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Journal of Chromatography B, 788 (2003) 45–55
JOURNAL OF CHROMATOGRAPHY B
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Determination of ragaglitazar, a novel dual acting peroxisome proliferator-activated receptor (PPAR) α and -γ agonist, in human plasma by high-performance liquid chromatography coupled with tandem mass spectrometry

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Received 10 June 2002; received in revised form 2 December 2002; accepted 9 December 2002

Abstract

A sensitive and specific LC/MS/MS method has been developed and validated for determination of ragaglitazar (NNC 61-0029 or DRF 2725) in human plasma. After solid-phase extraction (SPEC® PLUS™ C_8) of plasma, separation was performed on a Symmetry Shield™ RP8 column (mobile phase: acetonitrile: 10 m*M* ammonium acetate, pH 5.6 (40:60 v/v)). Two ranges were validated having LLOQs of either 0.500 or 100 ng/ml and linearity up to either 500 or 50 000 ng/ml. The intra-assay precision and accuracy were 1.1% to 15.7% and 85.8% to 118.2% (range 0.500–500 ng/ml) and 2.0% to 8.8% and 92.9% to 104.8% (range 100–50 000 ng/ml). The method was applied for determination of ragaglitazar in plasma from phase 1 and 2 clinical studies.

Keywords: Ragaglitazar

1. Introduction

Ragaglitazar ((*S*) 3-[4-[2-(phenoxazin-10-yl)ethoxy]phenyl]-2-ethoxy-propanoic acid), $C_{25}H_{25}NO_5$, (I, Fig. 1) is a novel dual peroxisome proliferator-activated receptor (PPAR) agonist in phase 3 clinical development for the treatment of patients with type 2 diabetes. The biological effect of ragaglitazar is exerted via two isoforms of the PPAR family, namely PPARγ and PPARα [1]. To be able to quantify the compound in plasma samples from clinical studies during development, an assay using high-performance liquid chromatography coupled with tandem mass spectrometry was developed. Liquid chromatography coupled with atmospheric pressure tandem mass spectrometry is a proven technique for the determination of pharmaceuticals in biological matrices providing speed, sensitivity and selectivity [2–5]. As sample preparation technique solid-phase extraction (SPE) was employed using microcolumn disc technology (SPEC) [6–9]. Ragaglitazar is the first drug of its class but general principles for solid-phase extraction and disk technology and quantitative analysis using liquid chromatography and ionspray tandem mass spectrometry were applied.

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1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(02)01014-0

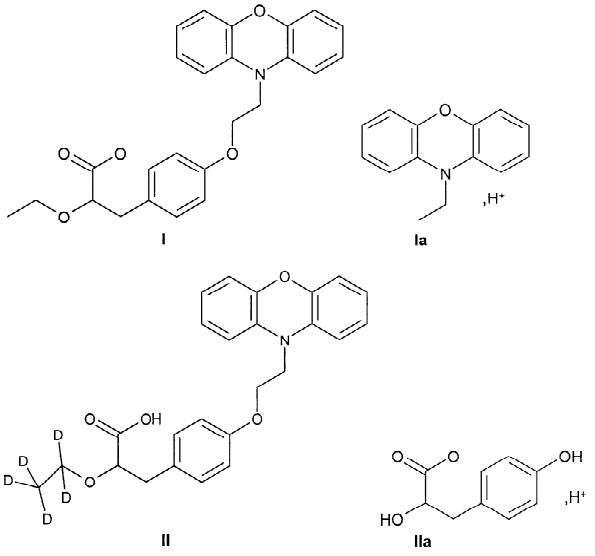

Fig. 1. Chemical structures of ragaglitazar (I) and its deuterated analogue (II) used as internal standard. The fragments monitored in SRM are shown for ragaglitazar (Ia, m/z: 210.0) and I.S. (IIa, m/z: 183.0).

As part of the documentation, the assay was validated according to current recommendations [10]. Initially a validation covering the concentration range 0.250–500 ng/ml was carried out with respect to intra-assay precision and accuracy, inter-assay precision, lower and upper limit of quantification (LLOQ and ULOQ), recovery, linearity, selectivity, ruggedness and stability. Having assayed samples from the first dose level of a phase 1 dose escalation clinical study it became obvious that the concentration range had to be extended. For a new range (50.0–50 000 ng/ml) only a limited validation was carried out testing the intra-assay precision and accuracy, lower and upper limit of quantification (LLOQ and ULOQ), linearity and ruggedness.

2. Experimental

2.1. Materials and reagents

The molecular mass of ragaglitazar is 419.5 g/mol. All plasma concentrations, stock solutions and dilutions are given in mass units, as free acid. The arginine salt of ragaglitazar (*Mw* 593.7 g/mol, purity: >99%) was used for preparation of stock solutions.

As an internal standard (IS), a deuterated analogue of ragaglitazar labelled in five positions ([^{2}H]-(*S*) 3-[4-[2-(phenoxazin-10-yl)-ethoxy]phenyl]-2-(1,1,2,2,2-pentadeutro-ethoxy)-propanoic acid), $C_{25}H_{20}{}^2H_5NO_5$, was used (II, Fig. 1, purity: >98%).

