from the individual blank human plasma and urine samples, therefore selectivity of the methods has been proved. Representative chromatograms of standards at LLOQ and blank plasma and urine extracts are presented in Figs. 3 and 4.

3.4. Linearity of the calibration curve

Back-calculated concentration, accuracy and precision of the calibration standards and calibration curve parameters (slope, intercept, correlation coefficient) are listed in Tables 1 and 2. The correlation coefficient was >0.998 for each calibration curve. The bias% for the back-calculated concentration of calibration standards ranged from −2.9 to 5.1%, and the CV% was ≤7.4% over the whole calibration range for all three analytes in both biological matrix. The standards showed a linear relationship between the response and concentration using weighted ($1/y^2$) least square linear regression.

3.5. Accuracy and precision, LLOQ, dilution integrity

As shown by the data in Table 3 for plasma and Table 4 for urine, the intra-batch accuracy (expressed as %inaccuracy) evaluated from low, medium and high level validation QC samples was within ±7.4%. The inter-batch accuracy evaluated at the same concentrations ranged from −4.3 to 2.9%. The intra- and inter-batch precision (expressed as CV%) was ≤7.5% and ≤6.7%, respectively.

LLOQ was characterized by intra- and inter-batch accuracy and precision data obtained for QC samples at concentration of 0.05 ng/ml for RGH-188 and 0.1 ng/ml for the metabolites in plasma, as well as 0.1 ng/ml for all three analytes in urine. The QC samples at LLOQ were determined with sufficient accuracy and precision (Tables 3 and 4).

Dilution integrity was investigated only for plasma. Since the QC samples at concentrations of 250 and 500 ng/ml were determined with sufficient accuracy and precision (Tables 3 and 4), the samples with concentrations above 25 ng/ml (the upper limit of the calibration range) can be reliably measured by 10- or 20-fold dilution with blank plasma.

