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Validated LC–MS/MS methods for the determination of risperidone and the enantiomers of 9-hydroxyrisperidone in human plasma and urine

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ABSTRACT

Two liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) methods are described, one for the quantitative determination of risperidone and the enantiomers of its active metabolite 9-hydroxyrisperidone (paliperidone) in human plasma and the other for the determination of the enantiomers of 9-hydroxyrisperidone in human urine. The plasma method is based on solid-phase extraction of 200 μl of sample on a mixed-mode sorbent, followed by separation on a cellulose-based LC column with a 13.5-min mobile phase gradient of hexane, isopropanol and ethanol. After post-column addition of 10 mM ammonium acetate in ethanol/water, detection takes place by ion-spray tandem mass spectrometry in the positive ion mode. Method validation results show that the method is sufficiently selective towards the enantiomers of 7-hydroxyrisperidone and capable of quantifying the analytes with good precision and accuracy in the concentration range of 0.2–100 ng/ml. An accelerated (run time of 4.3 min) and equally valid method for the enantiomers of 9-hydroxyrisperidone alone in plasma is obtained by increasing the mobile phase flow-rate from 1.0 to 2.0 ml/min and slightly adapting the gradient conditions. The urine method is based on the same solid-phase extraction and chromatographic approach as the accelerated plasma method. Using 100 μl of sample, (+)- and (−)-9-hydroxyrisperidone can be quantified in the concentration range 1–2000 ng/ml. The accelerated method for plasma and the method for urine can be used only when paliperidone is administered instead of risperidone, as there is insufficient separation of the 9-hydroxy enantiomers from the 7-hydroxy enantiomers, the latter ones being present only after risperidone administration.

1. Introduction

Risperidone is an atypical antipsychotic drug, which blocks serotonin 5-HT_2 and dopamine D_2 receptors and is widely used in the treatment of schizophrenia. The main *in vivo* metabolic pathway of risperidone is 9-hydroxylation, while 7-hydroxylation and *N*-dealkylation are involved to a much lesser extent [1,2]. The major metabolite, 9-hydroxyrisperidone, is equipotent to the parent drug in terms of pharmacological activity, when the plasma concentrations of risperidone plus 9-hydroxyrisperidone have been reported as the "active moiety" in studies with risperidone [3,4]. Because of its antipsychotic activity, 9-hydroxyrisperidone itself has also been investigated for the treatment of schizophrenia under the name paliperidone [5]. Since the early 1990s, the pharmacokinetics of risperidone and 9-hydroxyrisperidone have been extensively studied, which was permitted only by the availability of a number of bioanalytical methods for the quantitation of risperidone and 9-hydroxyrisperidone in plasma. Initially, radioimmunoassay and high-performance liquid chromatography (HPLC) were used [6–8], but more recently liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has emerged as the technique of choice because of its superior sensitivity and selectivity [9,10]. A rigorously validated LC–MS/MS method, with a lower limit of quantitation (LLOQ) of 0.1 ng/ml for both analytes, has been described by our groups [11] and has been applied to tens of thousands of samples over the past years.

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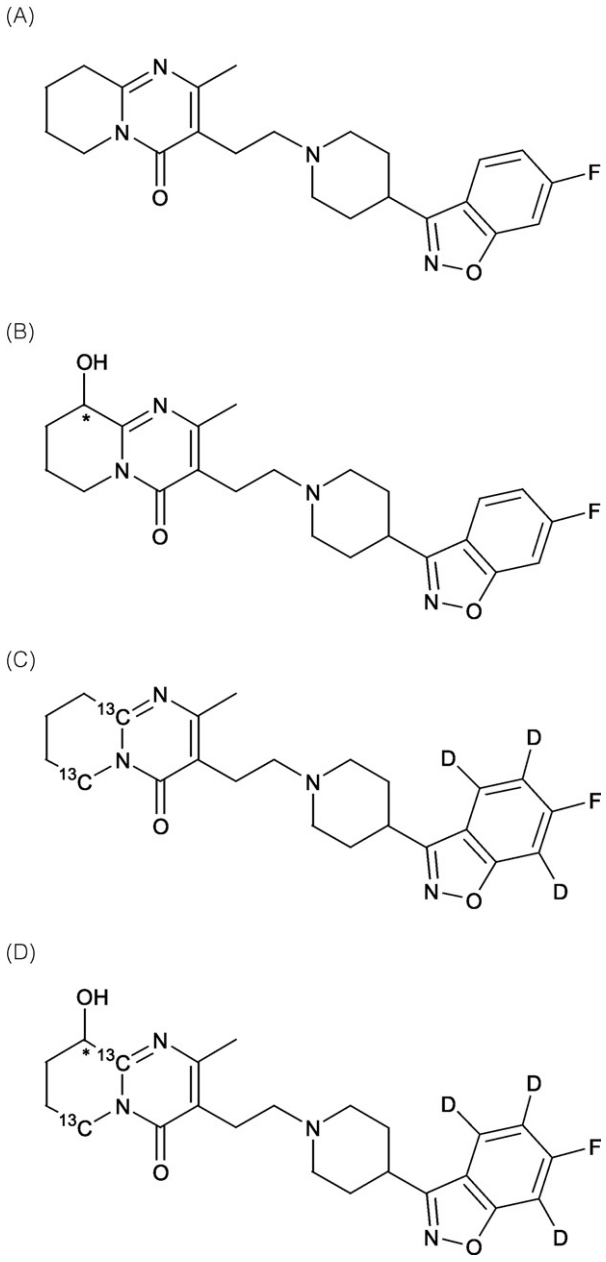

Fig. 1. Structures of risperidone (A) and 9-hydroxyrisperidone (B) and the stable isotope labelled internal standards for risperidone (C) and 9-hydroxyrisperidone (D), * indicates position of chiral carbon atom.