Deionised water was obtained from a Milli-Q system (Millipore S.A., Molsheim, France). LiChrosolv acetonitrile and methanol, 25% ammonia solution, ammonium acetate and acetic acid were all obtained from Merck, Darmstadt, Germany, and were of analytical grade. Sodium hydroxide 1 N of analytical grade was from Bie & Berntsen, Copenhagen, Denmark, SPE-columns (SPEC PLUS C_8; 3 ml–15 mg), were from Ansys Diagnostics, Lake Forest, CA, USA, 96 well plates, (polypropylene; 0.3 ml) were obtained from Nunc, Roskilde, Denmark, and Scotch Pad film for 96 well plates was from 3M.

2.1.1. Instrumentation

The assay was set up on an LC/MS/MS system consisting of a Perkin-Elmer Series 200 LC HPLC pump (Perkin-Elmer™ Instruments, Norwalk, CT, USA), a Kontron 480 column oven (Kontron Instruments, Switzerland), a Gilson 233 XL autosampler and a 402 Syringe pump (Gilson, Villier Le Bel, France) and a Sciex API 3000 mass spectrometer (MDS Sciex, Thornhill, Ontario, Canada). Tuning of the mass spectrometer was done using a Harvard Model 1140-001 infusion pump (Harvard Apparatus, Southmatick, MA, USA). For sample preparation a Heraeus Megafuge 3.0R centrifuge (Heraeus Instruments, Ostende, Germany) and a TurboVap® LV Evaporator (Zymark, Hopkinton, MA, USA) were used.

2.2. Stock solutions, calibration standards and quality controls

For the first part of the validation three stock solutions of analyte were made, one in methanol at 1.00 mg/ml for calibration standards (stability at −20 °C investigated for 3 months and due to evaporation of methanol shown to be stable for only 1 month) and two in water at 1.00 mg/ml for QC samples and 1.25 mg/ml for spiked samples (stock

solutions in water should be prepared fresh just before use). Working solutions were made fresh by dilution with water.

Calibration standards at concentrations of 0.250, 0.500, 1.00, 2.50, 10.0, 25.0, 100, 250 and 500 ng/ml were made by adding plasma to 200 μl standard working solutions to a final volume of 10.0 ml. Aliquots of 250 μl were stored frozen (−20 °C).

QC-samples were made at 1.00, 20.0 and 400 ng/ml by adding plasma to 400 μl QC working solution to a final volume of 20.0 ml. Aliquots of 600 μl were frozen (−20 °C).

Spiked plasma samples at concentrations of 0.250, 0.500, 1.00, 20.0 and 500 ng/ml were prepared by adding plasma to 100 μl working solution to a final volume of 5.0 ml. Suitable aliquots were frozen (−20 °C).

An I.S. stock solution of 0.5 mg/ml was made in methanol (stability investigated for 3 months at −20 °C. Though slightly increasing concentration with time due to evaporation of methanol, the stability was set to be 3 months). A working solution in methanol at a concentration of 0.2 μg/ml was determined to be stable for at least 1 week at 4 °C.

In the extended validation covering the concentration range 50.0–50 000 ng/ml, stock solutions were made fresh in water (2.50 mg/ml for calibration standards and spiked samples and 2.00 mg/ml for QC-samples). Calibration standards, QC-samples and spiked plasma samples were made similar to those above in concentrations of 50.0, 100, 150, 250, 1000, 2500, 10 000, 25 000 and 50 000 ng/ml for calibration standards, 200, 3000 and 40 000 ng/ml for QC-samples and 50.0, 100, 150, 2000, 25 000 and 50 000 ng/ml for spiked plasma samples. The I.S. working solution in this case was at a concentration of 10 μg/ml (stability: determined to be at least 1 week at 4 °C).

2.3. Sample preparation

Sample clean up was carried out by SPE. All SPE steps were carried out by centrifugation (conditioning at 500 rpm~51 *g* for 2 min, and the remaining steps at 1000 rpm~204 *g* for 3 min). The SPE columns were conditioned with 1×0.5 ml methanol followed by 1×0.5 ml water. A volume of 250 μl plasma was mixed with 750 μl 0.2 N NaOH, and 100 μl I.S. working solution, before the total volume was applied to the SPE column. The samples were washed with 1×1 ml water:methanol (95:5 v/v) and eluted with 2×500 μl methanol:25% ammonia solution (99:1 v/v). The eluate was evaporated to dryness in a TurboVap (50 °C, ~30 min). The samples were reconstituted in 150 μl of acetonitrile:water (20:80 v/v). Following centrifugation (3500 rpm for 2 min~2520 *g*), 100 μl was transferred to a 96 well plate.

In the extended range assay the evaporation step was omitted. Instead 10 μl of eluate was transferred to a 96 well plate and 190 μl of acetonitrile:water (20:80 v/v) was added.

To assess recovery of analyte, IS was not added to the extracted calibration standards until after elution, when they were evaporated to dryness. Non-extracted reference calibration standards were prepared by adding 50 μl of standard working solution and 50 μl of I.S. to dried extracts of control plasma, evaporated to dryness and reconstituted in 150 μl of acetonitrile:water (20:80 v/v).

2.4. Chromatographic conditions

The injection volume was 30 μl, the flow rate was set at 1.0 ml/min and the column temperature was maintained at 35 °C. The mobile phase consisted of acetonitrile:0.010 *M* ammonium acetate buffer (pH 5.6) (40:60 v/v). The autosampler needle wash solution was methanol:25% ammonia solution (99:1 v/v), and 2 post- and 1 pre-injection cycles each of 475 μl were programmed. Separation was performed on a Waters Symmetry Shield™ RP8, 3.9×50 mm, 5 μm, guarded by a Waters Symmetry Shield RP8, 3.9×20 mm, 5 μm, (Waters, Milford, MA, USA). The run time was 3 min and the retention time 2 min 25 s for ragaglitazar as well as for the I.S.

