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Determination of risperidone and enantiomers of 9-hydroxyrisperidone in plasma by LC-MS/MS

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Abstract

A robust and validated liquid–liquid extraction LC-MS/MS method was developed for population pharmacokinetic analysis and therapeutic drug monitoring of risperidone and the enantiomers of its major active metabolite (+)-and (−)9-hydroxyrisperidone in pediatric patients. The method was rapid, sensitive and used a low sample amount (200 μL), which is very desirable for the pediatric population. The assay was validated from 0.2 to 50 ng/mL in plasma for all analytes. LLOQ for all analytes was 0.2 ng/mL. The extracts were analyzed by normal phase LC-MS/MS. The sample run time was 8 min. Intra- and interday precision for all analytes was ≤6%; method accuracy was between 89 and 99%. Additional experiments were performed to analyze matrix effects and identify a proper internal standard for each analyte. The validated method was used to study risperidone and its enantiomer metabolites in plasma as part of a population pharmacokinetic study in pediatric patients with pervasive developmental disorder (PDD).

Keywords: Risperidone; 9-Hydroxyrisperidone enantiomers; LC-MS/MS method; Matrix effects

1. Introduction

Risperidone (RIS) is a benzisoxazole derivate belonging to the class of 'atypical' neuroleptics, reported to have fewer side effects than traditional agents. RIS selectively antagonizes dopamine (D2) and serotonin (5HT2) receptor systems in the brain and has a lower propensity to induce extrapyramidal adverse events at therapeutic doses [1,2]. It is effective in the treatment of schizophrenia and other psychiatric illnesses in adults and children including pervasive developmental disorders (PDD), autism and attention-deficit disorder (ADD) [3,4]. Despite the widespread use of RIS in children with PDD pharmacokinetic data in this patient population are lacking [5]. The only well documented pharmacokinetic data available are from pre-marketing studies in healthy subjects and adult patients. In adults, risperidone is rapidly and completely absorbed after oral administration. The drug is metabolized mainly by the liver; less than 1% is excreted unchanged in the feces. The major pathways include, in order of preference, 9-hydroxylation, *N*-dealkylation, and 7-hydroxylation [6]. The principal metabolite 9-hydroxyrisperidone has been shown nearly equipotent to risperidone in animal studies. Other metabolites do not exhibit significant pharmacological activities [7]. Hydroxylation of risperidone is catalyzed by CYP2D6 and is subject to the same polymorphism as debrisoquine and dextromethorphan. In poor metabolizers (PM) the half-live of RIS was approximately 19 h as compared to 3 h in extensive metabolizers (EM). However, as the pharmacological activity of 9-hydroxyrisperidone is almost similar to that of the parent compound the half-life of the "active moiety" (RIS + 9-hydroxyrisperidone) was found to be approximately 20 h both in PM and EM. Risperidone exhibits linear elimination kinetics with steady-state levels within 1 day for RIS and within 5 days for the active fraction (RIS + 9-hydroxyrisperidone). However, large inter-individual variability in RIS and 9-hydroxyrisperidone concentrations have been described for adult patients during routine therapeutic drug monitoring [8].

Early methods for the quantification of RIS and 9-hydroxyrisperidone have mostly used HPLC with ultraviolet (UV) or electrochemical detection [8,9]. Although these

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methods may be suited for therapeutic drug monitoring of target concentrations in the range of 4–30 ng/mL for risperidone and 15–60 ng/mL for the sum of parent drug and 9-hydroxymetabolites ('active moiety'), their lower levels of quantification (0.5 and 5 ng/mL, respectively) are not particularly suited for pharmacokinetic studies. In recent years several mass spectrometric methods have been developed that all have much improved sensitivity and specificity [10–15].

Recently it was found that the metabolism of RIS results in the formation of two enantiomers, (+)9-hydroxyrisperidone and (−)9-hydroxyrisperidone, and that the enantioselective hydroxylation is catalyzed by CYP2D6 and CYP3A4, with the CYP3A4 pathway metabolizing at a slower formation rate [16]. As part of their paper the authors briefly described the LC-MS/MS assay used for separation of the 9-hydroxyrisperidone's optical isomers, but details were lacking and no validation results were presented. As both enantiomers are considered pharmacologically active, pharmacogenetic differences in their metabolism may contribute in different ways to the overall efficacy and side effect profiles in our pediatric patients.

