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Determination of the enantiomeric composition of a new insulin sensitizer in plasma samples from non-clinical and clinical investigations using chiral HPLC with electrospray tandem mass spectrometric detection

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Abstract

Two drug assays were developed and applied to assess the enantiomeric composition of an insulin sensitizer drug in plasma after administration of its racemate to man, and in human and animal plasma and serum samples generated after in vitro experiments. The sample preparation for the assays consisted either of protein precipitation and column-switching, or liquid–liquid extraction and direct injection. Subsequently, both assays employed chiral HPLC coupled to atmospheric pressure ionization mass spectrometry. An interconversion of the racemate to a mixture enriched with the (+)-enantiomer could be confirmed for all species and biological matrices. The individual enantiomers could be quantified in the concentration range 0.5–500 ng/ml, starting with a 100-μl plasma aliquot. Inter- and intra-assay precision and accuracy were in the range 0.1–7.9 and 88.8–106.0%, respectively. Run times of 5 min for a single sample allows the analysis of more than 200 samples overnight.

Keywords: Ionspray; Chiral drug assay; Glitazones; Interconversion

1. Introduction

Type 2 diabetes is a chronic, progressive and multisystem disorder, leading not only to hyperglycemia, but also to dyslipidemia, hypertension, obesity and accelerated atherosclerosis. A further characteristic of Type 2 diabetes is an increase in endogenous glucose production, which significantly contributes to maintaining a hyperglycemic state. Insulin resistance is one of the first hallmarks of this disease, which is initially compensated by an increase in insulin production, followed by an insulin deficiency. The latter results from a progressive pancreatic failure.

World Health Organization (WHO) estimated that, in 2000, about 99 million people in the developed countries live with diabetes, and in 2025 more then 227 million people will suffer from this disease [1]. Thus, there is a high social and economical requirement for an adequate treatment of this disease.

R483, a new insulin sensitizer, belongs to the thiazolidinediones (or glitazones), representing a new class of orally active drugs that reduces insulin resistance, and hence increases glucose uptake in skeletal muscle and adipose tissue, as well as decreasing hepatic glucose production. R483, (*R*,*S*)-5-[[4-[2-(5-methyl-2-phenyl-oxazol-4-yl)ethoxy]benzo [2] thiophen-7-yl]methyl]-thiazolidine-2,4-dione is administered as a racemate (Fig. 1). For the development of a racemic drug, the pharmacological effects of each enantiomer has to be evaluated, since it is well known and recognized that different enantiomers can show different pharmacodynamic effects. In the best case, one of the enantiomers has an equal or less pronounced effect than the other, and in the worst case it is toxic [3,4].

Enantiomers can be separated using different approaches, such as formation of diastereomeric derivatives, prior separation on non-chiral columns [5], addition of chiral additives such as cyclodextrins and formation of diastereomeric complexes [6,7] with separation on a non-chiral stationary phase, or the separation of the enantiomers on a chiral stationary phase [8–10]. Chiral separations of drugs have been reported using the following methodologies: GC [11,12], HPLC [13], electrophoresis [14,15] and supercritical fluid chromatography [16] coupled to atmospheric pressure ionization mass spectrometry. Enantioselective separations together with tandem mass spectrometric

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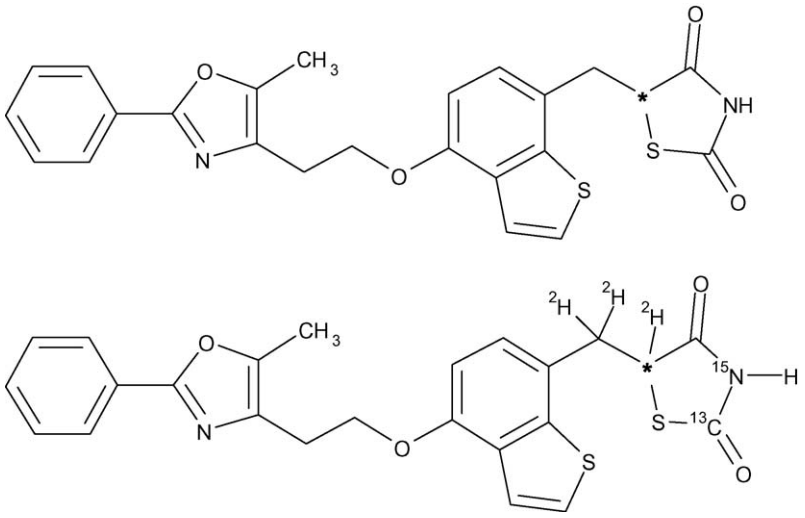

Fig. 1. Structures of the analyte (top) and the internal standard (bottom).

detection with ESI (electrospray ionization) or with atmospheric pressure chemical ionization (APCI) have been reported in either normal phase [17–20] or in reversed phase mode [21].