The 9-hydroxylation of risperidone, just as its 7-hydroxylation, leads to the formation of a chiral carbon atom resulting in two enantiomers: the (+)- and the (−)-form (Fig. 1). Both *in vitro* and *in vivo* results suggest that the cytochrome P-450 isoenzyme CYP2D6 catalyzes the formation of the (+)-enantiomer, whereas CYP3A4 and CYP3A5 play an important role in the (−)-9-hydroxylation [12,13]. However, detailed information about the kinetics of formation of the (+)- and (−)-9-hydroxy metabolites of risperidone, as well as about the further disposition of the enantiomers is scarce. An important reason for this is that, for the enantiomers of 9-hydroxyrisperidone, no method has been described for their determination in urine (relevant concentrations down to low-ng/ml) and, until recently, neither for their determination at the relevant concentrations found in plasma (typically low- to sub-ng/ml). One method has been published for plasma [12], which is based on separation of the enantiomers on a chiral α-1 acid glycoprotein column. In this method, UV detection was performed and, therefore, the sensitivity was limited. The LLOQ of 25 nM (about 10 ng/ml) restricted the use of the method to *in vitro* metabolism studies and the determination in plasma of top levels of the analytes only. Recently, Čabovska et al. described an LC–MS/MS method for risperidone and the enantiomers of 9-hydroxyrisperidone in plasma with an LLOQ of 0.2 ng/ml for all analytes [14]. A limited validation of the method was performed, but no details of application to clinical samples were shown.

The present paper reports an LC–MS/MS method for the determination of risperidone and the enantiomers of 9-hydroxyrisperidone in human plasma down to 0.2 ng/ml as well as a method for the enantiomers of 9-hydroxyrisperidone in human urine down to 1 ng/ml. The results of a thorough validation of the methods are presented and a comparison is given of the chiral plasma method with the non-chiral LC–MS/MS plasma method for risperidone and 9-hydroxyrisperidone published earlier [11]. Finally, the suitability of the methods for routine bioanalysis is illustrated by their application to pharmacokinetic samples for various clinical trials.

2. Experimental

2.1. Chemicals

Risperidone, racemic 9-hydroxyrisperidone, the separate (+)- and (−)-enantiomers of 9-hydroxyrisperidone, racemic 7-hydroxyrisperidone, $^{2}H_2$-$^{13}C_2$-risperidone and racemic $^{2}H_2$-$^{13}C_2$-9-hydroxyrisperidone were provided by Johnson and Johnson Pharmaceutical Research and Development (Beerse, Belgium). Ethanol, methanol, hexane, ammonium acetate, acetic acid, potassium dihydrogen phosphate, potassium hydroxide and ammonium hydroxide (25%) were obtained from Merck (Darmstadt, Germany), heptane from Riedel-de Haen (Seelze, Germany) and isopropanol from VWR (Amsterdam, the Netherlands). HPLC grade water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Analyte-free human heparin plasma was obtained from Biomedia (Boussens, France) and analyte-free human heparin whole blood and analyte-free human urine from Janssen Pharmaceutica (Beerse, Belgium).

2.2. Standard solutions

2.2.1. Plasma method

Methanolic stock solutions at 100 μg/ml were prepared separately for risperidone, racemic 9-hydroxyrisperidone and their internal standards. Combined calibration solutions were prepared for risperidone at 0.0400, 0.100, 0.200, 0.400, 1.00, 2.00, 4.00, 10.0 and 20.0 and for 9-hydroxyrisperidone at 0.0800, 0.200, 0.400, 0.800, 2.00, 4.00, 8.00, 20.0 and 40.0 ng per 100 μl methanol. A combined internal standard working solution was prepared in methanol at 1.00 ng $^{2}H_2$-$^{13}C_2$-risperidone and 2.00 ng $^{2}H_2$-$^{13}C_2$-9-hydroxyrisperidone per 100 μl. All stock solutions were stored at −20 °C and all calibration and working solutions at +4 °C.

Calibration samples at 0.200, 0.500, 1.00, 2.00, 5.00, 10.0, 20.0, 50.0 and 100 ng/ml for risperidone and each of the 9-hydroxyrisperidone enantiomers were prepared freshly every day by adding 100 μl of the appropriate calibration solution to 200-μl aliquots of analyte-free plasma. Validation samples were prepared in plasma at 0.200, 0.520, 5.20 and 76.0 ng/ml (0.200, 0.570, 5.10 and 78.1 ng/ml for the accelerated method) and stored at −20 °C in polyethylene tubes. Calibration and validation samples were prepared from separate stock solutions.

2.2.2. Urine method

Stock solutions were used as described for the plasma method. Calibration solutions were prepared for 9-hydroxyrisperidone at 0.200, 0.400, 1.00, 2.00, 4.00, 10.0, 20.0, 40.0, 100, 200 and 400 ng per 100 μl methanol. An internal standard working solution was prepared in methanol at 10.0 ng $^{2}H_{2}$-$^{13}C_{2}$-9-hydroxyrisperidone per 100 μl. Calibration samples at 1.00, 2.00, 5.00, 10.0, 20.0, 50.0, 100, 200, 500, 1000 and 2000 ng/ml for each of the 9-hydroxyrisperidone enantiomers were prepared freshly every day by adding 100 μl of the appropriate calibration solution to 100-μl aliquots of analyte-free urine. Validation samples were prepared in urine at 1.00, 2.70, 50.0 and 1560 ng/ml and stored at −20 °C in glass tubes. Calibration and validation samples were prepared from separate stock solutions.

2.3. Sample preparation

Sample preparation for plasma was essentially as described for the non-chiral method [11]. Briefly, 200 μl of plasma was mixed with 3.0 ml 0.1 M phosphate buffer (pH 6.0), 100 μl methanol (or calibration solution) and 100 μl internal standard working solution and transferred to a 10 cm^3/130 mg Bond Elut Certify solid-phase extraction (SPE) column, obtained from Varian (Walnut Creek, CA, USA), which had been conditioned with 3.0 ml of methanol, 3.0 ml of water and 1.0 ml of 0.1 M phosphate buffer (pH 6.0). The SPE column was washed with 3.0 ml of water, 1.0 ml of 1 M acetic acid solution and 3.0 ml of methanol and subsequently eluted with 3.0 ml of a mixture of methanol and 25% ammonium hydroxide (98:2, v/v). The eluate was evaporated to dryness under nitrogen at 65 °C and the residue was dissolved in 200 μl heptane–0.01 M ammonium acetate in ethanol (50:50, v/v). For urine, the sample preparation procedure was similar, except that 100 μl of urine was used and that 200 μl of blank plasma was added to the mixture before SPE.