The purpose of this study was to develop a validated, low sample volume, high sensitivity, LC-MS/MS assay for the determination of risperidone and 9-hydroxyrisperidone optical isomer concentrations in pediatric samples. The method was used to survey therapeutic plasma concentrations of RIS and 9-hydroxyrisperidone enantiomers and explore safety/adverse event data in relation to enantiomer concentrations as part of a population pharmacokinetic study in pediatric PDD patients while on risperidone maintenance treatment and will be published separately.

2. Experimental

2.1. Chemicals

Hexane, methanol, 2-propanol, ammonium acetate, ethanol were purchased from Fisher Scientific (Pittsburgh, PA). Risperidone, R068808, (−)9-hydroxyrisperidone and (+)9-hydroxyrisperidone were kindly donated by Janssen Research Foundation (Beerse, Belgium). Internal standard, racemic mixture of deuterated 9-hydroxyrisperidone-d_4 was obtained from Toronto Research Chemicals (North York, Ontario, Canada). *tert*-Butyl methyl ether was purchased from Sigma–Aldrich (Milwaukee, WI). Drug free plasma was obtained from Hoxworth Blood Center (Cincinnati, OH).

2.2. Equipment

The LC-ESI-MS/MS system consisted of a Waters Acquity Ultra Performance LC and Micromass Quattro Micro API (Waters, Milford, MA). Separation was achieved on Chiralcel OJ 4.6 mm × 50 mm column (Chiral Technologies Inc., West Chester, PA). The sample tray was held at 5 °C. Mobile phase A was 10 mM ammonium acetate in a 50:50 (v:v) ethanol/propanol mixture. Mobile phase B was pure hexane. The gradient started at 20% A which was increased to 90% A over 4.5 min, then held constant for an additional 2 min. At 6.6 min the column was returned to 20% A and re-equilibrated for 1.5 min. The on-column flow rate was 1 mL/min, with split flow before the detector with approximately half of the flow (0.5 mL/min) going to waste. Sample injection volume was 20 μL. Collision gas was argon (Praxair, Cincinnati, OH) with pressure of 8 μbar. Drying gas and nebulizing gas was nitrogen (Praxair, Cincinnati, OH). The following detection parameters were used: 800 L/h desolvation gas flow, 3.5 kV capillary voltage, 35 V cone voltage, 450 °C source temperature and 30 V collision energy. Detection of ions was done in positive ionization mode with the following transitions in multiple reaction monitoring mode (MRM): m/z 411.1 → 191.1 for risperidone, m/z 421.1 → 201.1 for R068808, 427.1 → 207.1 for (+/−)9-hydroxyrisperidones and m/z 431.1 → 221.1 for deuterated 9-hydroxyrisperidones (Fig. 1). An injection was performed every 8 min. The MS data were collected between 1.5 and 5 min during the run in order to reduce the file size and data collected.

2.3. Standard solutions

Standard solutions of 1 mg/mL of risperidone, R068808, (−)9-hydroxyrisperidone, (+)9-hydroxyrisperidone and deuterated 9-hydroxyrisperidones each were prepared in methanol Working solutions for spiking were prepared by serial dilutions with methanol. The final concentration of racemic deuterated 9-hydroxyrisperidone-d_4 and R068808 in the solutions employed for internal standard spiking was 100 ng/mL. All solutions were stored at −20 °C before analysis. Calibration standards and quality control samples were prepared by spiking blank (drug free) human plasma and 200 μL aliquots were stored at -20 °C until analysis. Risperidone and (+/−)-9-hydroxyrisperidone concentration in the calibration samples were 0.2, 1, 2, 5, 10, 20, and 50 ng/mL and 0.6, 8, and 40 ng/mL in the quality control samples.

2.4. Sample preparation

A 10 μL aliquot of the internal standard spiking solution was added to 200 μL of the plasma sample. Then, 1 mL of *tert*-butyl methyl ether was added into the polypropylene centrifuge tube and was shaken for 10 min on a shaker. A similar sample extraction using 0.5 mL of plasma has been described by Zhang et al. [15], but in our method we used a lower sample and solvent volume. Subsequently, the mixture was centrifuged at 10,000 rpm. Supernatant was transferred to glass tubes and dried under nitrogen at 40 °C. The dry residue was reconstituted in 200 μL of 5 mM ammonium acetate solution in heptane/ethanol/propanol (50:25:25, by volume).

2.5. Validation experiments

2.5.1. Matrix effects

Following a suggestion in a recent article by Matuszewski [17], relative matrix effects were evaluated using calibration standards in five different lots of blank plasma. The

first experiment was done using only deuterated (+/−)9-hydroxyrisperidones as an internal standard to evaluate applicability of (+)9-hydroxyrisperidone-d_4 as an internal standard for risperidone. The second set used three internal standards: R068808, (+)9-hydroxyrisperidone-d_4 and (−)9-hydroxyrisperidone-d_4.

