Investigating the Chiral Metabolism of an Achiral Drug Using Agilent SFC-MS/MS Technology

Application Note

Pharma Discovery

Authors

Siji Joseph
Agilent Technologies, Inc.
Bangalore, India

Rajendra Prasad T, Prashant Kole, and Prabhakar KR
Biocon BMS R&D Center
Syngene International Ltd.
Bangalore, India

Abstract

A robust SFC-MS/MS method was developed for the quantification of achiral risperidone and its enantiomeric hydroxy metabolite. The method was fast, and offered efficient separation of risperidone and the enantiomers of 7- and 9-hydroxy risperidone for sensitive quantification. The total separation time was 6 minutes, and trazodone was used as an internal standard. Experiments were performed to assess specificity and carryover for each analyte. The method was tested from 0.9 to 15,000 nM using rat liver microsomes as the matrix for risperidone and enantiomers of its major metabolite, 9-hydroxy risperidone. For the minor metabolite, 7-hydroxy risperidone, the method was found to be linear from 0.5 to 7,500 nM. Method accuracy was calculated using quality control samples, and was found to be within 100 ±10 %. The method was used to study risperidone *in vitro* metabolism, and quantitate the formation of hydroxy metabolite enantiomers.

Agilent Technologies

Introduction

Risperidone is predominantly metabolized by CYP2D6 to 9-hydroxy risperidone (Paliperidone), which is the major and active metabolite. The plasma concentrations of the parent compound plus the 9-hydroxymetabolite has been reported as the active moiety in several publications. Risperidone is achiral, and its hydroxylation results in the formation of two enantiomer pairs of 9- and 7-hydroxy metabolites[1,2]. The principal metabolite 9-hydroxyrisperidone has been shown to be nearly equipotent in its pharmacological activity to that of risperidone in animal studies[3,4]. CYP2D6 is a polymorphic enzyme, and it is important to understand its contribution to the overall metabolism, especially if the metabolite is active, as in the case of risperidone.

Supercritical fluid chromatography (SFC) is considered a green chromatographic technique for the separation of chiral and achiral compounds, and can be used with various detection modes.

SFC provides additional advantages, including rapid separations compared to conventional normal phase chromatography. Mass spectrometry detection further increases sensitivity. This Application Note describes the chiral separation and sensitive quantification of hydroxy metabolites of risperidone from biological matrices using SFC coupled with triple quadrupole mass spectrometry.

Experimental

Standards and chemicals

Methanol, ammonium acetate, β-nicotinamide adenine dinucleotide phosphate (NADPH), formic acid, risperidone standard, and trazodone internal standard were purchased from Sigma-Aldrich (Bangalore, India). A racemic mixture of 7-hydroxy risperidone, (–)-9-hydroxyrisperidone, and (+)-9-hydroxyrisperidone were purchased from Carbosynth India (Chennai, India). Male Sprague Drawely Rat liver microsomes (RLM) were procured from Corning Life Sciences (MA, USA). Recombinant cDNA individually expressed human CYP enzymes (CYP2D6, CYP3A4, and CYP3A5) prepared from a baculovirus-infected insect cell system (Supersomes) were procured from BD Biosciences Company (NY, USA).

Instrumentation

An Agilent 1260 Infinity Analytical SFC system (G4301A) coupled with an Agilent 6460 Triple Quadrupole LC/MS system was used for the studies. A flow splitter was installed, through which one part of the column flow was directed to the LC/MS, and another part to the backpressure regulator (BPR) of the SFC module to minimize peak broadening. Mobile phase A was super critical CO_2, and mobile phase B was methanol with 40 mM ammonium acetate and 0.1 % formic acid. Table 1 lists the SFC instrumentation and gradient parameters, and Table 2 lists the 6460 LC/MS parameters. MRM detection in positive ionization was performed to acquire the data. Protonated precursors of risperidone and hydroxy metabolites were selected for MRM based quantification. The MRM transitions used for the data acquisitions are tabulated in Table 3.