2.4. Separation and detection

2.4.1. Plasma method

The chromatographic system consisted of an Agilent (Santa Clara, CA, USA) HP-1100 LC system for injection and pumping of the mobile phase, a Daicel (Osaka, Japan) Chiralcel OJ column (50 mm × 4.6 mm, 10-μm particle size), conditioned at 40 °C in a water bath, and an MDS Sciex (Foster City, CA, USA) API 3000 mass spectrometer, equipped with a Turboionspray™ interface at 50 °C. Aliquots of 15 μl of the pre-treated samples were injected. The mobile phase, a mixture of hexane (solvent A), 0.01 M ammonium acetate in isopropanol (solvent B) and 0.01 M ammonium acetate in ethanol (solvent C) was delivered at 1.0 ml/min. Isocratic elution was performed until 7.0 min after injection using a mixture of 80% A, 10% B and 10% C, after which a step gradient was applied with 10% A, 45% B and 45% C until 10.6 min; finally the column was re-equilibrated at 80% A, 10% B and 10% C until 13.5 min. Post-column addition of 0.01 M ammonium acetate in ethanol-water (94:6, v/v) was performed at 0.5 ml/min. From this total flow of 1.5, 0.1 ml/min was directed to the mass spectrometer and the remainder was split off to waste.

Quantitation was achieved by MS/MS detection in the positive ion mode. Detection of the ions was performed by multiple reaction monitoring (MRM) of the transition of the *m*/*z* 411.2 precursor ion to the *m*/*z* 191.0 product ion for risperidone, the *m*/*z* 427.2 precursor ion to the *m*/*z* 207.0 product ion for 9-hydroxyrisperidone, the *m*/*z* 415.2 precursor ion to the *m*/*z* 209.0 product ion for the internal standard for risperidone and the *m*/*z* 431.2 precursor ion to the *m*/*z* 209.0 product ion for the internal standard for 9-hydroxyrisperidone.

2.4.2. Accelerated plasma method and urine method

A faster version of the plasma method was obtained by adapting the elution characteristics. The flow rate was 2.0 ml/min and from 0 to 2.3 min, a mixture of 70% A, 25% B and 5% C was used, followed by a mixture of 10% A, 45% B and 45% C from 2.3 to 3.5 min and re-equilibration at the initial composition from 3.5 to 4.3 min. The post-column solution was pumped at 1.0 ml/min. A flow of 0.3 ml/min was directed to the mass spectrometer, with the Turboionspray™ interface operating at 200 °C. Other experimental conditions were similar to those described above. For the urine method, the same settings were used as for the accelerated plasma method.

2.5. Validation

The methods were validated in accordance with the FDA guidance for bioanalytical method validation [15] and also based on the paper of Shah et al. [16]. A full validation was performed for the plasma and urine methods and a partial validation (precision, accuracy and selectivity) for the accelerated plasma method. For all methods, a linear model was used to describe the relation between (log-transformed) analyte concentration and instrument response (log-transformed peak area ratio of analyte over internal standard). The selectivity towards endogenous plasma and urine components was determined by analysing six different sources of non-pooled, analyte-free matrix, unspiked and spiked at the LLOQ level.

The precision and accuracy of the methods were assessed at four concentration levels by analysis of the validation samples (see Sections 2.2.1 and 2.2.2) in duplicate in five analytical batches. The within-batch accuracy was calculated for each batch as the mean concentration found for that batch relative to the theoretical concentration. The overall accuracy was defined as the mean of the within-batch accuracies. The within-batch precision was calculated for each batch as the coefficient of variation (CV) over the two results and the overall CV over all ten results. The performance of the accelerated plasma method was determined by analysing the validation samples in six-fold in one analytical batch and calculating the (within-batch) precision and accuracy.

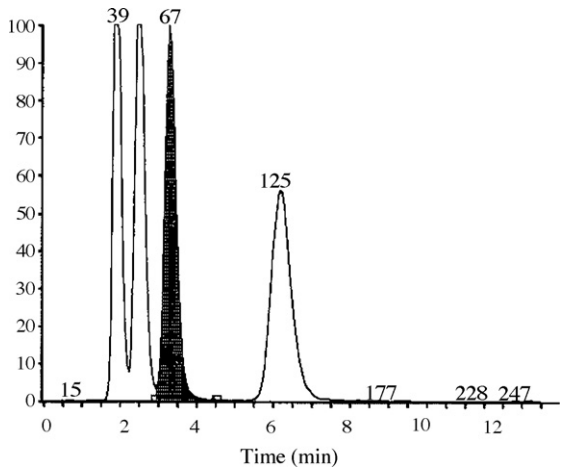

Fig. 2. chromatogram showing the mass transition for 9-hydroxyrisperidone in a test solution containing equal concentrations of the racemates of 7- and 9-hydroxyrisperidone, using the original plasma method; elution order of the enantiomers is (+)-7-hydroxy, (−)-7-hydroxy, (+)-9-hydroxy and (−)-9-hydroxy.