2.5.2. Precision and accuracy

The intra-day precision and accuracy for the method was determined by analyzing five sets of quality control samples at three different levels (0.6, 8 and 40 ng/mL) on the same day. For inter-day precision and accuracy, three sets of quality control samples at three different levels were analyzed on four separate days following the internal guidelines in our laboratory. According to FDA guidelines, precision for quality controls must be within 15%, and accuracy between 85 and 115% [18].

2.5.3. Selectivity

Selectivity of the method was determined by analyzing plasma from six different sources to test for interferences at the retention times of analytes and internal standards.

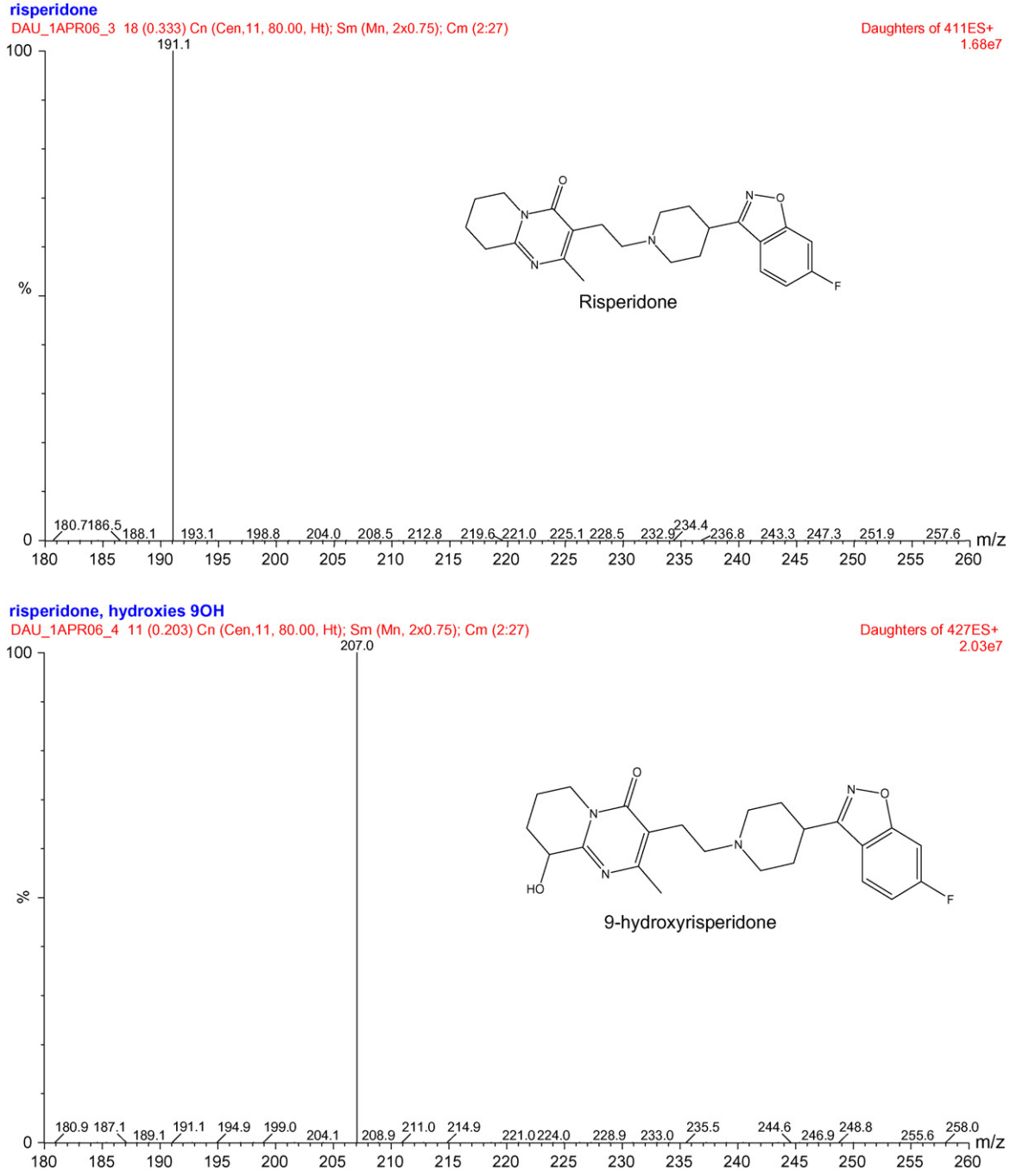

Fig. 1. Fragment ion mass spectra of analytes with parent ion structure.