Standard solutions

Individual standard solutions of risperidone, R-(–)-9-hydroxyrisperidone, S-(+)-9-hydroxy risperidone, and (R/S)-(±)-7-hydroxyrisperidone at 2 mM were prepared in methanol. Calibration standard stock solution was then prepared by mixing 2 mM stock solutions of these individual standard solutions in methanol to obtain 150 µm each of the analytes (13.3x dilution). This calibration standard stock was then ½ log diluted in methanol to obtain working solutions ranging from 9.2 to 150,000 nM. Spiked working solutions were prepared by diluting 10x in rat liver microsomes to obtain calibration standards (0.92 to 15,000 nM). The linearity range for each of the 7-hydroxyrisperidone was corrected to 0.46 to 7,500 nM, assuming equimolar levels of each of the R and S enantiomers in racemic standard used as the standard in this study.

To prepare QC samples, calibration standard stock was diluted in methanol to obtain working QC solutions of 27.5, 5,000, and 70,000 nM. Working QC solutions were prepared by diluting 10x in RLM to obtain QC samples 2.7 nM (LQC), 500 nM (MQC), and 7,000 nM (HQC). Concentrations of each isomer of 7-hydroxy risperidone in QC samples were 1.35 (LQC), 250 (MQC), and 3,500 (HQC) nM. Internal standard (Trazodone) was spiked at 20 nM concentration in the precipitation reagent (methanol).

Experimentation

The methods were tested for parameters such as selectivity, carryover, precision, accuracy, linearity, and reproducibility for each analyte. The method selectivity towards the *in vitro* matrix was determined by comparing the response of the least concentrated sample (LLOQ) with the RLM blank matrix. The method was considered selective if the signal at the retention time of the respective analytes and internal standard in blank samples was less than 20 % of the signal of the analytes in the LLOQ sample. Carryover was calculated from the analyte peak area in a blank injection, which was acquired immediately following an

injection of the highest concentration calibration standard (ULOQ). The percentage of the analyte peak area observed in blank trace was compared to the corresponding peak area from ULOQ as the analyte carryover.

The intra-day precision and accuracy of the methods were assessed by analyzing the area and retention time precision of six repeat preparations of quality control samples at three different levels (LQC, MQC, and HQC). A linear regression model was used to plot the linearity relation between analyte concentration and instrument response ratio of the analyte to that of the internal standard. The overall accuracy was calculated from six repeat preparations of QC samples using the linearity curve equations. The method robustness was evaluated by measuring analyte area and retention time consistency over 300 repeated injections using a spike mixture at 25 nM concentration of all the analytes.

In-vitro samples and cleanup

Risperidone (10 µm) was incubated with rat liver microsomes (1 mg/mL) in the presence of 1 mM NADPH at 37 °C in 100 mM potassium phosphate buffer, and aliquots (100 µL) were taken at 0, 5, 10, 20, 30, and 60 minutes, and quenched with four volumes of methanol containing internal standard. The reaction mixture was then centrifuged at 10,000 rpm for 6 minutes, and supernatant was used for the SFC/MS/MS analysis.

Risperidone (1 µm) was incubated with recombinant CYP2D6, CYP3A4, and CYP3A5 supersomes (25 pmol/mL) in the presence of 1 mM NADPH at 37 °C in 100 mM potassium phosphate buffer, and aliquots (100 µL) were taken at 0 and 60 minutes, and quenched with four volumes of methanol containing internal standard. The reaction mixture was then centrifuged at 10,000 rpm for 6 minutes, and supernatant was used for the SFC/MS/MS analysis. Concentrations of the metabolites in these enzyme preparations were calculated from the corresponding linear equations. Figure 1 shows the sample preparation protocol for calibrations standards, QCs, and samples.

Table 1. Agilent 1260 Infinity SFC method parameters.