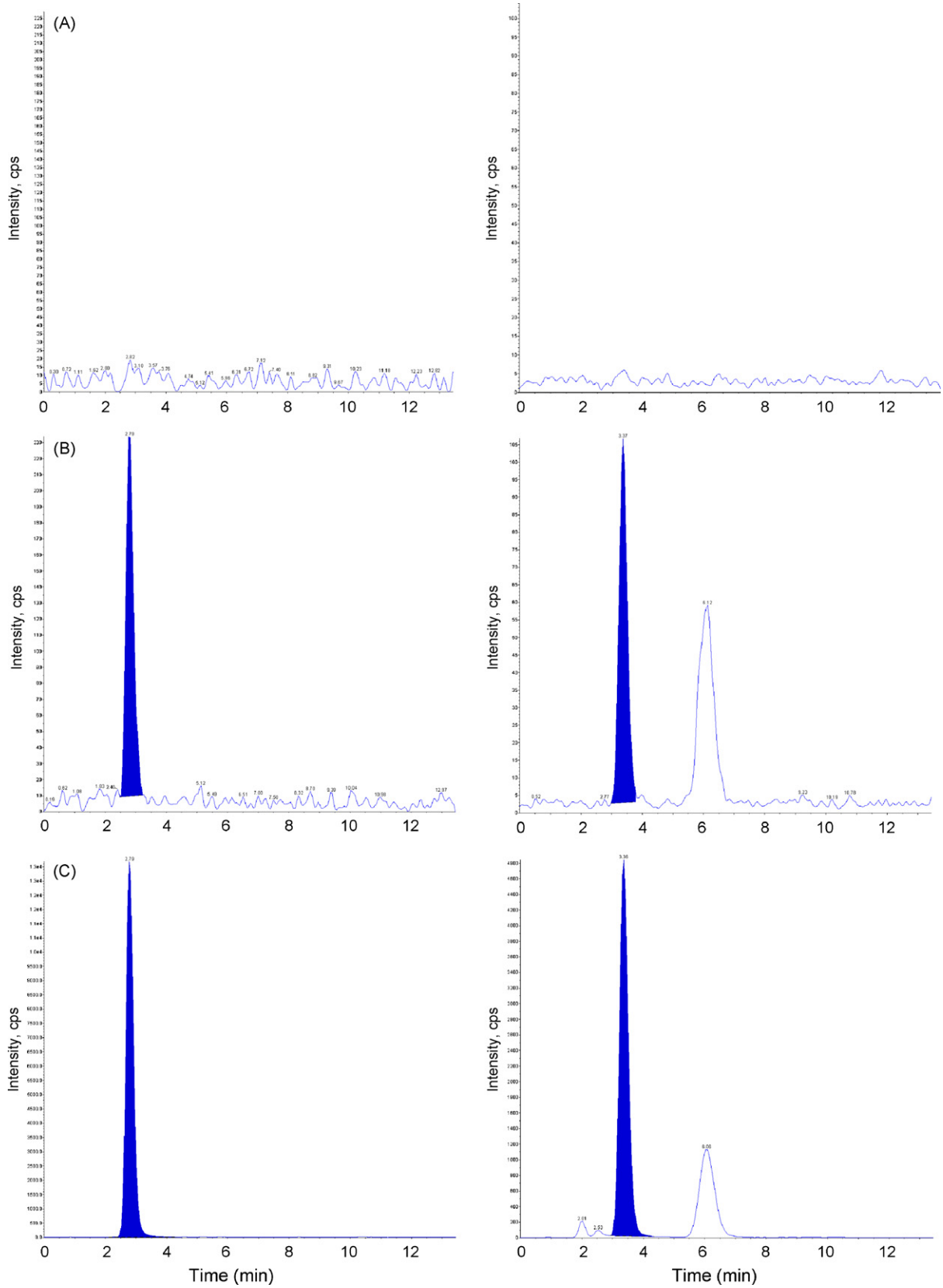

Fig. 3. Chromatograms showing the mass transition for risperidone (left panels) and 9-hydroxyrisperidone (right panels) in (A) analyte-free plasma, (B) plasma spiked at 0.200 ng/ml and (C) plasma taken from a patient 1 h after a 0.25 mg oral dose of risperidone during a twice daily dose regimen; concentrations: risperidone: 11.1 ng/ml; (+)-9-hydroxyrisperidone: 10.4 ng/ml, (−)-9-hydroxyrisperidone: 4.14 ng/ml; elution order of the enantiomers is (+) and (−).

For the enantiomers of 9-hydroxyrisperidone, the accuracy of the results was also determined at the possible *in vivo* concentration ratios of 1:10 and 10:1. In plasma, 0.500 ng/ml of one enantiomer was determined in the presence of 5.00 ng/ml of the other and vice versa; in urine, these concentrations were 2.00 and 20.0 ng/ml, respectively. The results were expressed as degree of inter-conversion, which was calculated as the difference in concentration of one enantiomer before and after storage relative to the concentration of the other enantiomer before storage.

The extraction recovery of the analytes was evaluated at three concentrations (1.00, 5.00 and 100 ng/ml for plasma and 5.00, 50.0 and 2000 ng/ml for urine) by comparing the peak areas, obtained from calibration samples after standard analysis to the peak areas, obtained from analyte-free samples, spiked after extraction. The extraction recovery of the internal standards was determined similarly at one concentration (5.00 ng/ml for plasma and 50.0 ng/ml for urine).

The stability of analytes and internal standards was evaluated under various conditions by analysis in triplicate. Stability of risperidone and 9-hydroxyrisperidone racemate in stock solution and in plasma and whole blood was determined earlier using the non-chiral method [11] and the assessment was not repeated here. Freeze/thaw stability, stability at ambient temperature and stability at −20 °C of the separately spiked enantiomers of 9-hydroxyrisperidone in plasma was assessed at two concentrations (1.06 and 77.1 ng/ml for the (+)-form and 1.07 and 77.5 ng/ml for the (−)-form) by comparing the mean concentration after storage to the one before storage. Similarly, the stability in whole blood was determined at two concentrations (0.984 and 225 ng/ml for the (+)-enantiomer and 0.989 and 228 ng/ml for the (−)-enantiomer) and stability at ambient temperature in processed plasma was determined at 0.520, 5.20 and 76.0 ng/ml. Stability in urine (at 37 °C, ambient temperature, 4, −20 °C and after repeatedly freezing and thawing) was assessed at two concentrations (5.00 and 1560 ng/ml) for samples containing the separate enantiomers of 9-hydroxyrisperidone. Likewise, stability in processed urine at ambient temperature was evaluated at 2.70, 50.0 and 1560 ng/ml. Stability of the separate enantiomers of 9-hydroxyrisperidone in stock solution (methanol) was determined with HPLC–UV at 100 μg/ml after storage at −20, 4 °C and ambient temperature, by comparing the peak areas found after injection of the stored stock solutions to those found for freshly prepared stock solutions.