The aim of this paper is to describe the development of methods for the assessment of the enantiomeric composition of a racemic drug from in vitro and in vivo investigations. Special emphasis was given to the investigation of the influence of the experimental parameters on the final enantiomeric composition.

2. Experimental

2.1. Chemicals

R483 (racemic), R483/604 (racemic, stable isotope labeled structural analogue of the drug used as internal standard), (−)-R483 and (+)-R483 (the enriched enantiomers of the racemic R483) were obtained from F. Hoffmann-La Roche, Basel (Switzerland) and from F. Hoffmann-La Roche, Mannheim (Germany), respectively. Acetonitrile, methanol, Titrisol buffer pH 6, 7 and 8, and formic acid were purchased from Merck (Merck, Darmstadt, Germany) and were either chromatography or p.a. grade. Ammonium formate was delivered by Fluka (Buchs, Switzerland), pro analysi grade. Water was doubly distilled by means of a Milli-Qplus 185 device from Millipore (Volketswil, Switzerland) or delivered by Merck (chromatography grade). *t*-Butyl methyl ether, synthesis grade, was obtained from Merck.

2.1.1. In vitro experiments

Titrisol buffer pH 6, 7 and 8, plasma from rat, cynomolgus monkey and human, as well as freshly prepared human serum were spiked separately to achieve a final drug concentration of 5 μg/ml of the racemic R483 and its enriched enantiomers, (−)-R483 and (+)-R483. These samples were placed in an incubator (with a CO_2 content of 5%) for up to 6 h at a temperature of 37 °C. Samples were taken at the beginning of incubation and at intervals of 15 min up to an hour, and then further at 1-h intervals. The pH of the incubation media was monitored during the time of incubation.

2.2. Sample preparation

2.2.1. In vitro samples

Plasma aliquots (100 μl) were placed into 1.5 ml Eppendorf vials. After addition of 10 μl of internal standard solution (100 ng/10 μl R483/604 in methanol containing 0.1% formic acid), extraction with 1 ml *t*-butyl methyl ether for 5 min by means of a rotating mixer was carried out. Then, the samples were centrifuged for 5 min at 13,000 × *g* for phase separation, and the organic layer was transferred to another vial. The samples were evaporated to dryness under a gentle stream of nitrogen at 40 °C, and thereafter reconstituted with 100 μl of mobile phase. A 20-μl aliquot of the sample was injected.

2.2.2. In vivo samples

Samples from clinical investigations were prepared in two steps, whereby the first step was carried out in a Clinical Pharmacology Unit (CPU) and the second in the analytical laboratory. Ex vivo plasma aliquots from volunteers (100 μl) were plasma precipitated at the CPU with 150 μl of a mixture composed of ethanol–acetonitrile–0.5 M $HClO_4$ (50:50:2, v/v/v) which contained 10 ng of the internal standard R483/604. After mixing, the samples were centrifuged at 14,000 × *g* for 10 min. The supernatant was separated from the precipitate and stored in a deep freezer at −20 °C until further sample preparation. Sample preparation in the analytical laboratory consisted of diluting 100 μl of the supernatant with 500 μl 0.1% formic acid. A 500-μl aliquot was subjected to the chiral LC–MS/MS system.

2.3. Preparation of calibration standards and QC samples for the quantification of the enantiomers

Drug free human plasma (0.5 ml aliquots) was treated with 0.75 ml of a mixture of acetonitrile–ethanol–0.5 M $HClO_4$ 50:50:2 (v/v/v) containing either 1000, 500, 100, 20, 10, 4, 2

or 1 ng of the racemic R483, and 50 ng of the internal standard (R483/604). After vigorous mixing, the sample was centrifuged at 14,000 × *g* for 10 min and the supernatant was transferred to another vial and stored at −20 °C until use. Aliquots (100 μl) of these mixtures with concentrations of 500, 250, 50, 10 5, 2, 1 and 0.5 ng/ml of each enantiomer were used as calibration standards. QC samples were prepared accordingly from an independently weighed stock at concentrations of 200, 40 and 1.5 ng/ml. Supernatant of the QCs were also stored at −20 °C until use. Aliquots (100 μl) from each stock of calibration standards and QC samples were processed according to the method for in vivo samples.