Parameter	Setting
SFC module	BPR: 120 bar (temperature: 60 °C)
Mobile phases	A) CO_2 B) 0.1 % formic acid in methanol with 40 mM ammonium acetate
Column	ChiralPak (Chiral Technologies, West Chester, PA, USA) AD-3 (3.0 × 100) mm, 3 µm column at 45 °C
Injection volume	3 µL, partial loop
Needle wash	Methanol
Flow rate	2.5 mL/min
Gradient	Time (min) %B 0 25 1.5 35 6 35
Post run	2 minutes

Table 2. Agilent 6460C LC/MS acquisition parameters.

Parameter	Setting
Acquisition mode	MRM
Ion polarity	Positive
Capillary voltage	30 V
Fragmentor voltage	140 V
Dwell time	50 ms
Cell accelerator voltage	7 V
Q1/Q2 resolution	Wide/Unit
Gas temperature	325 °C
Gas flow	8 L/min
Nebulizer	25 psi
Sheath gas	375 °C
Sheath gas flow	12 L/min
V-Cap	3,500 V

Table 3. MRM transitions used for LC/MS data acquisition.

Compound	Precursor	Quantifier product ion	Qualifier product ion
Risperidone (RIS)	411.1	191.1	110
R-(–)-9-hydroxyrisperidone (R-9-OH)	427.1	207.1	110.2
S-(+)-9-hydroxyrisperidone (S-9-OH)	427.1	207.1	110.2
7-hydroxyrisperidone (7-OH)	427.1	207.1	189.1
Trazodone (ISTD)	372.1	176.2	

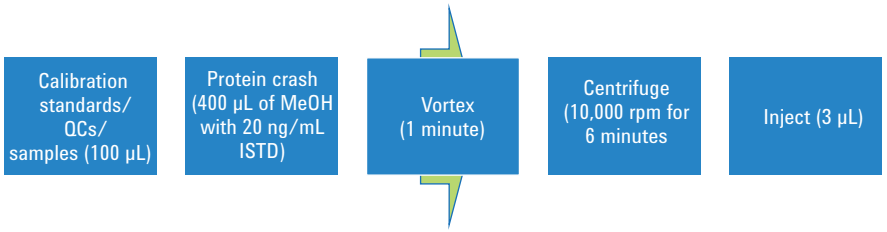

Figure 1. Sample cleanup protocol.

Results and Discussion

Metabolite elution profile

Chiral SFC analysis using CHIRALPAK AD-3 column was able to resolve enantiomers of both 7- and 9-hydroxy risperidone as well as the parent drug and internal standard. The total chromatographic separation time for the stereospecific profiling of risperidone and hydroxylated metabolites was less than 6 minutes by the SFC/MS/MS method. Excellent baseline separation of all analytes was observed with good method reproducibility (Figure 2). The sample preparation procedure used for the quantification of risperidone and enantiomeric metabolites was quick, simple, and efficient.