2.6. Cross-validation with non-chiral method

A cross-validation of the non-chiral LC–MS/MS method [11] and the chiral plasma method described here was performed in order to investigate the consistency of both methods. A set of 1308 plasma samples, obtained from dosed subjects, was analysed with both methods and the results of the samples with quantifiable results for each of the three analytes were used. For 9-hydroxyrisperidone, the results of the enantiomers obtained with the chiral method were summated and compared to the racemate results obtained with the non-chiral method, while for risperidone the concentrations obtained with both methods were directly compared. Non-weighted linear regression analysis was conducted (on log-transformed *x*- and *y*-axes) and the regression parameters were calculated for both risperidone and 9-hydroxyrisperidone. In addition, the mean relative difference between the chiral and non-chiral results was determined for each compound by averaging all relative differences, which were calculated by dividing the (absolute) concentration difference between the two results by the corresponding mean concentration.

3. Results and discussion

3.1. Sample preparation

Because of its good performance, the sample-preparation procedure employed for the determination of risperidone and racemic 9-hydroxyrisperidone [11] was used here as well. The mixed-mode SPE phase traps the analytes in their cationic forms (at pH 6), mainly because of ion-exchange interactions. The selectivity of sample preparation is thus complementary to the chiral selectivity of the subsequent LC separation. The extraction recovery was almost complete for all analytes, in both plasma and urine.

For urine, a small loss of the analytes was observed, when spiked samples were stored in polyethylene tubes, most probably because of adsorption onto the tube wall. When spiked urine samples were transferred five times, each time into a new 20-ml tube, an analyte loss of ca. 3.5% per cycle was observed for polyethylene, while this was only about 1% per cycle for glass. The, much larger, polypropylene collection and storage containers used in clinical trials showed no significant adsorption after storage for 24 h. Therefore, spiked quality control samples were stored in glass tubes prior to analysis, while study samples were stored in polypropylene. None of these effects were observed for plasma, which leads to the conclusion that the presence of solubilizing plasma proteins helps to prevent the adsorption of the, rather non-polar, analytes during the various stages of sample storage and analysis. Therefore, in order to avoid possible adsorption effects during sample preparation, a two-fold excess of analyte-free plasma was added to the pipetted urine aliquots.

3.2. Separation

Just as for the non-chiral method, an important issue is the potential interference of the minor metabolite 7-hydroxyrisperidone, which cannot be discriminated from the 9-hydroxy form by mass spectrometry and must, therefore, be chromatographically separated. For the present chiral method, this means that not only the enantiomers of 9-hydroxyrisperidone have to be properly resolved but also the enantiomers of 7-hydroxyrisperidone have to be separated from those of 9-hydroxyrisperidone. With a short (50 mm) chiral column, packed with a cellulose tris(4-methylbenzoate) stationary phase and a mobile phase of 80% hexane, 10% isopropanol and 10% ethanol (the latter two containing 0.01 M ammonium acetate) delivered at 1.0 ml/min, the enantiomers of 9-hydroxyrisperidone could be very well resolved (R_s = 3.4) within 7 min. Under these conditions, acceptable separation (R_s = 1.5) of the latest eluting 7-hydroxyrisperidone enantiomer (the (−)-form) from the first eluting 9-hydroxyrisperidone enantiomer (the (+)-form) was also achieved (Fig. 2). Including a step gradient at the end of each run to elute possible remaining sample components off the column and subsequent re-equilibration, the total chromatographic run time was 13.5 min.

The speed of analysis could be significantly increased by doubling the mobile phase flow-rate and adapting the mobile phase composition to 70% hexane, 25% isopropanol and 5% ethanol. Despite a loss of separation efficiency (plate number as calculated for (+)-9-hydroxyrisperidone dropped from 480 to 270), the enantiomers of 9-hydroxyrisperidone were well resolved (R_s = 2.7) within just 2.5 min. The total run time was only 4.3 min, including a short step gradient and re-equilibration time. Separation of the enantiomers of 9-hydroxyrisperidone from those of 7-hydroxyrisperidone is not relevant for the accelerated plasma method, since this method is only used to sup-