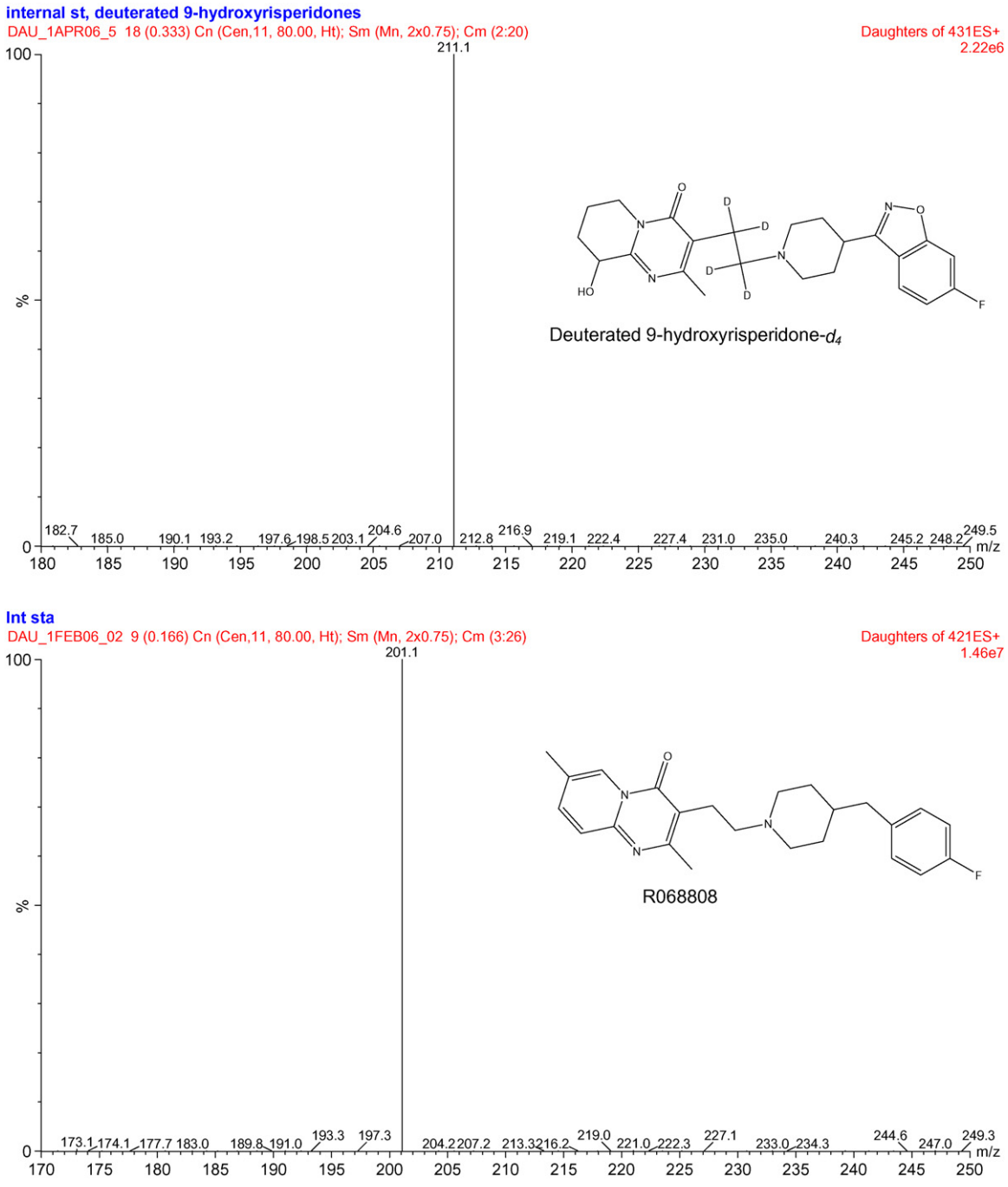

Fig. 1. (*Continued*).

2.5.4. Recovery and ion suppression

The experiments were done in replicates of five at each concentration level. Ion suppression experiments were performed at two concentrations. The 10 μL of IS solution and 20 μL of either 6 or 400 ng/mL risperidone and 9-hydroxyrisperidones were dried down and then reconstituted with heptane:ethanol:propanol mixture as described in sample preparation section (to achieve the amount of analyte present in 200 μL of plasma samples at conc. 0.6 or 40 ng/mL). These samples represent matrix free results.