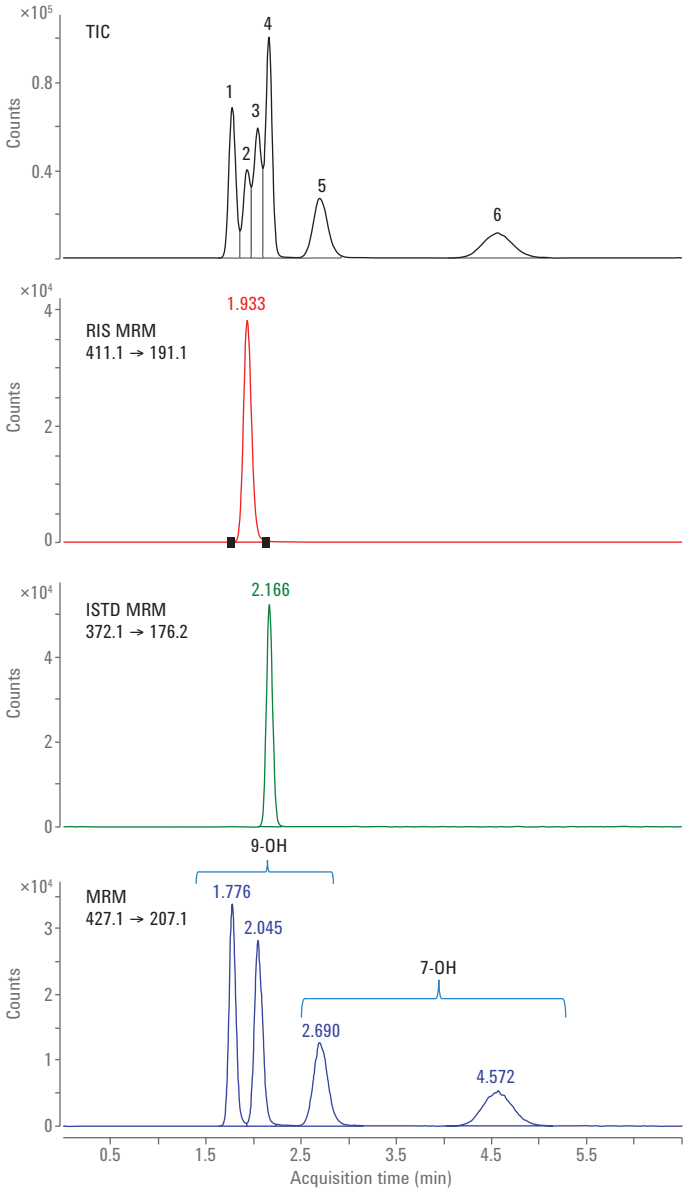

Figure 2. Elution profile of ISTD, risperidone, and hydroxy metabolites. The top trace is the total ion chromatogram (TIC), and below it are individual MRM traces of each analyte.

Method selectivity

To assess the selectivity of the method, the target MRM transition at the least concentrated calibration (LLOQ) level was compared with that of blank trace, and no significant interfering peaks were observed. Analyte peaks in matrix blank were found to be insignificant (< 20 %) compared to peak areas at the LLOQ calibration level (Figure 3). The observed height and signal-to-noise (S/N) comparison between matrix blank and the LLOQ level are shown in Figure 4A and 4B. These results confirmed that analyte peaks even at LLOQ levels are clear and significant, demonstrating the sensitivity of the SFC-MS method.

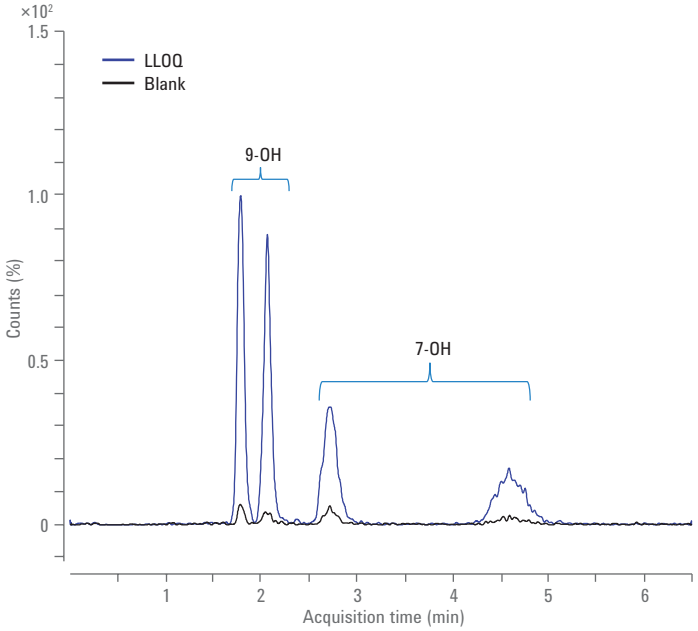

Figure 3. Overlay of MRM traces of hydroxy metabolites from LLOQ level with RLM matrix blank trace to evaluate method selectivity. Analyte peaks in matrix blank were found to be < 20 % compared to peak areas in LLOQ calibration level.