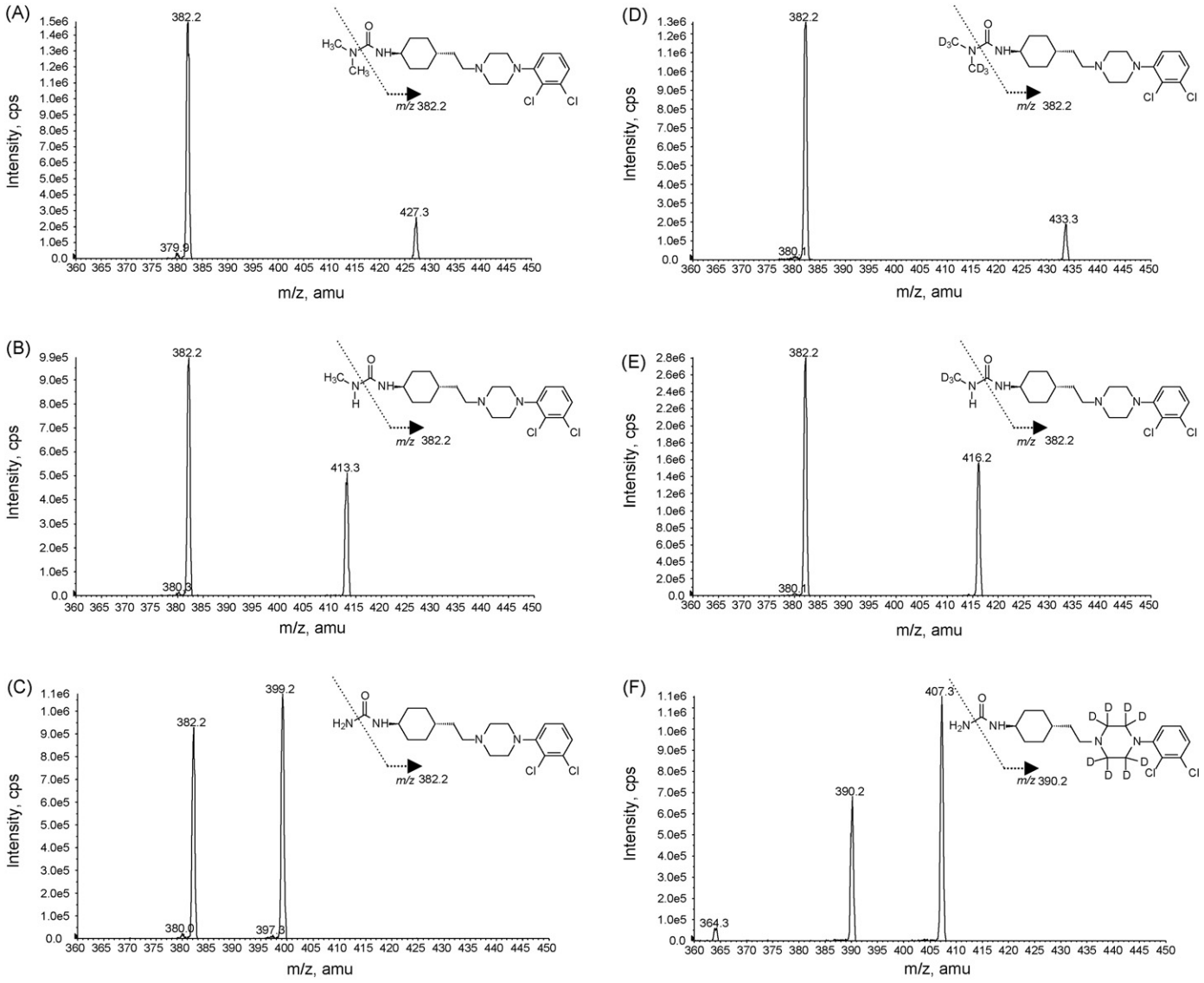

Fig. 2. (+)ESI–MS/MS spectra for RGH-188 (A), desmethyl- (B) and didesmethyl-RGH-188 (C) and their IS, i.e. [$^2H_6$]–RGH–188 (D), [$^2H_3$]-desmethyl-RGH–188 (E) and [$^2H_8$]-didesmethyl–RGH–188 (F).

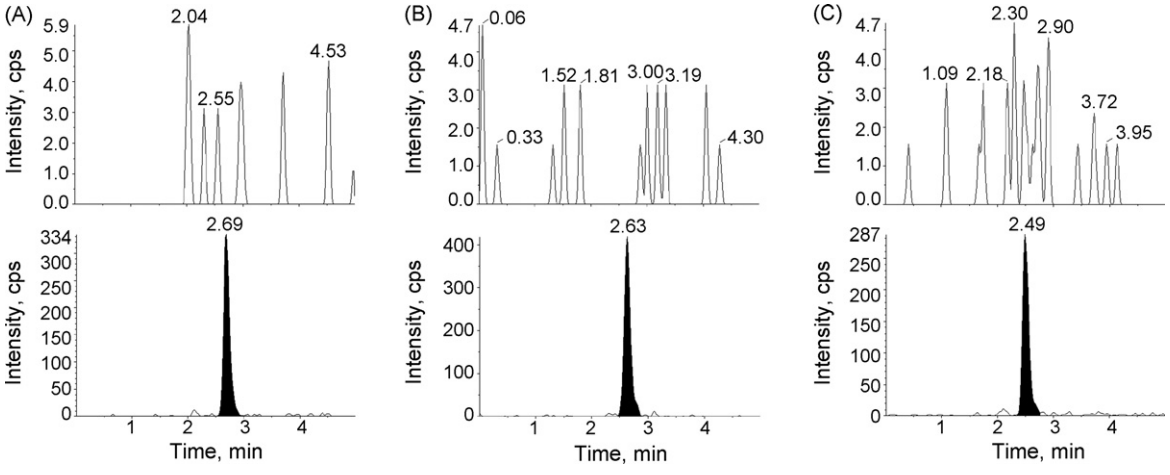

Fig. 3. Representative chromatograms of calibration standards at LLOQ and blank plasma extracts for RGH-188 (A), desmethyl- (B) and didesmethyl-RGH-188 (C).