2.5. Mass spectrometry

A PE Sciex mass spectrometer (API 3000) equipped with a TurboIonspray® interface was used as a detector. It was operated in positive SRM mode having precursor ions at *m*/*z* 420.1 (ragaglitazar) and *m*/*z* 425.1 (I.S.), and product ions of *m*/*z* 210.0 (ragaglitazar) and *m*/*z* 183.0 (I.S.), suggested products and product ion scans shown in Figs. 1 and 2, respectively. The ionspray voltage was 4000 V, orifice voltage was 40 V, and collision energy was 31

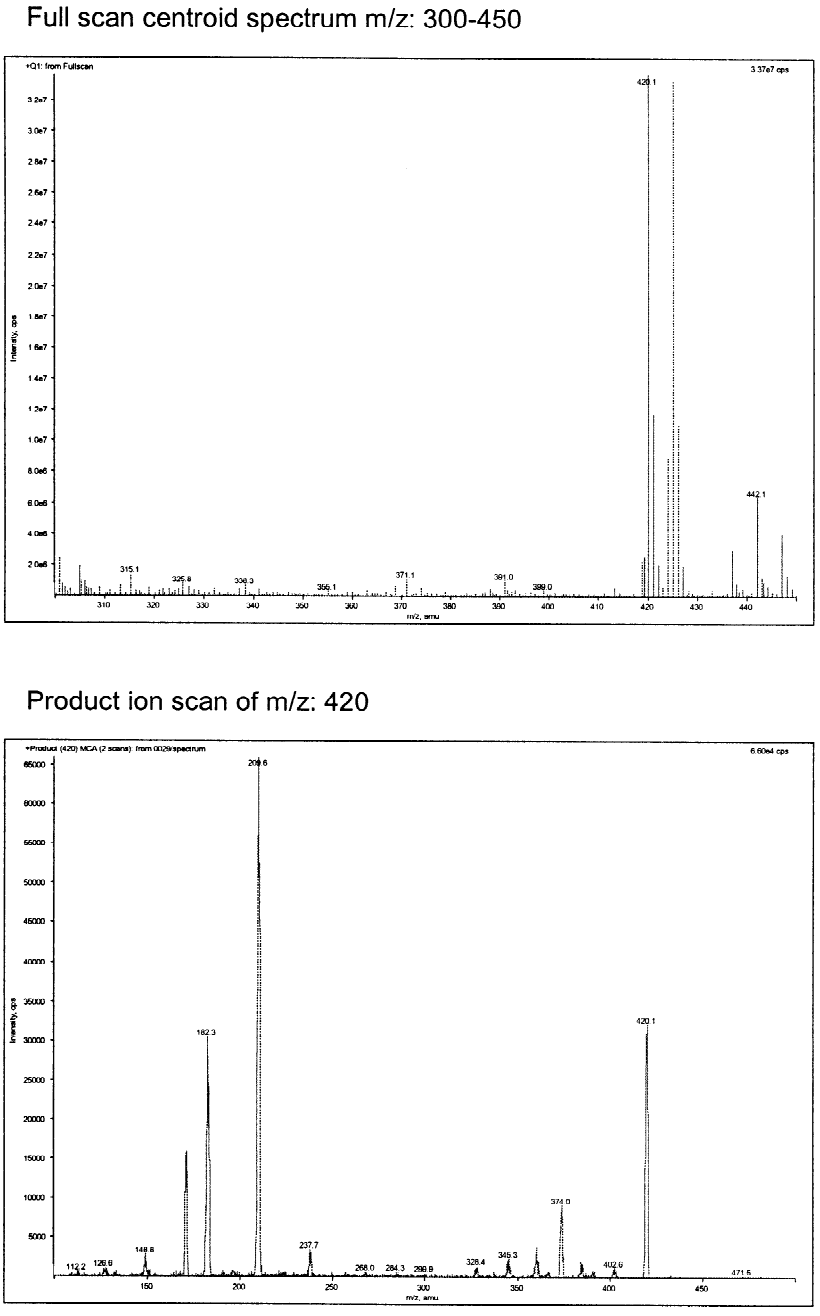

Fig. 2. Full scan centroid spectrum of ragaglitazar (m/z: 420) and I.S. (m/z: 425) (top) and product ion spectra of ragaglitazar (products of m/z: 420) (mid) and I.S. (products of m/z: 425) (bottom). Tune solution: 1000 ng/ml (full scan) or 25 ng/ml (product ion scans) of ragaglitazar and I.S. in methanol:0.01 *M* ammonium acetate (50:50 v/v). Infusion rate: 3.00 ml/h.