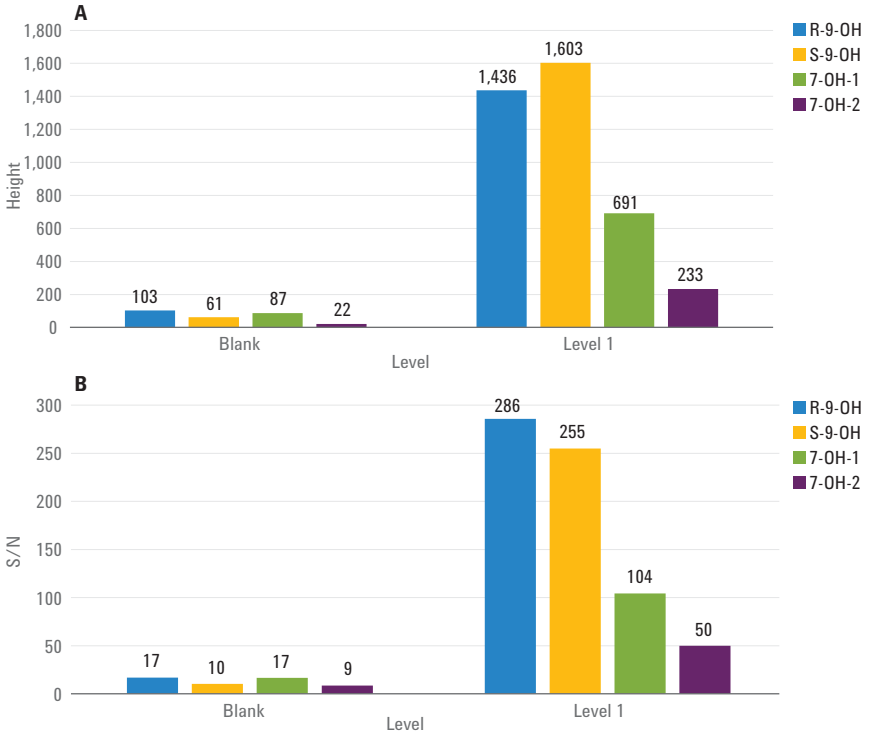

Figure 4. A) Comparison of height observed in MRM traces of hydroxy metabolites for the LLOQ level with that from matrix blank trace. B) Comparison of S/N observed in MRM traces of hydroxy metabolites for the LLOQ level with that from matrix blank trace. The analyte peak height and S/N values observed in matrix blank are well within <20 % limit compared to values from LLOQ trace.

Method carryover

Method carryover was established as the percentage area of each hydroxy metabolite signal observed in the matrix blank, which was injected immediately after the injection of the ULOQ, and found to be <0.008 % (Figure 5).

Linearity

The linearity curve for each metabolite was plotted from the LLOQ level to the upper linearity level. To determine the best linearity response function, various regression models were evaluated, and the best calibration model was with Type: Linear, Origin: Ignore, Weight: 1/x. This method was found to be linear across more than four orders of magnitude ($R^2 > 0.99$). Figure 6 gives the linearity curve of R-9-hydroxy risperidone as an example, and Table 4 summarizes the linearity results out of three different preparations.