2.4. Instrumentation

2.4.1. HPLC instrumentation for in vitro samples

The extracted samples were injected by means of a Gilson 234 autoinjector equipped with an external 20 μl loop. The loop as well as the injection needle was purged before and after injection with a 1:1 acetonitrile–methanol mixture. The mobile phase, consisting of acetonitrile–methanol–10 mM ammonium acetate–acetic acid 70:20:10:1 (v/v/v/v), was delivered by a Kontron 420 HPLC pump (Kontron Instruments, Zuerich, Switzerland) in isocratic mode at 1 ml/min. Separation of the enantiomers was carried out on a DAICEL Chiracel OJ-R column, 4.6 mm i.d. × 150 mm long (Mallinckrodt Baker B.V., Deventer, The Netherlands). The flow rate to the ion spray ion source was reduced to 0.4 ml/min by means of a splitter and a restriction capillary.

2.4.2. HPLC instrumentation for in vivo samples

The column-switching narrow-bore HPLC system was composed of a 2.1 mm i.d. × 10 mm long trapping column (XTerra RP-18, 5 μm particle size, Waters AG, Rupperswil, Switzerland) which was installed on a combination of two high pressure switching valves (H.S. Valve 7000E, LabSource, Reinach, Switzerland). Injection of the samples was carried out using an HTS PAL autoinjector (Brechbühler, Schlieren, Switzerland) equipped with a 1-ml syringe and a 500-μl external loop. Separation of the enantiomers was carried out on a DAICEL Chiracel OJ-R column, 2 mm i.d. × 125 mm long (Grom Analytik + HPLC GmbH, Herrenberg, Germany). The trapping process, the washing procedure as well as the reconditioning of the trapping column was performed by means of a Merck-Hitachi L6200A HPLC-pump (Merck Kg, Darmstadt, Germany) equipped with a low pressure gradient module. The chiral analytical column (Daicel Chiracel OJ-R) was installed on the same valve as the trapping column so as to elute the analytes off the trapping column in back-flush mode. A guard column (SecurityGuard™ Octadecyl, 4 mm × 2 mm, Phenomenex, Torrance, CA, USA) protected the analytical column. Elution from the trapping column and separation on the analytical column was achieved with a high pressure gradient HPLC system, which was composed of two Shimadzu LC10AD HPLC-pumps (Burkhard Instrumente, Geroldswil, Switzerland), both connected to a 75-μl dynamic mixer (Portmann Instruments, Biel-Benken, Switzerland). The two pumps formed the isocratic mixture for separation were controlled by a system controller SCL-10Avp (also from Shimadzu). Samples were washed after injection for 0.7 min with water–acetonitrile–methanol–formic acid 85:7.5:7.5:0.1 (v/v/v/v) in front-flush mode and for an additional 0.3 min in back-flush mode at a flow rate of 2 ml/min. Then, the samples were transferred to the chiral analytical column with acetonitrile–methanol–water–formic acid 70:20:10:0.1 (v/v/v/v) at a flow rate of 0.4 ml/min. The valve was kept in this position for 0.7 min, then, flushing of the trapping column with acetonitrile–methanol–formic acid 50:50:0.1 (v/v/v) was performed for 0.7 min at a flow rate of 2 ml/min. The initial conditions were re-established 2.4 min after injection.

2.5. Mass spectrometric detection

Mass spectrometric detection was performed on an atmospheric pressure ionization triple quadrupole instrument API3000 (Applied Biosystems Sciex, Concord, ON, CA) in selected reaction monitoring (SRM) mode. Protonated molecules of the analytes and the internal standards were formed with ESI by means of a TurboIonSpray™ ion source. The protonated molecules were subjected to collision induced dissociation (CID) in a high-pressure collision cell containing nitrogen at a pressure of about 2.6×10^{-5} bar. A collision energy of 38 eV was applied to the protonated molecules to form abundant fragment ions which were selected in Q3 before detection in the electron multiplier. Nitrogen was used as nebulizing and collision gas, and air as auxiliary gas. A temperature of 350 °C was applied to the auxiliary gas to support ion evaporation. Both quadrupole mass filters were tuned to unit mass resolution (full width half max, FWHM definition) corresponding to 0.7 mass units peak width over the mass range *m/z* 75–915 using a mixture of eight quaternary alkyl ammonium salts.