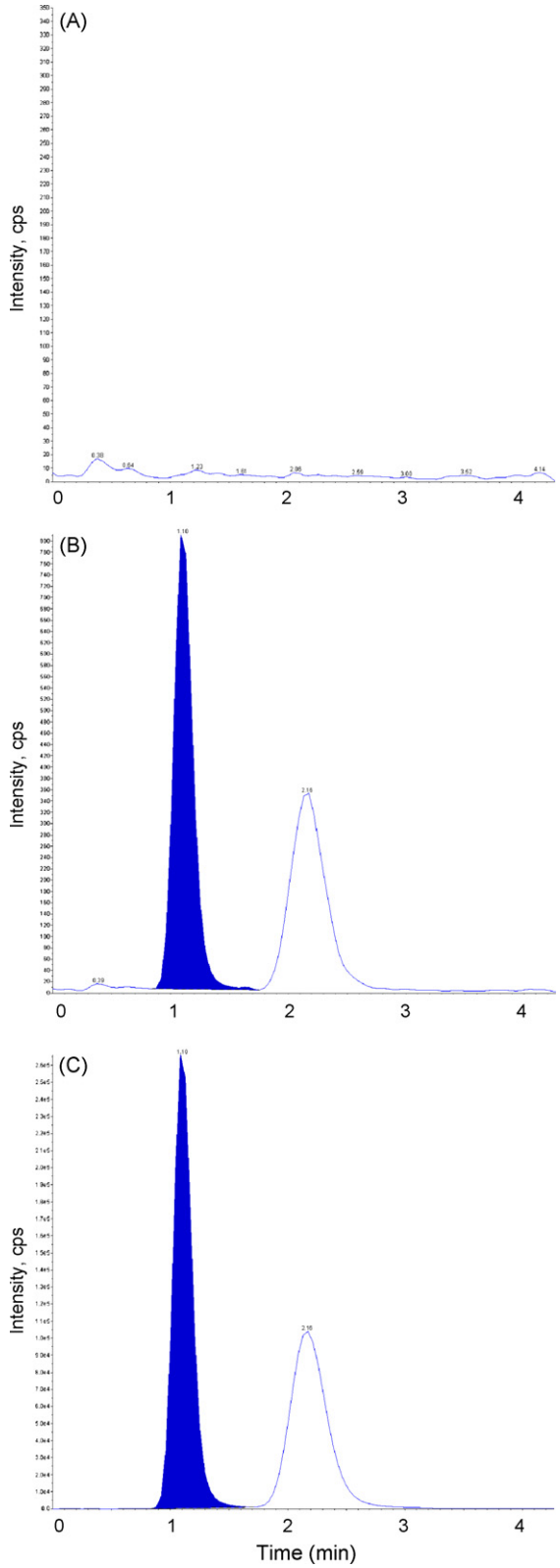

Fig. 4. Chromatograms, obtained with the accelerated method, showing the mass transition for 9-hydroxyrisperidone in (A) analyte-free urine, (B) urine spiked at 1.00 ng/ml and (C) urine collected from a healthy volunteer 8–12 h after a single oral dose of 6 mg ER OROS® paliperidone, concentrations: (+)-enantiomer: 265 ng/ml, (−)-enantiomer: 276 ng/ml; elution order of the enantiomers is (+) and (−).

port studies with administered paliperidone and the 7-hydroxy metabolite of risperidone will not be present in these plasma samples.

3.3. Detection

The molecular ions of risperidone (*m*/*z* 411.2) and 9-hydroxyrisperidone (*m*/*z* 427.2) and their internal standards (*m*/*z* 415.2 and *m*/*z* 431.2, respectively) are fragmented almost exclusively by cleavage of the ethyl-piperidinyl bond. The analytes loose a mass fragment of *m*/*z* 220.2 and the internal standards a mass fragment of *m*/*z* 222.2 and produce product ion signals at *m*/*z* 191.0, *m*/*z* 207.0, *m*/*z* 193.0 and *m*/*z* 209.0, respectively.

The sensitivity of detection, however, was limited in the presence of the large proportion of hexane (70–80%) necessary to effect a rapid and efficient chromatographic separation. By post-column addition of 0.01 M ammonium acetate in an ethanol/water mixture, the ionisation efficiency could be increased three-fold. The water content of the post-column addition solvent was varied between 3% and 6% (v/v), but no differences in sensitivity were observed. Since the MS signal was found to be most stable upon addition of an ethanol/water mixture of 94/6 (v/v), this composition was selected. Altogether, ionisation and fragmentation were found to be highly efficient and a considerable detector response was obtained at the LLOQ levels (0.200 ng/ml in plasma and 1.00 ng/ml in urine) using just 200 μl of plasma or 100 μl of urine (Figs. 3B and 4B).

3.4. Validation results

For all calibration curves recorded, back-calculated concentrations of the calibrators were within 10% of the theoretical value, which was well within the requirements of international guidelines [15] and indicated the acceptability of the linear calibration model.

The methods were sufficiently selective towards endogenous plasma and urine components and possible impurities of the chemicals used. None of the tested lots of analyte-free plasma or urine contained any unacceptable interference, as is exemplified in Figs. 3A and 4A. In addition, accuracy and precision found for the six different lots of plasma and urine, spiked at the LLOQ level, was fully satisfactory (Table 1), which indicates that-although the magnitude of the matrix effect was not determined—differences in the plasma and urine composition do not affect the quality of the results. For risperidone, (+)- and (−)-9-hydroxyrisperidone in plasma, the mean values for accuracy were 109.8%, 105.6% and 110.4%, respectively, while the CV was 8.4%, 13.4% and 8.9%, respectively. For the (+)- and (−)-enantiomers of 9-hydroxyrisperidone in urine, the results were 97.4% and 97,7%, respectively, for accuracy and 3.0% and 3.9%, respectively, for precision. Altogether, these results show that quantitation down to 0.200 ng/ml in plasma (Fig. 3B) and down to 1.00 ng/ml in urine (Fig. 4B) is not affected by interference from the sample matrix or the chemicals used.

Table 1
Matrix effect: precision and accuracy ($n = 6$) using six different lots of matrix

Matrix	Theoretical concentration (ng/ml)	Analyte	Accuracy (%)	Precision (%CV)
Plasma	0.200	Risperidone	109.8	8.4
	0.200	(+)-9-Hydroxyrisperidone	105.6	13.4
	0.200	(−)-9-Hydroxyrisperidone	110.4	8.9
Urine	1.00	(+)-9-Hydroxyrisperidone	97.4	3.0
	1.00	(−)-9-Hydroxyrisperidone	97.7	3.9

Table 2
Summary of precision and accuracy results (plasma method, $n = 10$)

Analyte	Theoretical concentration (ng/ml)	Overall accuracy (%)	Overall precision (%CV)
Risperidone	0.200	110.0	11.4
	0.520	96.4	6.0
	5.20	94.5	3.5
	76.0	91.6	5.7
(+)-9-Hydroxyrisperidone	0.200	107.0	9.4
	0.520	95.7	7.2
	5.20	97.2	4.1
	76.0	94.2	5.6
(−)-9-Hydroxyrisperidone	0.200	105.1	10.1
	0.520	95.2	4.8
	5.20	95.7	3.1
	76.0	94.4	5.5

Accuracy and precision results are summarized in Table 2 for the plasma method, Table 3 for the accelerated plasma method and Table 4 for the urine method. Values for accuracy were between 90% and 110% over the entire concentration range, while the results for precision (%CV) with one exception did not exceed 12%, which was well within the requirements [15]. The results for the accelerated plasma method were comparable to those of the original plasma method, which showed that a three-fold higher sample throughput (from 13.5 to 4.3 min per sample) can be obtained with equally valid results.