To 10 blank plasma samples, 1 mL of TBME was added and then extracted as described in Section 2.4. After transferring the supernatant into glass tubes for drying, 10 μL of IS solution and 20 μL of either 6 or 400 ng/mL risperidone and 9-hydroxyrisperidones were added. The solutions were dried down and reconstituted as described in sample preparation section.

The average areas for the risperidone, (−)9-hydroxyrisperidone and (+)9-hydroxyrisperidone then were compared for both groups of samples to evaluate ion suppression.

For recovery, the analytes' responses from extracted samples at known concentrations at the lower and higher end of the working range (0.6 and 40 ng/mL) were compared with responses from the spiked matrix. Spiked matrix was obtained by extracting blank plasma samples, then adding the correspond-

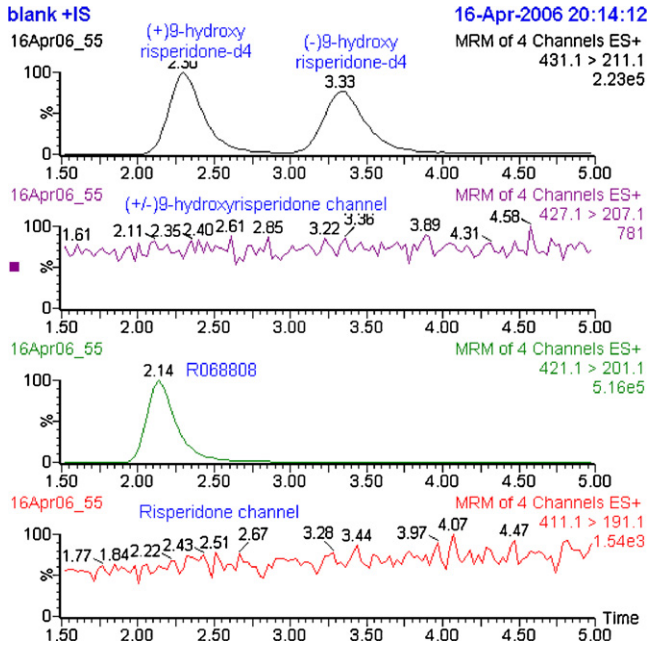

Fig. 2. Chromatogram of a blank plasma sample with all three internal standards (100 ng/mL). Mobile phase: hexane and 10 mM ammonium acetate in ethanol:propanol (50:50) gradient. Flow rate 1 mL/min.

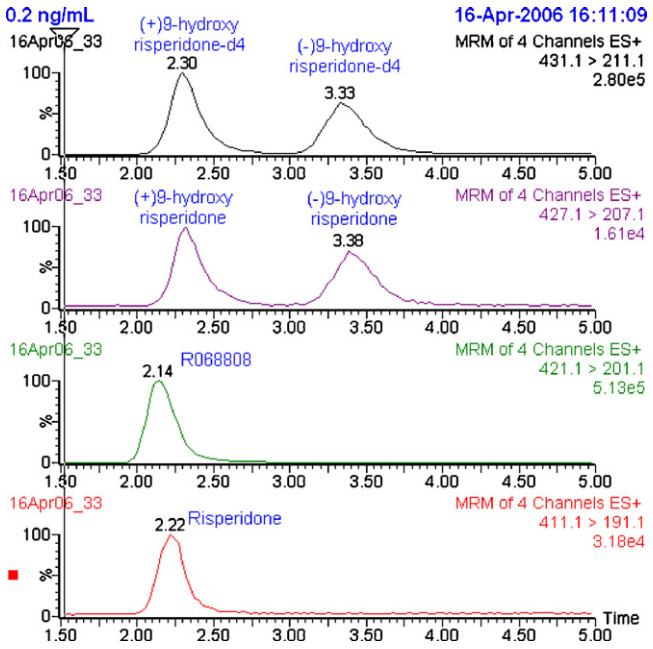

Fig. 3. Chromatogram of the lowest calibrator, 0.2 ng/mL with all three internal standards (100 ng/mL). Mobile phase: hexane and 10 mM ammonium acetate in ethanol:propanol (50:50) gradient. Flow rate 1 mL/min.

ing concentrations of analytes to *tert*-butyl methyl ether before drying down step.