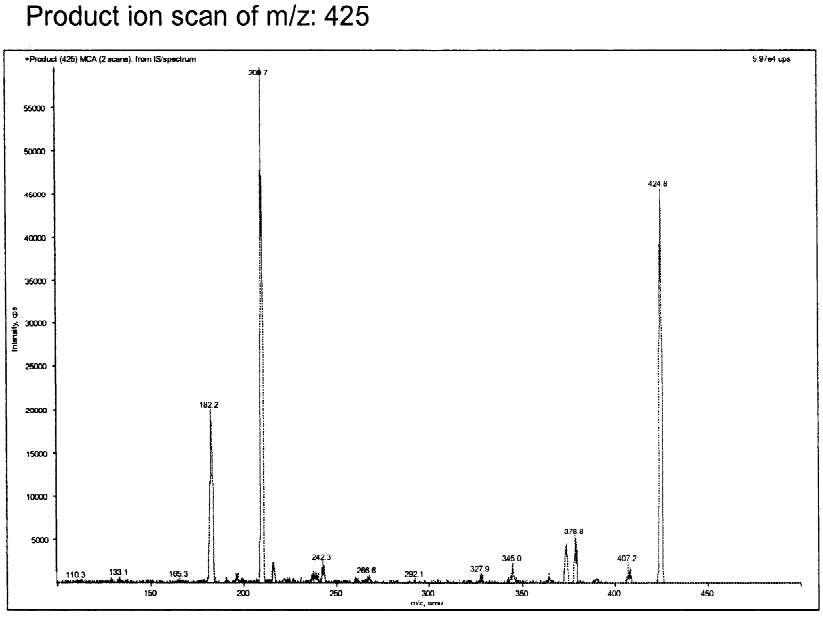

Fig. 2. (*continued*)

V. The interface temperature was 250 °C, and the split flow into the mass spectrometer was 200 μl/min. Tuning and resolution (unit mass) of the mass spectrometer was checked before each assay series by flow injection (Harvard Apparatus, infusion rate 3.00 ml/h) of tuning solution.

2.6. Clinical phase 1 single dose study

The validated assay was used for determination of ragaglitazar in plasma samples from a randomised, double-blind, placebo-controlled, dose escalation, oral single-dose phase 1 trial in healthy male subjects [11]. Six groups, each of 6 ragaglitazar treated subjects and 2 placebo treated subjects, except one group of only five ragaglitazar treated and 2 placebo treated subjects, were included in the study. The subjects were dosed once orally with a water solution of ragaglitazar at dose levels of 1, 5, 15, 45, 90 and 120 mg. The subjects were fasted from at least 10 h before until 4 h after drug administration. Blood was drawn into tubes containing EDTA pre dose (time 0) and 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12, 16, 24, 36, 48, 60 and 72 h post dosing. Plasma was separated from the whole blood and stored frozen (−18 °C) pending analysis.

3. Results and discussion

3.1. Validation

3.1.1. Intra-assay precision and accuracy — estimation of limits of quantification

The intra-assay precision and accuracy and the lower (LLOQ) and upper (ULOQ) limit of quantification were estimated in three assay batches carried out by three technicians in the initial validation and in two batches carried out by two technicians in the extended range. Spiked plasma samples were assayed in six replicates at each concentration level (0.250, 0.500, 1.00, 20.0, 500 ng/ml in the initial and 50.0, 100, 150, 2000, 25 000 and 50 000 ng/ml in the extended range validation). The guidelines [2] require the intra-assay precision (C.V.) to be below 15%, and the accuracy to be within 85–115%, except in the LLOQ range, where precision should be below 20% and accuracy within 80–120%. In the concentration range 0.500–500 ng/ml the intra-assay precision ranged from 1.1% to 15.7% and the accuracy from 86% to 118%. In the initial validation, the LLOQ accuracy and precision criteria were met in two of three intra-assay experiments at 0.250 ng/ml and in all three experiments at 0.500 ng/ml.