3. Results and discussion

3.1. Mass spectrometry

The analytes, as well their internal standards, formed in ESI abundant protonated molecules at *m/z* 465 and 470, respectively. The highest signal intensity for the signals of the protonated molecules at *m/z* 465 and 470 was achieved with an orifice voltage of 55 V, a sprayer voltage of 5500 V and a turbo gas temperature of 450 °C. After CID, abundant fragment ions at *m/z* 186 were produced which were employed for the quantification in SRM mode. A collision energy of 38 eV showed the highest abundance for the product ion at *m/z* 186. A representative product ion spectrum of the drug is shown in Fig. 2.

3.2. Chiral separations

Several chiral separations have been reported using normal phase HPLC conditions [17,22] showing baseline separation within the time frame of 3 min [20] to about 12 min [18,19,23]. All of these methods required the transfer of the analytes from aqueous phase into a non-aqueous normal phase, and later the careful control of the water content of the mobile phase to main-

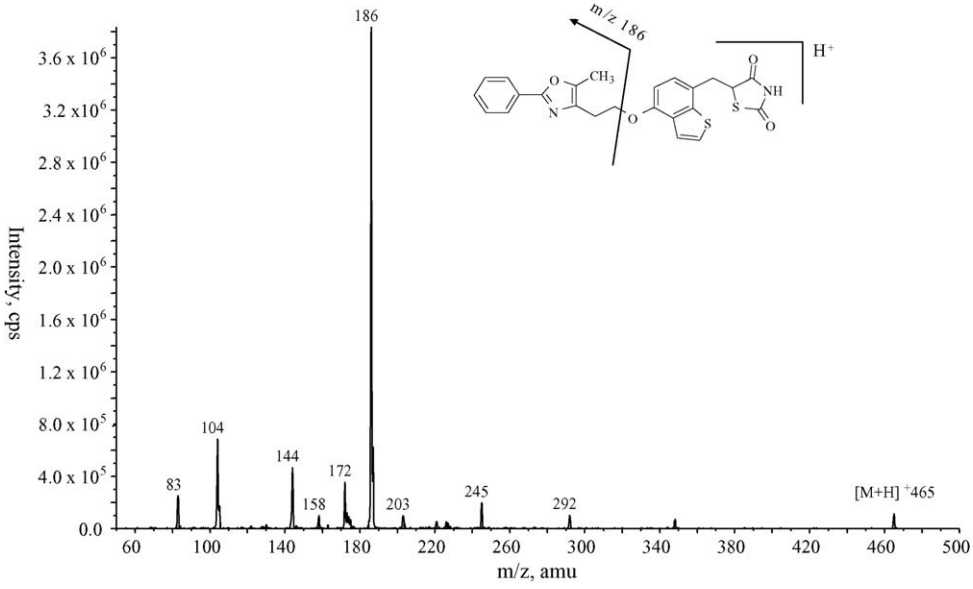

Fig. 2. Product ion spectrum of the drug.

tain separation efficiency. Due to the easier control of reversed phase mobile phases with respect to constant elution conditions and better compatibility with the electrospray process, we decided to use reversed phase mobile phases together with ESI. After testing several types of chiral columns, we obtained the best separations with a Chiracel OJ-R column. We started with a mobile phase composition of acetonitrile–methanol–10 mM ammonium acetate–acetic acid 70:20:10:1 (v/v/v/v) which gave a baseline separation. However, we achieved a seven-fold increase in sensitivity by replacing the ammonium acetate phase by water and the acetic acid phase by formic acid. This intensity gain was achieved without loss of chromatographic resolution.