2.5.5. *Autosampler stability*

There was no racemization observed for (−)9-hydroxyrisperidone and (+)9-hydroxyrisperidone in plasma samples kept at −20 °C for 6 months. Racemization of the enantiomers has been observed in strong alkaline or acidic milieu. During bioanalysis at slightly acidic (pH 6) or slightly alkaline conditions racemization is neglectable [Personal communication Janssen Pharmaceutica N.V. Beerse, Belgium]. Since the short term and long term stability of risperidone and total 9-hydroxyrisperidone in plasma, as well as their stock solution stability in methanol, has been already reported in the literature [14], only sample stability in the autosampler was determined during the validation process. Five sets of quality control samples (0.6, 8 and 40 ng/mL) were analyzed, then kept in the autosampler tray for 24 h at 5 °C and re-analyzed. The differences in results were compared using the Student *t*-test.

3. Results and discussion

According to the FDA guidelines for bioanalytical method validation it is necessary for matrix effects to be assessed [18] because they affect detection limits, precision and accuracy for a method [19]. However, it is not described how to ensure that a method is free from matrix interferences. Matuszewski [17] in his work nicely demonstrates that determination of calibration slopes in five different batches of a biofluid helps to quantify relative matrix effects. He also observed that stable isotope-labeled analogues, used as internal standards, eliminate relative matrix effects.

R068808 has been used as the internal standard in several assays for risperidone [13,16,20]. Therefore, the initial method development involved the use of R068808 as the only internal standard for all three analytes. However, early on it was observed that R068808 does not compensate for (−)9-hydroxyrisperidone's matrix effects. It was clear that an internal standard is needed which would co-elute not only with risperidone and (+)9-hydroxyrisperidone,

Table 1
Slope differences in 5 different lots of plasma for all analytes

Compound	A		B	
	Slopes CV (%)	Slope difference (%)[a]	Slopes CV (%)	Slope difference (%)[a]
Risperidone	28.3	98.6	3.8	11.2
(+)9-Hydroxyrisperidone	2.1	5.1	2.1	5.1
(−)9-Hydroxyrisperidone	4.0	9.0	1.1	3.2

A: Internal standard for risperidone and (+)9-hydroxyrisperidone is deuterated (+)9-hydroxyrisperidone-d_4, for (−)9-hydroxyrisperidone is deuterated (−)9-hydroxyrisperidone-d_4. B: Internal standard for risperidone is R068808, for (+)9-hydroxyrisperidone is deuterated (+)9-hydroxyrisperidone-d_4, for (−)9-hydroxyrisperidone is deuterated (−)9-hydroxyrisperidone-d_4.

[a] Maximum difference between the highest and the lowest slope values divided by the lowest slope value and multiplied by 100.