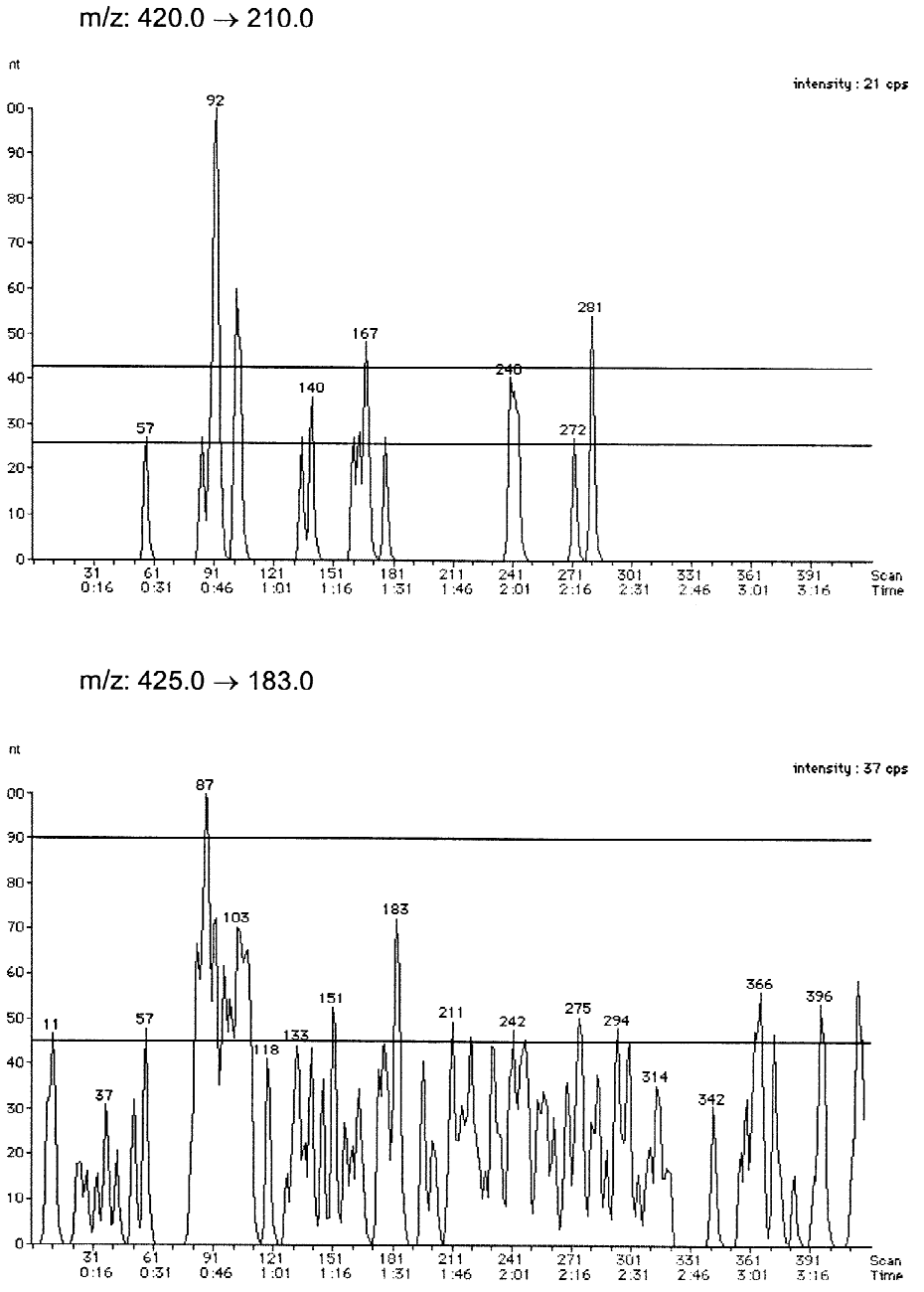

Fig. 3. SRM chromatograms of blank plasma. Ragaglitazar (m/z: 420.1→210.0) (top), I.S. (m/z: 425.1→183.0) (bottom). Background noise ragaglitazar: ~ 10 cps, background noise I.S.: ~ 20 cps.

Consequently, the LLOQ was set to be 0.500 ng/ml (C.V. 6.5%, 9.0% and 5.4%, accuracy 86%, 90% and 118%) and the ULOQ to be 500 ng/ml (C.V. 4.3%, 3.4% and 1.1%, accuracy 96%, 103% and 93%). The extended range validation, in the concentration range of 100–50 000 ng/ml, had an intra-assay precision from 2.0% to 8.8% and an accuracy from 93% to 105%. The LLOQ accuracy and precision criteria were met in both experiments at 100 ng/ml, but only in one at 50.0 ng/ml. Consequently, the LLOQ was

set to be 100 ng/ml (C.V. 8.0% and 8.8%, accuracy 93% and 98%) and the ULOQ to be 50 000 ng/ml (C.V. 2.1% and 2.0%, accuracy 103% and 105%). Chromatograms from the initial validation of blank and spiked plasma at the LLOQ level 0.500 ng/ml containing 20 ng I.S. are shown in Figs. 3 and 4, respectively. The background noise of the chromatograms is approximately 10 and 20 cps for ragag-