3.2.1. In vitro investigations

Incubations of the racemic drug as well as its individual enriched enantiomers in buffer at pH values of 6, 7 and 8 showed that the enantiomeric composition remains stable even after storage for 6 h at 37 °C. In addition, storage in the acidified injection solution for 5 h at room temperature did not show any change. Extraction recovery was determined for the racemic mixture, as well as for the enriched enantiomers, to exclude its possible impact on the results of the in vitro experiments. The extraction recovery was found to be between 89.8 and 94.9%. The elution order was determined by injection of the enriched enantiomers, (−)-R483and (+)-R483. The (+)-enantiomer eluted first with a retention time of 3.35 min followed by the (−)-enantiomer at 4.31 min. A representative SRM ion chromatogram of the racemic mixture of the two enantiomers exhibiting a resolution R of 1.3 is shown in Fig. 5 ($R = 2$ (t (+) − t (−))/(w(+) + w(−)) where t (+) and t (−) represent the retention time of the (+) and (−)-enantiomer and w(+) and w(−) represent the base-width of the individual enantiomers). The racemic drug as well as the enriched enantiomers were incubated with rat and cynomolgus monkey plasma, both stabilized with EDTA/NaF, and with human plasma, stabilized either with heparin or citrate, as well as with freshly prepared human serum. The results revealed that the enantiomeric excess (ee, ee = (C(+) − C(−))/(C(+) + C(−) × 100%, assuming (+) > (−)), where C(+) and C(−) represent the concentrations of the particular enantiomers, of the racemate changed within 5 h at 37 °C in rat plasma from 2 to 21%, and in cynomolgus monkey plasma from 4 to 30%, together with a slight increase of the pH from 7.73 to 8.05 for rat, and from 7.73 to 8.10 for cynomolgus monkey plasma. The evaluation of the behavior of the mixtures enriched with enantiomers in rat plasma showed that the ee of the (+)-enantiomer decreased from 53 to 35%, whereas the ee for the (−)-enantiomer increased from (−)55 to (+)20% under the same conditions applied to the racemic drug. In cynomolgus monkey plasma, the ee of the (+)-enantiomer decreased from 51 to 39% while the ee of the (−)-enantiomer increased from (−)52 to (+)26%. The same experimental parameters were applied to human plasma stabilized with citrate and heparin as well as to freshly prepared human serum. The results for human plasma and serum showed the same tendency for interconversion of

Table 1
Results from incubations of human plasma and serum with the racemic drug and the enriched enantiomers (+) and (−)

	ee (%) = ((+) − (−))/((+) + (−)) × 100					
	Racemate (0 h)	Racemate (6 h)	(+)-Enantiomer (0 h)	(+)-Enantiomer (6 h)	(−)-Enantiomer (0 h)	(−)-Enantiomer (6 h)
Plasma (heparin)	6	47	64	54	−60	44
Plasma (citrate)	1	36	68	41	−68	36
Serum	5	44	62	51	−59	42

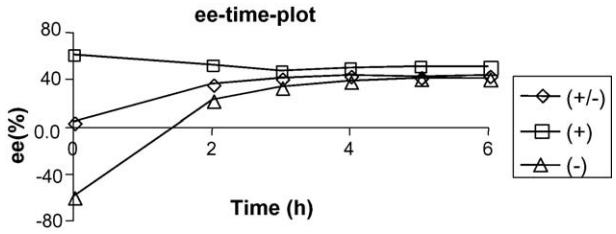

Fig. 3. ee–Time profile of an incubation of the racemate and the mixtures enriched with enantiomers in freshly prepared human serum.

the racemic drug as well as of its enriched enantiomers. The results are compiled in Table 1, values were recorded in duplicate with reasonable values for the deviation from the mean (mean 4.2%, range 0.1–13.8%, $N=42$ for the human serum experiment). A ee–time plot showing the ee values of the racemate and of the enriched enantiomers incubated in freshly prepared human serum is drawn in Fig. 3.