The *in vivo* concentration ratio of the enantiomers of 9-hydroxyrisperidone in plasma and urine may vary from 1:10 to 10:1. If, in such a case, inter-conversion of the enantiomers occurs, the accuracy of the result for the enantiomer present at the lowest concentration may be compromised. Since this phenomenon is not picked up during the normal accuracy assessment, with the enantiomers spiked at equal concentrations, the degree of inter-conversion was determined by analysing samples spiked with one enantiomer at 0.500 ng/ml and the other at 5.00 ng/ml and vice versa (plasma) or 2.00 and 20.0 ng/ml (urine). It appeared that the overestimation due to possible inter-conversion during sample extraction and analysis amounted to no more than 1.2% for plasma and to no more than 0.2% for urine. This indicates that the enantiomers of 9-hydroxyrisperidone can be quantified within the normal criteria for accuracy, also if relative their concentrations differ up to a factor 10.

Table 3
Summary of precision and accuracy results (accelerated plasma method, $n = 6$)

Analyte	Theoretical concentration (ng/ml)	Accuracy (%)	Precision (%CV)
(+)-9-Hydroxyrisperidone	0.200	90.0	4.0
	0.570	90.0	2.2
	5.10	92.9	2.3
	78.1	99.2	2.1
(−)-9-Hydroxyrisperidone	0.200	95.5	17.2
	0.570	90.4	6.4
	5.10	93.7	2.1
	78.1	98.6	2.2

Table 4
Summary of precision and accuracy results (urine method, $n = 10$)

Analyte	Theoretical concentration (ng/ml)	Overall accuracy (%)	Overall precision (%CV)
(+)-9-Hydroxyrisperidone	1.00	95.4	4.1
	2.70	93.3	3.4
	50.0	92.4	2.9
	1560	99.4	2.3
(−)-9-Hydroxyrisperidone	1.00	95.6	3.4
	2.70	94.4	2.5
	50.0	95.4	2.6
	1560	101.3	2.4

The extraction recovery was consistent over the calibration range and essentially complete for all analytes and both matrices. For plasma, the mean recovery for risperidone was 100.4%, for (+)- and (−)-9-hydroxyrisperidone, it was 99.9% and 89.9%, respectively. Mean recoveries for the internal standards of the three analytes were 102.7%, 102.4% and 92.9%, respectively. For urine, the results were comparable: for (+)- and (−)-9-hydroxyrisperidone mean recoveries were found of 97.7% and 98.0%; for the corresponding internal standards, the results were 100.7% and 101.1%, respectively. The somewhat lower recovery for (−)-9-hydroxyrisperidone and its internal standard in plasma is possibly related to the fact that recovery is calculated using peak area, whereas all other parameters are determined using peak area ratio over internal standard. This might lead to a higher variation in response, as no correction is provided by the internal standard.

The stability results, summarized in Table 5, show that the analytes are stable under all conditions investigated. For risperidone, stability in plasma, whole blood and stock solution (methanol) was already found to be acceptable in an earlier investigation [11]. The only parameter that was investigated for risperidone in the present study was stability in processed plasma, because the injection matrix (a mixture of heptane and ethanol) for the current chiral method was different from the one used for the non-chiral method (a mixture of water and acetonitrile). For most of the tests, the concentrations did not deviate by more than 5% from those of the reference samples. Whole blood containing the analytes may therefore be kept at 37 °C for up to 2 h and at ambient temperature or 4 °C for up to 24 h, before plasma is prepared. Plasma can be stored at −20 °C for up to 324 days and at ambient temperature for up to 72 h prior to sample preparation; in addition, it may be thawed and refrozen up to four times. Plasma extracts may be stored at ambient temperature for up to 5 days, or up to 6 days when only 9-hydroxyrisperidone is present. Similar results were found for urine: the enantiomers of 9-hydroxyrisperidone were demonstrated to be stable in urine for 1 h at 37 °C, for 24 h at 4 °C and for 72 h at ambient temperature. Storage at −20 °C up to 280 days and four consecutive freeze-thaw cycles are also allowed. In addition, urine extracts can safely be stored for up to 6 days at ambient conditions. Finally, methanolic stock solutions of both enantiomers of 9-hydroxyrisperidone can be used for 6 months when stored at −20 °C, for 1 month when stored at 4 °C and for 3 days when stored at room temperature, both protected and unprotected from light.

3.5. Cross-comparison with non-chiral method

A total of 1308 plasma samples, taken from dosed subjects, were analysed for risperidone and 9-hydroxyrisperidone with both the non-chiral method, described earlier [11], and the current chiral method. Fig. 5 shows the correlation plots obtained for risperidone (799 quantifiable results) and 9-hydroxyrisperidone (1009 quantifiable results). The results from both methods correlated very well, with correlation coefficients of 0.9942 for risperidone and 0.9940 for 9-hydroxyrisperidone. The slopes of the regression lines were 0.923 and 0.943 for risperidone and 9-hydroxyrisperidone, respectively, which indicates that, overall, the results obtained with the chiral method were slightly lower than those found with the

Table 5
Summary of stability results for low/(medium)/high concentrations, n.d. = not determined

Matrix	Temperature (°C)	Period	Mean deviation from $t = 0$ (%)		
			Risperidone	(+)-9-Hydroxyrisperidone	(−)-9-Hydroxyrisperidone
Plasma	−20	324 days	n.d.	−2.8/−3.4	−0.6/−8.5
	Ambient	72 h		−4.0/−1.2	−3.5/−0.5
	Freeze/thaw	4 Cycles		−2.6/−1.4	−0.3/+1.2
Whole blood	+4	24 h	n.d.	+1.8/+0.4	−1.0/+6.4
	Ambient	24 h		−8.0/−5.3	−8.6/−10.0
	37	2 h		−4.9/−7.7	−11.9/−5.0
Processed plasma	Ambient	5 days	−4.7/−10.2/−9.5	n.d.	n.d.
		6 days	n.d.	−5.3/+0.8/−2.0	+5.3/+0.4/+0.8
Urine	−20	280 days	n.d.	−7.4/−5.3	−9.9/−7.3
	+4	24 h		+1.6/0.0	−2.4/0.0
	Ambient	72 h		−6.7/−10.5	−11.4/−8.2
	+37	1 h		+1.1/−1.3	−1.3/+0.7
	Freeze/thaw	4 Cycles		+1.1/+0.7	−3.4/+0.7
Processed urine	Ambient	6 days	n.d.	+3.3/+1.8/+4.1	−2.2/+3.1/+2.3
Methanol	−20	6 month	n.d.	+2.5	−0.7
	+4	1 month		−1.6	−0.5
	Ambient (dark)	3 days		−1.2	+1.6
	Ambient (light)	3 days		−2.2	+3.3