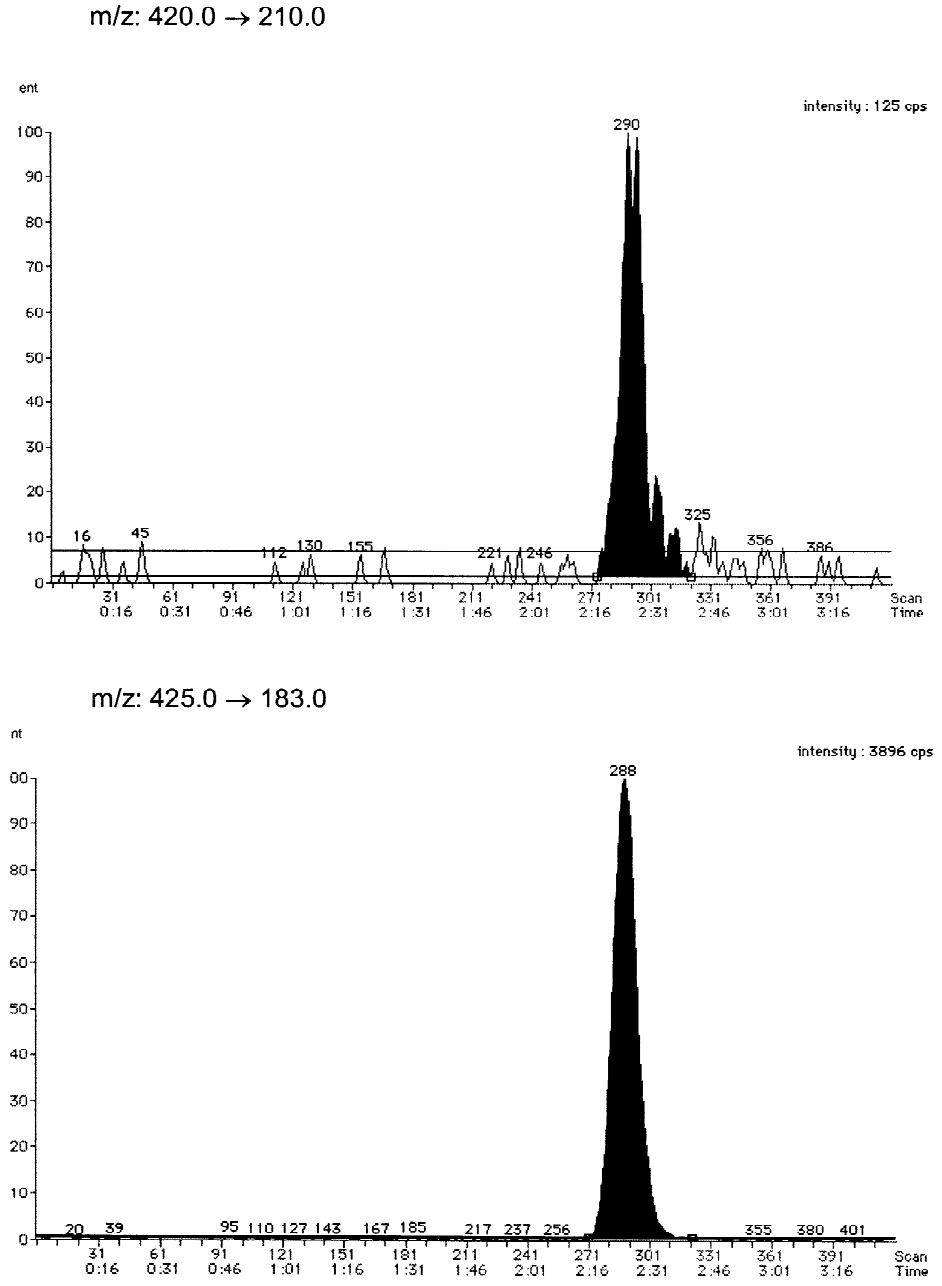

Fig. 4. SRM chromatograms of ragaglitazar (m/z: 420.1→210.0) (top) and internal standard (m/z: 425.1→183.0) (bottom) from plasma spiked with 0.500 ng/ml ragaglitazar and added 20 ng I.S. (80 ng I.S. per ml plasma). Peak height ragaglitazar: 125 cps, s/n ratio: 12.5. Peak height I.S.: 3896 cps, s/n ratio: 195.

litazar and I.S., respectively. An LLOQ peak has a height of 125 cps, giving a signal-to-noise ratio of 12.5, and that of the I.S. a height of 3896 cps, giving a signal-to-noise ratio of 195.

3.1.2. Total inter-assay precision

The total inter-assay precision was estimated in the initial validation on the mean intra-assay estimate from three intra-assay experiments. The total inter-assay precision ranged from 1.3% to 17.9% and the accuracy from 84.1% to 98.0% (Table 1).

3.1.3. Recovery

The recovery of ragaglitazar in the extraction procedure was estimated in the initial validation by assaying extracted calibration standards (I.S. added after extraction) and corresponding non-extracted standards in one assay series. Calibration graphs were constructed by linear regression (area ratio of analyte to I.S.) and the recoveries estimated as the slope ratio of extracted to non-extracted calibration standards:

$$\text{Recovery (analyte)} = \text{slope extracted}/\text{slope non-extracted} \cdot 100\%$$

The recovery was estimated to be 59%.

3.1.4. Linearity

The linearity of the assay and the reproducibility of the calibration graph (peak area ratio analyte/I.S. versus analyte concentration) in the plasma concentration range 0.500–500 ng/ml in the initial and 100–50 000 ng/ml in the extended range validation were evaluated from the determination coefficients of the weighted ($1/x$ calibration graphs from all the study batches (data from the initial validation shown in Table 2). A negligible difference in slope between batches and small y-intercepts was observed. Based on the determination coefficients ($r^2 = 0.998$ in both validations) of the calibration graphs, the assay was considered linear in each plasma concentration range (0.500–500 ng/ml and 100–50 000 ng/ml, respectively).

3.1.5. Selectivity

No peaks from endogenous substances were observed in the chromatograms of blank plasma extracts from six different humans. A representative chromatogram of a blank plasma extract is shown in Fig. 3.

3.1.6. Stability

The plasma stability was investigated both short- and long-term using quality control samples (1.00–20.0–400 ng/ml). A mean assay result in the range 80–120% of nominal was considered as indicating stability. In plasma, ragaglitazar was stable for at least 24 h at 4 °C as well as at room temperature. Further, the compound was stable in plasma during at least 3 freeze/thaw cycles. In dry plasma extracts,

Table 1
Total inter-assay precision and accuracy assessed from mean intra-assay estimates

Batch	Assay results (ng/ml) Spiked concentration level (ng/ml)				
	0.250	0.500	1.00	20.0	500
1	0.184	0.431	0.953	17.2	480
2	0.227	0.448	0.936	19.2	516
3	0.220	0.591	0.930	17.2	465
Mean	0.210	0.490	0.940	17.9	487
SD	0.023	0.088	0.012	1.2	26
C.V. (%)	11.0	17.9	1.3	6.5	5.4
Accuracy (%)	84.1	98.0	94.0	89.3	97.4

Table 2
Calibration graph data: Values having batch ID code ending -1 are from the beginning and those with the ending -2 are from the end of an assay series of 93 or 114 samples

Batch ID	Parameter		
	Slope	y-Intercept	r^2
990720UAL-1	0.0619	0.00632	0.9988
990720UAL-2	0.0613	0.00686	0.9985
990721UAL	0.0636	0.02484	0.9991
990722UAL-1	0.0654	0.00356	0.9986
990722UAL-2	0.0636	0.00962	0.9988
990727	0.0687	0.00624	0.9995
Mean	0.0641	0.00957	0.9989
SD	0.0027	0.00772	–
C.V. (%)	4.2	–	–

ragaglitazar was stable for at least 2 days at 4 °C as well as at room temperature. The long-term stability of ragaglitazar in plasma kept in a freezer (−18 °C) was investigated for 13 months. At 20.0 and 400 ng/ml no decline was observed within the period. At 1.00 ng/ml the concentration was within 80–120% of nominal until 6 months, but below 80% later than 6 months.