3.2.2. *Chiral drug assay for in vivo investigations*

To follow the interconversion of the administered racemic drug under in vivo conditions a chiral quantitative drug assay was necessary. The assay was developed with regard to the in vitro findings concerning the stability of the enantiomeric composition in acidified organic solution. Therefore, clinical personnel were instructed to preserve the in vivo enantiomeric composition of the plasma after sampling by immediate addition of the acidified internal standard solution. The assay at the analytical laboratory site then required only dilution of the organic supernatant with acidified water to reduce the elution power of the sample solution before on-line SPE. For further sample analysis, column switching using trapping and washing of the sample in front-flush combined with a second wash step in back-flush mode was employed. This approach has been used in our laboratory for several years [24–27], and has proved to be beneficial for extended analytical column lifetime, which is of special importance for the expensive chiral columns. The assay developed in our laboratory was linear from 0.5 to 500 ng/ml using (initially) a 100-μl plasma aliquot (corresponding to a 40-μl plasma equivalent applied to column). Data for inter-assay precision and accuracy are compiled in Tables 2 and 3. Inter-assay precision and accuracy were calculated by analysis of a set of calibration samples in duplicate on five different occasions. The first set, on each occasion, was used to establish the regression equation, which was applied to the second set which was treated like QC samples in order to qualify the method. Intra-assay precision and accuracy, calculated from independently prepared QC samples processed on one occasion at concentrations of 1.5, 40 and 200 ng/ml, were in the ranges 0.1–4.6 and 88.8–96.4% ($n=4$), respectively.

The specificity of the method was demonstrated by the absence of any interference in the ion chromatogram of the ana-

Table 2
Inter-assay precision and accuracy for (+)-R483

(+)-R483	Concentration added (ng/ml)	Average concentration found (ng/ml)	R.S.D. (%)	Accuracy (%)	*N*
C01	0.5	0.461	5.0	92.2	5
C02	1	1.05	7.5	105.3	5
C03	2	2.04	6.6	101.8	5
C04	5	5.16	2.6	103.3	5
C05	10	10.2	1.1	101.6	5
C06	50	47.7	3.3	95.3	5
C07	250	248	1.5	99.3	5
C08	500	473	2.0	94.6	5

Table 3
Inter-assay precision and accuracy for (−)-R483

(−)-R483	Concentration added (ng/ml)	Average concentration found (ng/ml)	R.S.D. (%)	Accuracy (%)	*N*
C01	0.5	0.492	7.9	98.3	5
C02	1	1.02	7.4	102.1	5
C03	2	2.12	4.0	106.0	5
C04	5	5.18	3.4	103.6	5
C05	10	10.5	1.4	104.6	5
C06	50	49.1	0.8	98.3	5
C07	250	247	1.8	98.9	5
C08	500	460	2.3	92.0	5

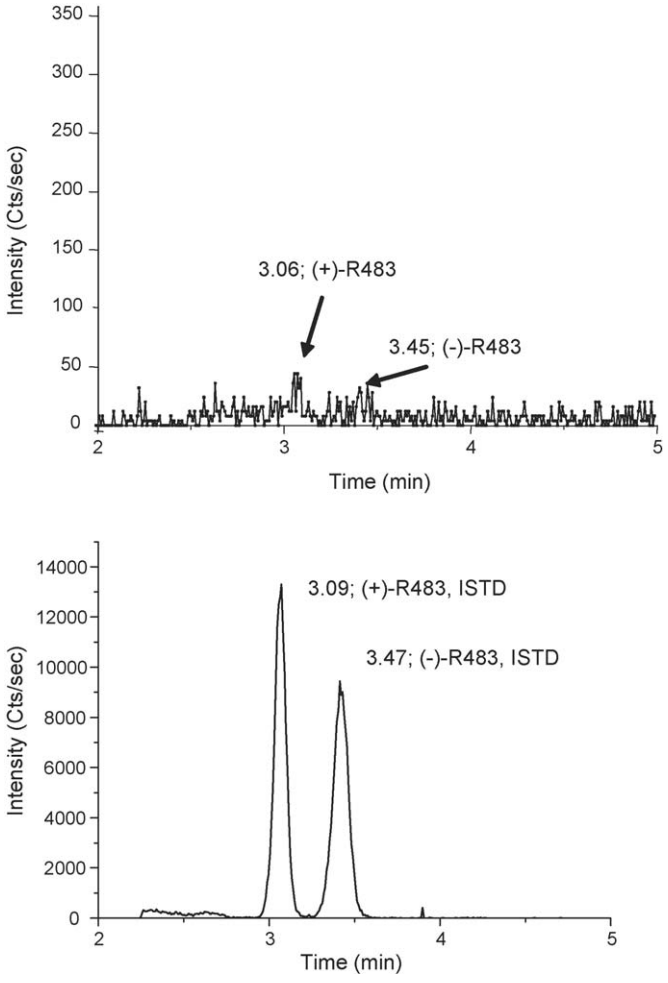

Fig. 4. Selected reaction ion chromatogram of a blank human plasma sample (analyte—top, internal standard (ISTD)—bottom).