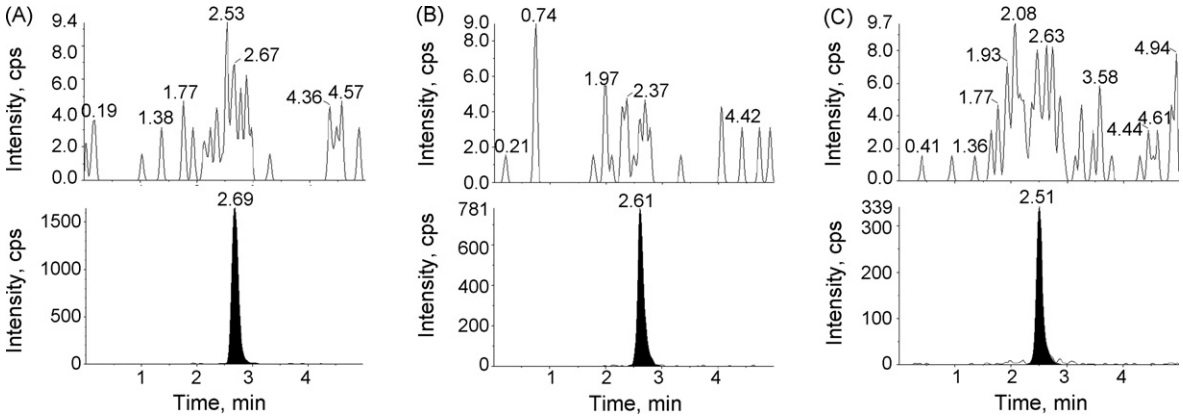

Fig. 4. Representative chromatograms of calibration standards at LLOQ and blank urine extracts for RGH-188 (A), desmethyl- (B) and didesmethyl-RGH-188 (C).

3.6. Matrix effect

There are a number of reports in literature dealing with the effect of the matrix on the determination of compounds from biological fluids using LC–MS/MS assays with minimal sample cleaning and short analysis time [3–5]. In order to decrease the potential ion suppression effect caused by co-eluting components of the sample extracts, internal standards should preferably be eluted with the same retention time as the analytes. It could be achieved with the usage of deuterated analytes as internal standards. For each analyte and both matrices the absolute peak area varied significantly from individual to individual, however the interference could be eliminated by the stable isotope IS since the matrix effect was the same for the analytes and the corresponding internal standards. As an example, Fig. 5 shows the peak area values for didesmethyl-RGH-188 when extracted from six

Table 1
Linearity of the calibration curves for RGH-188, desmethyl- and didesmethyl-RGH-188 in human plasma

Nominal concentration (ng/ml)	Concentration found (ng/ml)					*n*	Mean	S.D.	CV%	Bias%
RGH-188										
0.05	0.0552	0.0469	0.0488	0.0477	0.0509	5	0.04990	0.003322	6.7	−0.2
0.1	0.101	0.0960	0.114	0.107	0.0989	5	0.1034	0.00718	6.9	3.4
0.2	0.199	0.188	0.204	0.201	0.196	5	0.1976	0.00611	3.1	−1.2
1	0.993	0.950	1.01	1.03	0.968	5	0.9902	0.03199	3.2	−1.0
5	5.07	5.08	4.68	5.05	4.95	5	4.966	0.1680	3.4	−0.7
20	20.5	19.6	21.1	19.8	18.6	5	19.92	0.947	4.8	−0.4
25	26.1	26.6	25.0	25.4	25.5	5	25.72	0.630	2.4	2.9
Slope	0.994									
Intercept	0.00136									
r	0.9985									
Desmethyl-RGH-188										
0.1	0.0992	0.113	0.100	0.0994	0.0997	5	0.10226	0.006011	5.9	2.3
0.2	0.190	0.188	0.197	0.196	0.201	5	0.1944	0.00532	2.7	−2.8
1	0.968	1.03	0.968	0.949	1.07	5	0.9970	0.05100	5.1	−0.3
5	4.92	5.17	4.89	4.90	5.11	5	4.998	0.1318	2.6	0.0
20	20.2	20.4	20.0	20.4	19.7	5	20.14	0.297	1.5	0.7
25	27.0	26.0	25.0	25.6	24.0	5	25.52	1.119	4.4	2.1
Slope	1.01									
Intercept	0.00220									
r	0.9989									
Didesmethyl-RGH-188										
0.1	0.0938	0.102	0.0944	0.104	0.110	5	0.1008	0.00682	6.8	0.8
0.2	0.189	0.183	0.204	0.217	0.213	5	0.2012	0.01481	7.4	0.6
1	0.957	1.07	1.02	0.975	1.01	5	1.006	0.0438	4.4	0.6
5	4.90	4.83	5.08	4.71	4.76	5	4.856	0.1443	3.0	−2.9
20	20.0	20.7	19.6	20.3	19.6	5	20.04	0.472	2.4	0.2
25	25.6	25.5	26.4	25.1	26.2	5	25.76	0.532	2.1	3.0
Slope	0.881									
Intercept	0.00014									
r	0.9984									