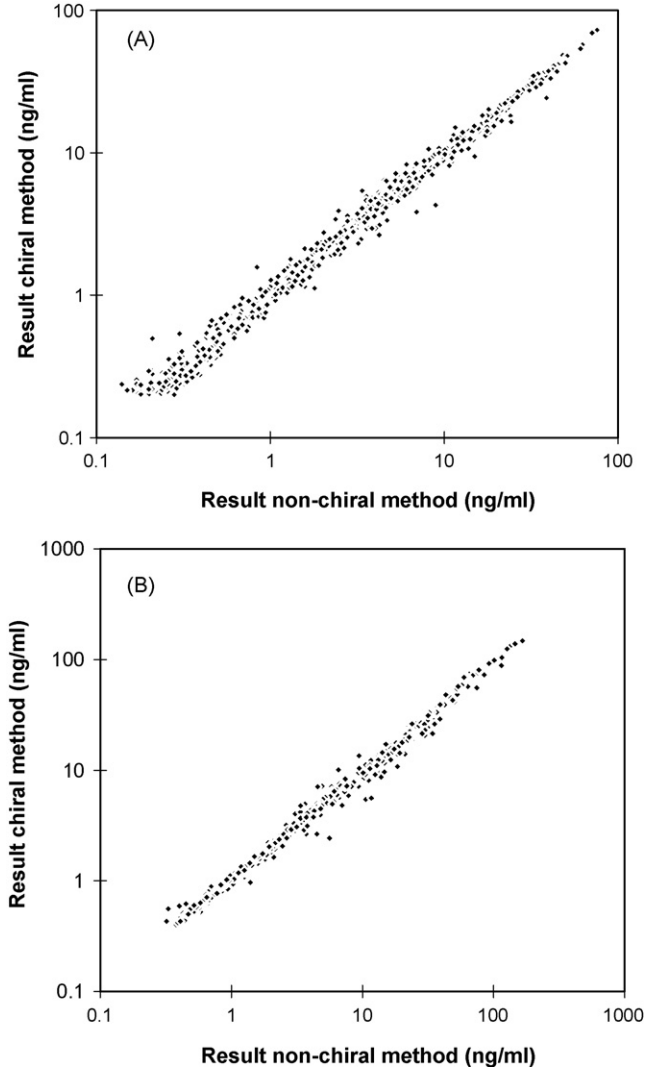

Fig. 5. Correlation plots showing the plasma concentrations as obtained by the chiral and the non-chiral methods for (A) risperidone and (B) 9-hydroxyrisperidone.

non-chiral method. The mean relative difference between the non-chiral and the chiral results was 11.7% for risperidone and 6.1% for 9-hydroxyrisperidone, which shows the consistency of both methods and the reliability of the results.

3.6. Application to pharmacokinetic studies

To date, more than 20,000 plasma samples and more than 2500 urine samples from clinical trials with risperidone and paliperidone have been analysed with the methods described. The average accuracy (expressed as %bias from the nominal concentration) and precision (expressed as %CV) found for spiked quality control samples are in the order of 7% for the low level and 5% for the medium and high levels, for all analytes. Representative chromatograms of samples taken from human subjects (plasma after dosing with risperidone and urine after dosing with paliperidone) are depicted in Figs. 3C and 4C, respectively. The former figure also shows that the enantiomers of 7-hydroxyrisperidone are well separated from those of 9-hydroxyrisperidone. As an illustration, Fig. 6 shows a plasma concentration versus time profile for the paliperidone enantiomers in a healthy volunteer, obtained after single oral dosing with racemic paliperidone.

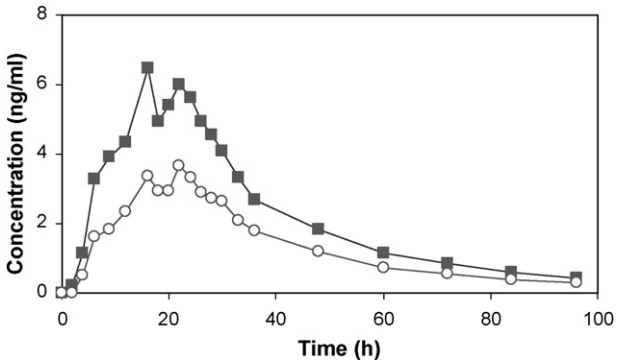

Fig. 6. Plasma concentration-time curves for (+)-9-hydroxyrisperidone (■) and (−)-9-hydroxyrisperidone (○) after a single oral dose of 6 mg ER OROS® paliperidone to a healthy volunteer.

4. Conclusion

The LC–MS/MS methods described in this paper allow the quantification of risperidone and the enantiomers of 9-hydroxyrisperidone (paliperidone) in human plasma down to 0.200 ng/ml as well as the quantification of the enantiomers of 9-hydroxyrisperidone (paliperidone) in human urine down to 1.00 ng/ml. Because of the good sensitivity and selectivity of the methods, the favourable accuracy and precision, the excellent stability of the analytes and the short analysis times, the methods are very suitable for the support of clinical trials with risperidone and paliperidone. Compared to previously described methods, they are more rigorously validated and have demonstrated their value in the analysis of thousands of samples. The plasma method offers up to 50-fold better sensitivity, while the urine method is the first one that has ever been described.

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