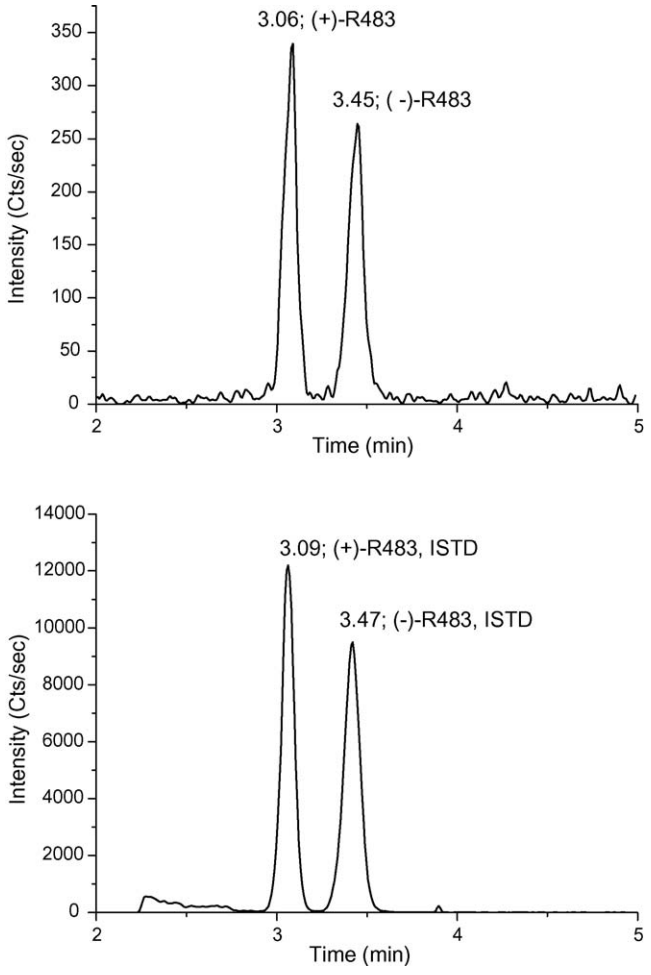

Fig. 5. Selected reaction ion chromatogram of a human plasma calibration sample C01 with 0.5 ng/ml of each enantiomer (analyte—top, internal standard—bottom).

lyte in a blank human plasma sample, as depicted in Fig. 4. The sensitivity of the assay is demonstrated by means of an extracted selected reaction ion chromatogram of the lowest calibration point at 0.5 ng/ml in Fig. 5. The analytical run time of 5 min per sample, together with minimal sample preparation in the analytical laboratory, allowed the analysis of about 200 samples per day. More then 800 injections were performed during assay development and application to a clinical study, without changes in retention time, resolution, or significant increase in back-pressure or replacement of any of the columns, showing the robustness of the assay. A comparison of the concentrations, determined with a non-chiral LC–single MS assay [28] applied in another laboratory, with the sum of both enantiomers quantified with the assay described above revealed an accuracy of 96.9%, with a relative standard deviation of 9.1% ($n = 114$). For this, samples processed on one day with the chiral method were compared with those analyzed with the non-chiral assay processed on different occasions. The evaluation of the results from the clinical study showed that the racemate was changed within a few hours to a mixture showing an (+)-enantiomeric excess of about $40 \pm 10\%$.

4. Conclusions

Drug assays were developed to assess the in vitro and in vivo behavior of a new racemic insulin sensitizer drug. In vitro investigations showed that the enantiomeric composition of the drug did not change in buffer solutions with pH between 6 and 8, and that in plasma of rats, cynomolgus monkey and human, and in human serum, an interconversion to a mixture enriched with the (+)-enantiomer occurred. This behavior could be confirmed after incubation of mixtures enriched with the particular enantiomers. A chiral quantitative drug assay for the determination of the particular enantiomers in the concentration range 0.5–500 ng/ml was developed and applied to a clinical study. For this assay a plasma equivalent of only 40 μl was necessary. The assay confirmed the in vitro results for the interconversion of the drug. The quantitative results from the chiral drug assay were in good agreement with results achieved with a non-chiral LC–single MS assay.

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