Table 2
Linearity of the calibration curves for RGH-188, desmethyl- and didesmethyl-RGH-188 in human urine

Nominal concentration (ng/ml)	Concentration found (ng/ml)					n	Mean	SD	CV%	Bias%
RGH-188										
0.1	0.0986	0.102	0.100	0.101	0.0987	5	0.1001	0.00147	1.5	0.1
0.2	0.191	0.208	0.196	0.200	0.208	5	0.2006	0.00747	3.7	0.3
1	0.993	1.02	0.977	1.02	0.974	5	0.9968	0.02238	2.2	−0.3
5	5.05	5.05	5.07	4.94	5.03	5	5.028	0.0512	1.0	0.6
20	20.4	20.0	19.7	19.8	20.9	5	20.16	0.493	2.4	0.8
25	24.8	24.7	24.7	24.8	24.7	5	24.74	0.055	0.2	−1.0
Slope	1.22									
Intercept	0.00335									
r	0.9997									
Desmethyl-RGH-188										
0.1	0.101	0.101	0.104	0.105	0.0925	5	0.1007	0.00492	4.9	0.7
0.2	0.192	0.199	0.205	0.202	0.194	5	0.1984	0.00541	2.7	−0.8
1	1.00	1.02	0.976	0.992	1.01	5	1.000	0.0169	1.7	0.0
5	5.08	5.00	5.05	4.91	4.96	5	5.000	0.0682	1.4	0.0
20	20.8	20.7	20.5	18.9	19.4	5	20.06	0.856	4.3	0.3
25	25.2	24.9	24.6	26.0	25.0	5	25.14	0.527	2.1	0.6
Slope	1.12									
Intercept	0.00422									
r	0.9993									
Didesmethyl-RGH-188										
0.1	0.0999	0.0980	0.105	0.0953	0.0914	5	0.09792	0.005085	5.2	−2.1
0.2	0.211	0.219	0.219	0.196	0.206	5	0.2102	0.00968	4.6	5.1
1	1.06	1.01	0.978	1.08	1.08	5	1.042	0.0456	4.4	4.2
5	5.11	4.91	5.13	4.96	4.90	5	5.002	0.1103	2.2	0.0
20	19.9	19.2	19.8	19.8	19.2	5	19.58	0.349	1.8	−2.1
25	24.7	24.6	24.5	22.5	25.7	5	24.40	1.166	4.8	−2.4
Slope	1.26									
Intercept	0.00662									
r	0.9984									

Table 3
Accuracy and precision of the validation QC samples for RGH-188, desmethyl- and didesmethyl-RGH-188 in human plasma