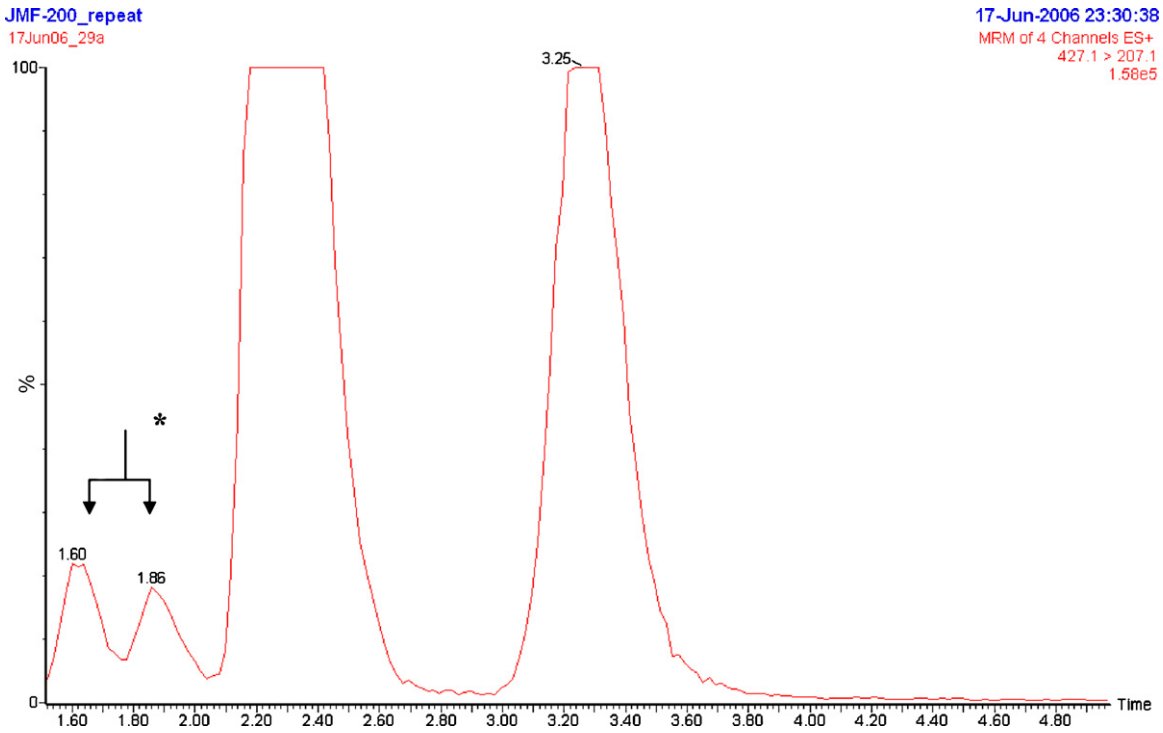

Fig. 4. 9-Hydroxyrisperidone MRM channel of a chromatogram of a patient's sample. Mobile phase: hexane and 10 mM ammonium acetate in ethanol:propanol (50:50) gradient. Flow rate 1 mL/min. * (+)-and (−)7-hydroxyrisperidone peaks.

Table 2
Intra- and inter-day precision and accuracy for quality controls and LLOQ

Analyte	Conc. of analyte (ng/mL)	Intra-day precision ($n=5$) (%)	Intra-day accuracy ($n=5$) (%)	Inter-day precision 4 days, ($n=12$) (%)	Inter-day accuracy 4 days, ($n=12$) (%)
Risperidone	0.2 (LLOQ)	5.0	−1.8	NA	NA
Risperidone	0.6	1.1	−8.1	3.3	−8.2
Risperidone	8	2.1	−1.7	4.9	−6.1
Risperidone	40	5.9	−1.1	2.7	−4.4
(+)9-OH Risperidone	0.2 (LLOQ)	5.5	1.5	NA	NA
(+)9-OH Risperidone	0.6	1.3	−7.7	3.2	−8.6
(+)9-OH Risperidone	8	2.6	−2.2	4.6	−5.0
(+)9-OH Risperidone	40	2.0	−4.3	3.1	−3.8
(−)9-OH risperidone	0.2 (LLOQ)	5.5	2.7	NA	NA
(−)9-OH Risperidone	0.6	2.2	−11.0	3.3	−9.8
(−)9-OH Risperidone	8	2.0	−4.3	3.7	−5.0
(−)9-OH Risperidone	40	6.0	−3.6	2.5	−4.6

NA: Not applicable because interday precision and accuracy was not determined for LLOQ.

but also with (−)9-hydroxyrisperidone. The next step in method development involved the use of a racemic mixture of deuterated 9-hydroxyrisperidone-d_4. Because deuterated (+)9-hydroxyrisperidone co-elutes with risperidone and (+)9-hydroxyrisperidone and its structure is analogue to risperidone, it was assumed that deuterated (+)9-hydroxyrisperidone can be used as internal standard for both of these analytes. Nevertheless, after analyzing 5 sets of calibration curves in different batches of blank plasma, coefficients of variation for the slopes of risperidone were unacceptably high using deuterated (+)9-hydroxyrisperidone as its internal standard (Table 1, case A). Therefore, it was decided to add back R068808 as the internal standard for risperidone. The use of R068808 greatly improved the coefficient of variation for risperidone slopes. Even the slope difference was within acceptable limits of our laboratory's standards, <15% (Table 1, case B). The five plasma batches used in both matrix effect experiments were identical. For all method validation experiments, all three internal standards were used: racemic deuterated (+/−)9-hydroxyrisperidone-d_4

Table 3
Recovery results for risperidone and 9-hydroxyrisperidone at high and low end of the curve

Analyte	For 0.6 ng/mL (%)	For 40 ng/mL (%)
Risperidone	88 ± 4	84 ± 8
(+)9-Hydroxyrisperidone	83 ± 4	78 ± 11
(−)9-Hydroxyrisperidone	81 ± 5	76 ± 9

Table 4
Stability data for the analytes using Student *t*-test ($p = 0.05$)