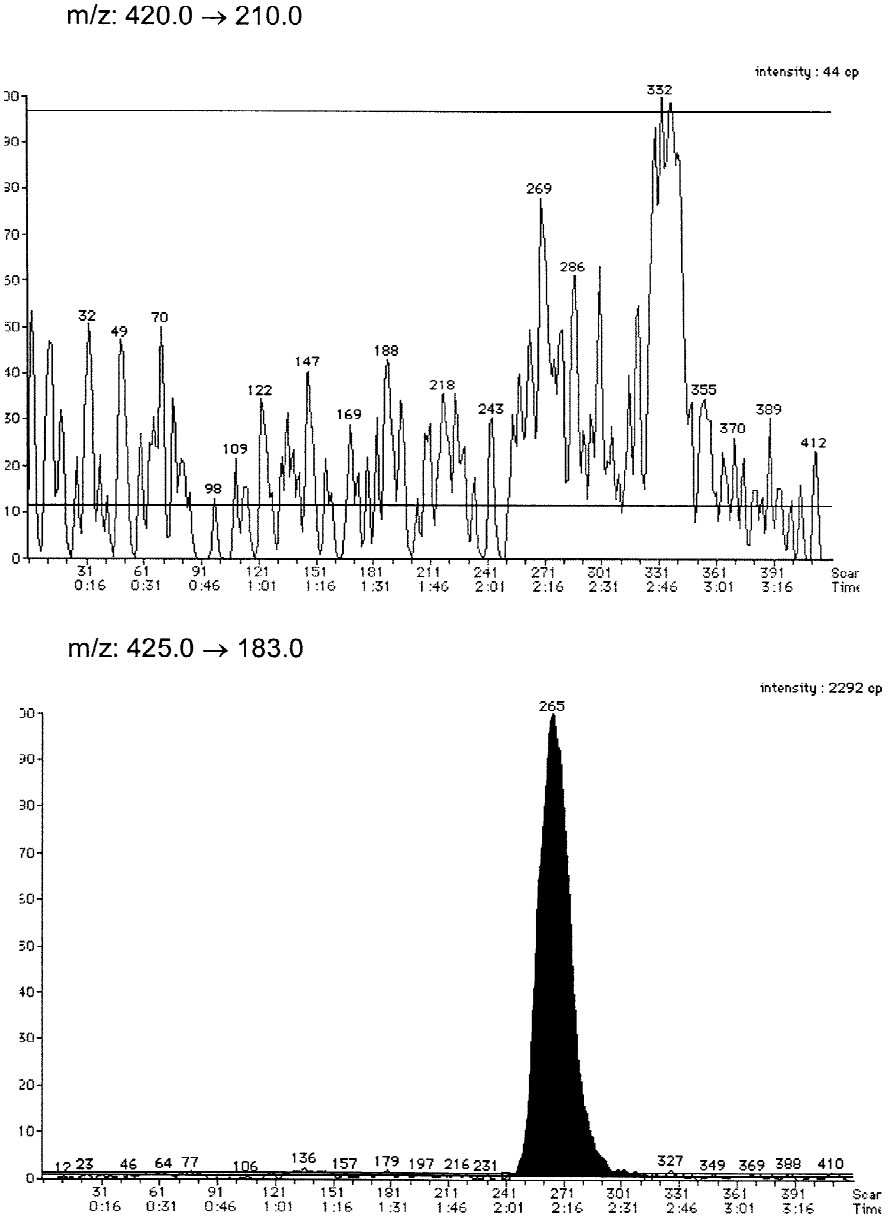

Fig. 5. SRM chromatograms of ragaglitazar (m/z: 420.1→210.0) (top) and internal standard (m/z: 425.1→183.0) (bottom) from plasma obtained pre-dose a subject dosed with 1 mg ragaglitazar.

3.2. Routine analysis of plasma samples from a clinical phase 1 study

During analysis of plasma samples (736 samples from 35 subjects) from the phase 1 single dose study, the acceptance criteria were met in all assay batches for both assay ranges ($n=6$, for each range). Chromatograms of a pre-dose and a 1 h post-dose plasma sample obtained from a subject dosed 1 mg ragaglitazar are shown in Figs. 5 and 6, respectively. The mean plasma concentration profiles for all dose levels are shown in Fig. 7.