QC level	Nominal concentration (ng/ml)	Intra-batch accuracy (%inaccuracy, $n = 5$, 3 days)	Inter-batch accuracy (%inaccuracy, $n = 15$)	Intra-batch precision (CV%, $n = 5$, 3 days)	Inter-batch precision (CV%, $n = 15$)
RGH-188					
LLOQ	0.05	Within ±11.0	8.2	≤10.1	8.0
Low 1	0.1	Within ±3.5	2.3	≤7.5	6.5
Low 2	0.25	Within ±3.2	−1.2	≤5.8	4.4
Medium	2.5	Within ±4.6	2.5	≤6.1	4.7
High	20	Within ±1.0	0.3	≤5.1	3.1
Dilution 1	250	Within ±2.2	0.7	≤3.1	2.7
Dilution 2	500	Within ±3.8	1.6	≤4.1	4.0
Desmethyl-RGH-188					
LLOQ	0.1	Within ±10.1	1.6	≤12.2	11.0
Low	0.25	Within ±5.9	−0.5	≤6.6	6.7
Medium	2.5	Within ±3.8	1.9	≤6.4	5.3
High	20	Within ±3.8	0.2	≤5.1	4.9
Dilution 1	250	Within ±4.0	0.7	≤2.9	3.6
Dilution 2	500	Within ±2.2	0.9	≤4.7	4.4
Didesmethyl-RGH-188					
LLOQ	0.1	Within ±12.7	5.2	≤10.9	8.7
Low	0.25	Within ±5.9	2.9	≤6.1	5.3
Medium	2.5	Within ±2.9	−1.4	≤5.3	4.4
High	20	Within ±7.4	−4.3	≤3.1	3.7
Dilution 1	250	Within ±6.7	−4.4	≤3.1	3.5
Dilution 2	500	Within ±4.4	−2.3	≤5.5	4.1

Table 4
Accuracy and precision of the validation QC samples for RGH-188, desmethyl- and didesmethyl-RGH-188 in human urine

QC level	Nominal concentration (ng/ml)	Intra-batch accuracy (%inaccuracy, $n=5$, 3 days)	Inter-batch accuracy (%inaccuracy, $n=15$)	Intra-batch precision (CV%, $n=5$, 3 days)	Inter-batch precision (CV%, $n=15$)
RGH-188					
LLOQ	0.1	Within ±8.1	−1.1	≤5.6	6.5
Low	0.25	Within ±2.4	−0.9	≤5.0	3.7
Medium	2.5	Within ±2.7	−0.9	≤4.7	3.9
High	20	Within ±3.5	−0.7	≤3.6	3.3
Desmethyl-RGH-188					
LLOQ	0.1	Within ±4.5	−4.0	≤8.9	7.0
Low	0.25	Within ±3.7	−1.6	≤4.7	4.1
Medium	2.5	Within ±2.3	0.4	≤4.2	3.1
High	20	Within ±3.7	−0.4	≤4.4	4.0
Didesmethyl-RGH-188					
LLOQ	0.1	Within ±7.3	0.8	≤10.7	10.2
Low	0.25	Within ±6.0	1.3	≤6.5	5.8
Medium	2.5	Within ±4.2	1.5	≤2.7	2.9
High	20	Within ±4.0	−1.3	≤4.6	4.1

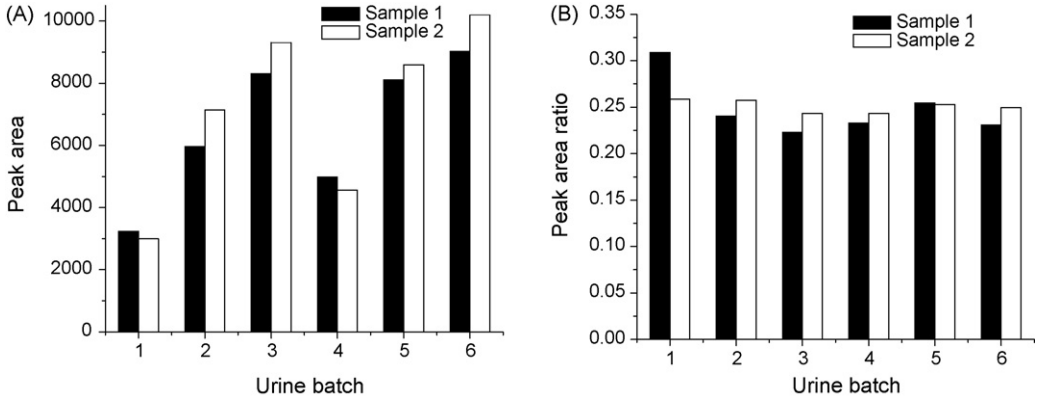

Fig. 5. Analyte peak area values (A) and the analyte/IS peak area ratios (B) obtained for didesmethyl-RGH-188 when extracted from six individual urine samples.

individual urine samples in duplicates and, for comparison, the analyte/IS peak area ratios for the same samples.