Analyte	Target conc. (ng/mL)	Conc. ng/mL for samples (after 24 h in autosampler)	Conc. ng/mL for control samples	T_{stat}	n	$T_{critical}$
Risperidone	0.6	0.56 ± 0.01	0.55 ± 0.01	0.674	5	2.776
	8	8.1 ± 0.2	7.9 ± 0.2	1.873	5	2.776
	40	40.6 ± 2.2	39.6 ± 2.3	0.522	5	2.776
(+)9-Hydroxyrisperidone	0.6	0.56 ± 0.01	0.55 ± 0.01	0.387	5	2.776
	8	7.9 ± 0.2	7.8 ± 0.2	0.976	5	2.776
	40	40.3 ± 2.6	39.6 ± 2.4	0.481	5	2.776
(-)9-Hydroxyrisperidone	0.6	0.54 ± 0.01	0.53 ± 0.01	1.074	5	2.776
	8	7.7 ± 0.2	7.7 ± 0.2	0.003	5	2.776
	40	38.8 ± 2.3	38.5 ± 2.3	0.189	5	2.776

for (+/−)9-hydroxyrisperidone and R068808 for risperidone. Representative chromatograms are shown in Figs. 2 and 3. It can be seen that there are no interference peaks present in the risperidone or 9-hydroxyrisperidone channel of the blank sample spiked with internal standards.

It has been reported that stable isotope-labeled internal standards compensate the best for matrix variability [17]. The difference between risperidone and 9-hydroxyrisperidone is just one hydroxyl group and one would assume that deuterated (+)9-hydroxyrisperidone would be an excellent internal standard for risperidone. Nevertheless, it was not the case as shown by our results using five plasma batches.

Remmerie et al. reported that it is necessary to separate 7-hydroxyrisperidone from 9-hydroxyrisperidones [14]. According to the authors, 7-hydroxyrisperidone is a minor metabolite in risperidones metabolism and its concentration can be up to 6% of 9-hydroxyrisperidone's concentration leading to overestimation of 9-hydroxyrisperidone concentration if not separated. Because a pure standard of 7-hydroxyrisperdione is not available, we used a patient sample with high concentrations of (+)-and (−)9-hydroxyrisperidone (30.3 and 11.7 ng/mL, respectively). The corresponding chromatogram is shown in Fig. 4. In the channel for the transition 427.1 → 207.1 for (+/−)9-hydroxyrisperidones, two small peaks were observed eluting before (+)9-hydroxyrisperidone. These peaks were not observed in any calibrator or QC sample. Assuming that (+/−)7-hydroxyrisperidone gives similar response as (+/−)9-hydroxyrisperidones and that the first peak represents (+)7-hydroxyrisperidone and the second is (−)7-hydroxyrisperidone, the relative amounts were 2.7 and 5.9%, respectively. Because MRM transitions for 7-hydroxyrisperidone would be identical to those for 9-hydroxyrisperidone, we assumed that these are the peaks of interest. The 7-hydroxyrisperidone is baseline separated from the analytes. If the 7-hydroxyrisperidone standard were available, then this method could be also validated for quantification of 7-hydroxyrisperidone.

Precision and accuracy data are summarized in Table 2. All the variations were within acceptable limits by the FDA guidelines [18]. Precision and accuracy for LLOQ was below 10%, which means that it would be possible to establish a lower LLOQ if necessary. For the present studies a 0.2 ng/mL limit of quantification was considered acceptable. Avenoso et al. report that, for daily dosages between 4 and 8 mg in adults, a quantification limit of 2 ng/mL is adequate [11]. There are some reports of a LLOQ of 0.1 ng/mL for risperidone and total 9-hydroxyrisperidone, but those assays involved use of larger sample volumes [14,21].

The absolute recovery results for all three analytes are shown in Table 3. Ion suppression on average was 12% for risperidone, 16% for (+)9-hydroxyrisperidone and 8% for (-)9-hydroxyrisperidone, which means that there is some matrix co-eluting with the peaks of interest. However, the internal standards compensate adequately for the suppression as revealed by the matrix effect experiments and shown by coefficients of variation for the slopes of calibration curves in five different batches of plasma. The samples were stable in the autosampler for 24 h at temperature 5 °C ($p = 0.05$) (Table 4).

4. Conclusions

The assay described was validated to be sensitive and selective for risperidone and enantiomers of 9-hydroxyrisperidone. This will allow for the first time in pediatric patients separate pharmacokinetic analysis of parent compound and its differentially metabolized 9-hydroxy-enantiomers in relation to clinical outcomes data. In addition, the separation of a minor risperidone metabolite 7-hydroxyrisperidone was achieved in a short sample run. The required sample volume was only 200 μL, easily applicable to pediatric populations for therapeutic drug monitoring. Matrix effects were evaluated to determine the best internal standards for the analytes. A separate internal standard for each analyte compensated best for matrix variability. It was determined that the deuterated (+)9-hydroxyrisperidone does not compensate for matrix effects for risperidone, which was a surprising fact since structurally hydroxyrisperidone is closer to risperidone than R068808.

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