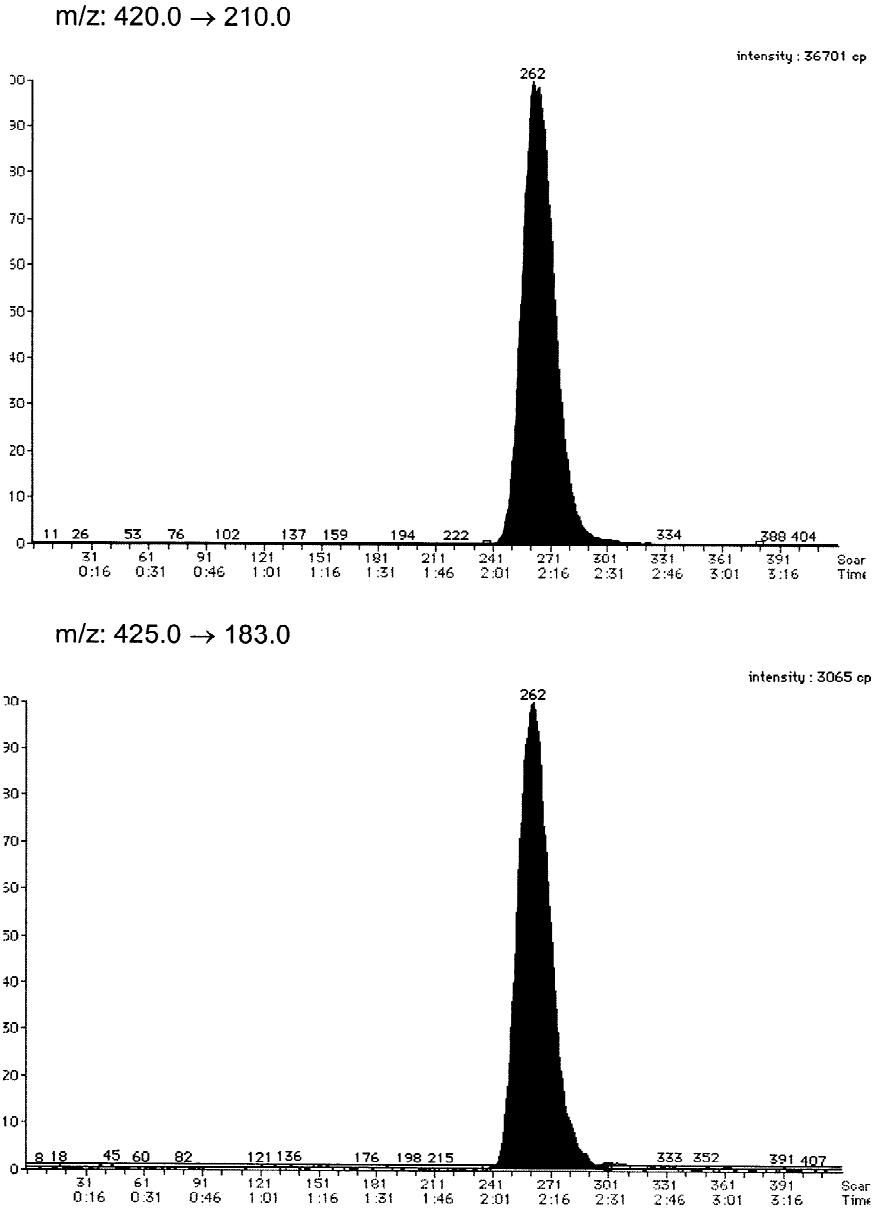

Fig. 6. SRM chromatograms of ragaglitazar (m/z: 420.1→210.0) (top) and internal standard (m/z: 425.1→183.0) (bottom) from plasma obtained 1 h post-dose from a subject dosed with 1 mg ragaglitazar. Ragaglitazar concentration: 219 ng/ml.

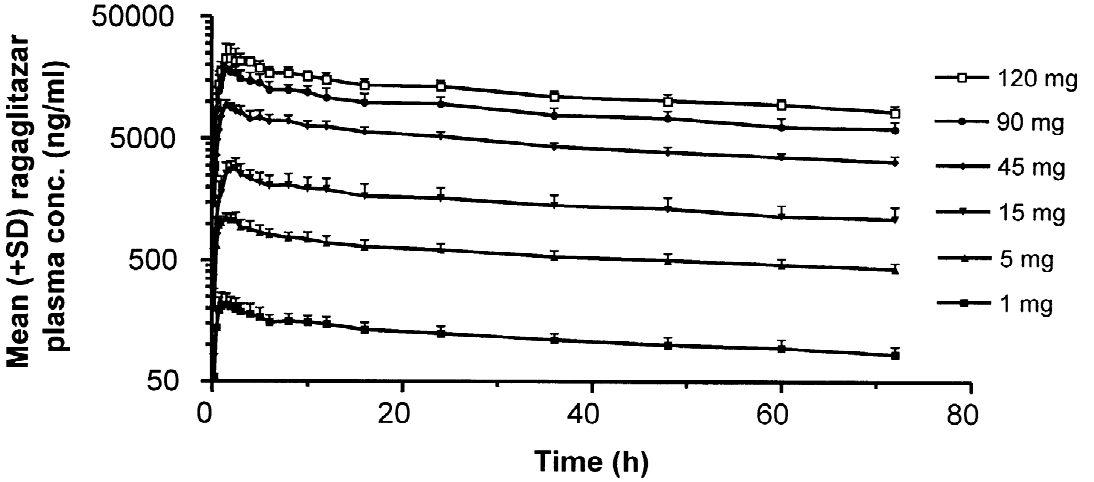

Fig. 7. Mean (+SD) plasma concentration–time plots of ragaglitazar from a phase 1 single oral dose escalation study.

4. Conclusion

The LC/MS/MS assay of ragaglitazar in human plasma was validated and found suitable for analysing samples in two concentration ranges; 0.500–500 ng/ml and 100–50 000 ng/ml. The intra-assay precision and accuracy, as well as total inter-assay precision, extraction recovery, linearity, selectivity, ruggedness and stability were found to be acceptable. The assay was successfully implemented for assay of plasma samples from clinical phase 1 and 2 studies.

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