3.7. Stability

The results of the stability assessments in human plasma and urine are summarized in Tables 5 and 6. As shown by the data, there was no degradation for RGH-188 and its metabolites after storage in human plasma and urine at room temperature for 3 h and after three freeze/thaw cycles. The reconstituted samples were considered stable for up to 24 h post-sample preparation when kept in the autosampler tray at approximately +10 °C. No decomposition of the analytes was found during 12- and 10-week storage below −20 °C in human plasma and urine, respectively.

Table 5
Stability of RGH-188, desmethyl- and didesmethyl-RGH-188 in human plasma (%difference from freshly prepared QC, $n=5$)

QC level	Nominal concentration (ng/ml)	Freeze and thaw stability (−20 °C, 3 cycles)	Short-term stability (room temperature, 3 h)	Post-preparative stability (+10 °C, 24 h)	Long-term stability (−20 °C, 12 weeks)
RGH-188					
Low	0.25	4.8	6.2	0.5	−0.7
High	20	−0.3	0.0	0.7	−1.7
Desmethyl-RGH-188					
Low	0.25	0.6	−0.8	3.3	−1.3
High	20	−0.3	2.7	1.6	2.7
Didesmethyl-RGH-188					
Low	0.25	−4.0	−4.2	0.3	0.4
High	20	0.3	−1.7	2.2	−0.2

Table 6
Stability of RGH-188, desmethyl- and didesmethyl-RGH-188 in human urine (%difference from freshly prepared QC, $n = 5$)

QC level	Nominal concentration (ng/ml)	Freeze and thaw stability (−20 °C, 3 cycles)	Short-term stability (room temperature, 3 h)	Post-preparative stability (+10 °C, 24 h)	Long-term stability (−20 °C, 12 weeks)
RGH-188					
Low	0.25	−0.4	−1.7	−1.5	−5.8
High	20	−2.1	−0.1	−0.4	−1.1
Desmethyl-RGH-188					
Low	0.25	−2.6	3.0	2.0	−1.7
High	20	−1.2	−0.5	1.5	−2.2
Didesmethyl-RGH-188					
Low	0.25	3.7	−0.7	5.7	−0.8
High	20	−0.4	−1.5	1.2	−4.2

3.8. Application of the methods to clinical sample analysis

The LC–MS/MS methods reported here were successfully applied in Phase I clinical studies for the investigation of the pharmacokinetic profile of RGH-188 and its active metabolites in man after single- and multiple-dose oral administration. More than 2000 plasma and urine samples were analysed so far and none of them raised any problems during quantifica-

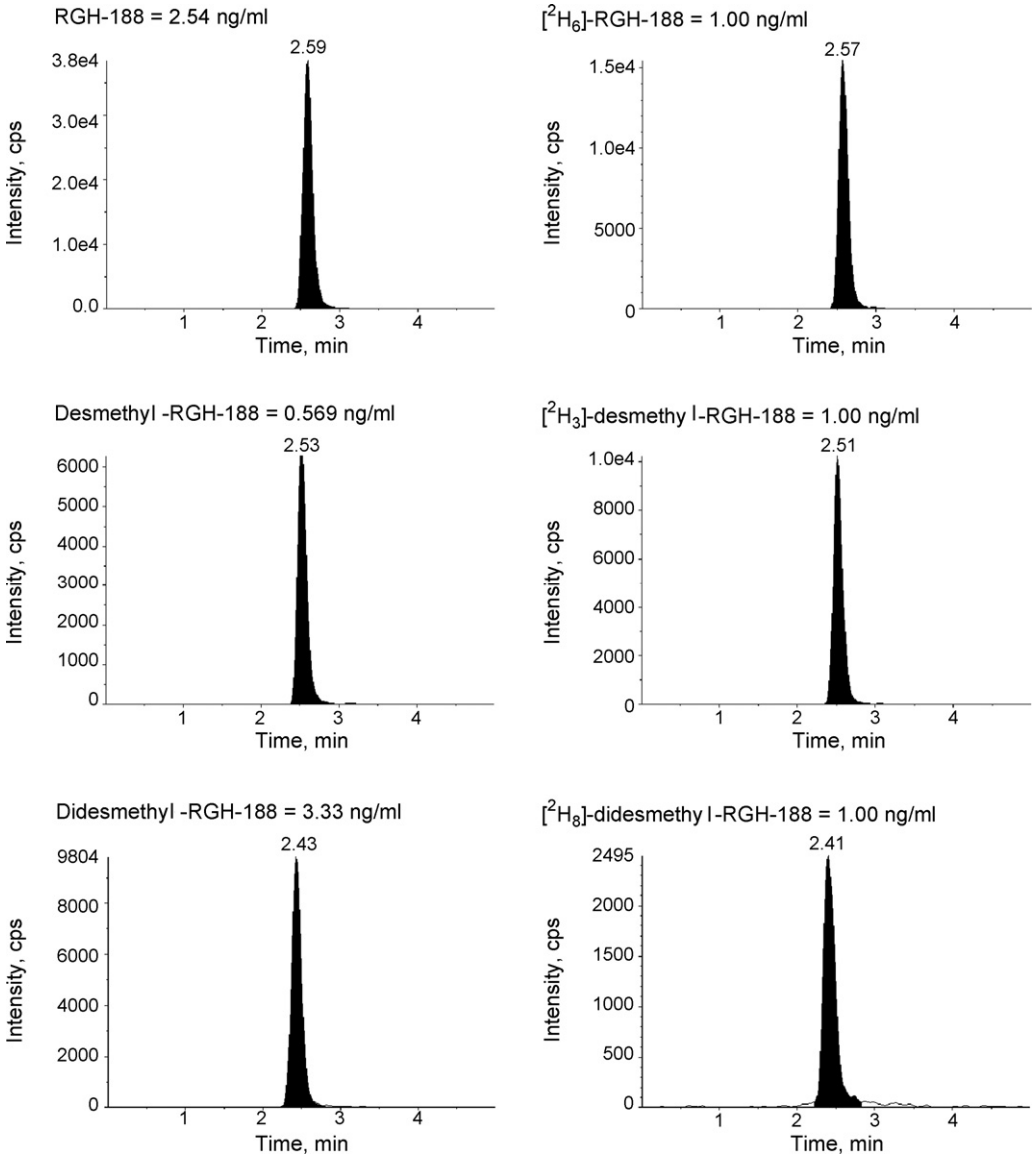

Fig. 6. Representative chromatograms of clinical plasma samples (Day 14, 2-h sample of a subject receiving 1 mg RGH-188 once daily).

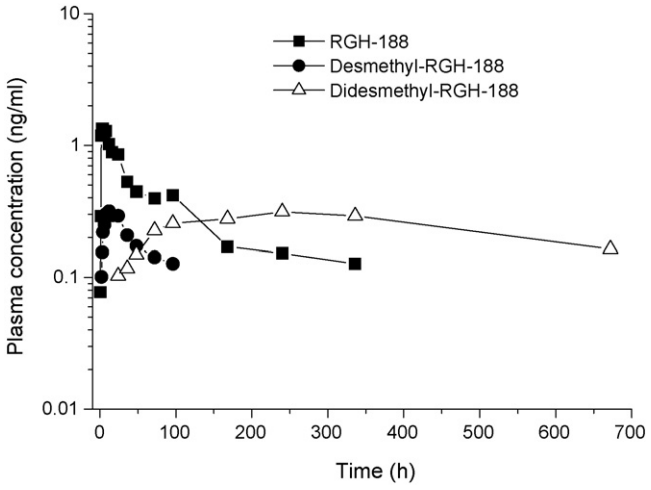

Fig. 7. Plasma concentration–time profile for RGH-188 and its metabolites after single oral administration of RGH-188 to a healthy subject at dose of 2.5 mg.

tion of the analytes. Fig. 6 shows representative chromatograms for real clinical samples and Fig. 7 presents the plasma concentration–time profile for RGH-188 and its metabolites after single oral administration of RGH-188 to a healthy subject at dose of 2.5 mg.

4. Conclusion

Sensitive and specific LC–MS/MS methods using LLE have been validated for simultaneous determination of RGH-188 and its metabolites in human plasma and urine. The results obtained during the validation fully met the criteria generally established for bioanalytical assays [6]. The methods are robust enough to support clinical development of RGH-188.

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