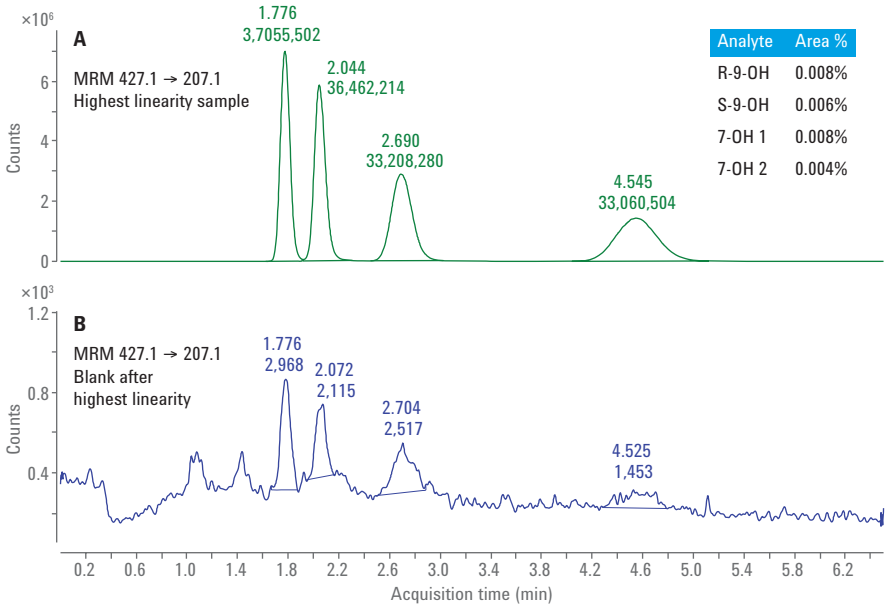

Figure 5. Carryover evaluation for hydroxy metabolites. The MRM trace for hydroxy metabolites at ULOQ level (5A) is compared with that of next immediate blank sample (5B).

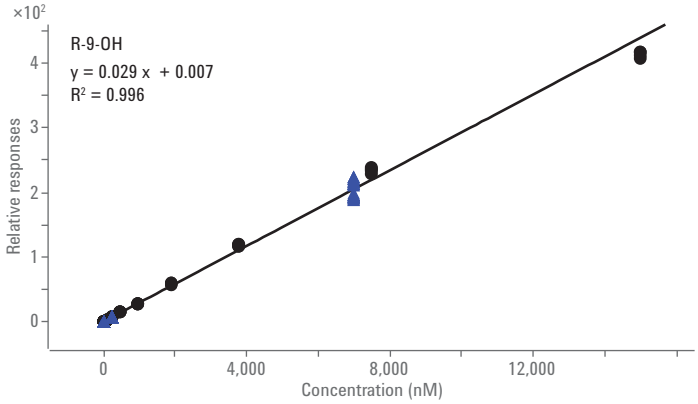

Figure 6. Linearity curve of R-9-hydroxyrisperidone from 0.9 to 15,000 nM.

Table 4. Linearity results summary for three different preparations.

No.	Sample	Concentration range	R^2 Value		
			Prep 1	Prep 2	Prep 3
1	RIS	0.9 to 15,000 nM	>0.996	>0.999	>0.998
2	R-9-OH	0.9 to 15,000 nM	>0.996	>0.999	>0.999
3	S-9-OH	0.9 to 15,000 nM	>0.996	>0.997	>0.999
4	7-OH-1	0.5 to 7,500 nM	>0.997	>0.998	>0.999
5	7-OH-2	0.5 to 7,500 nM	>0.996	>0.995	>0.999

Precision and accuracy

Precision was determined by measuring the relative standard deviation (RSD) of retention time (RT) and peak area of replicate injections for six different preparations of QC samples (LQC, MQC, and HQC). Excellent RT and peak area ratio precision values for all samples were observed. The RT RSD was < 0.7 %, and area ratio RSD was < 3.0 % for all three QC samples (Figures 7A and 7B).

Accuracy values were calculated for all QC samples and calibration levels. Observed accuracy values for all six preparations of three levels of QC samples were between 90 and 110 %. Observed accuracy values at least concentrated calibration level for all analyses were within 100 ±20 %, whereas for all other linearity levels, the values were within 100 ±15 %.

Method robustness

To evaluate method robustness, a spike mix of risperidone and all four hydroxy metabolites at a concentration of 25 nM were injected over 300 replicate injections continuously, and area and RT consistency were calculated. The results are graphically summarized in Figures 8A and 8B. From these data, it is evident that this SFC/MS/MS method is robust and reproducible.

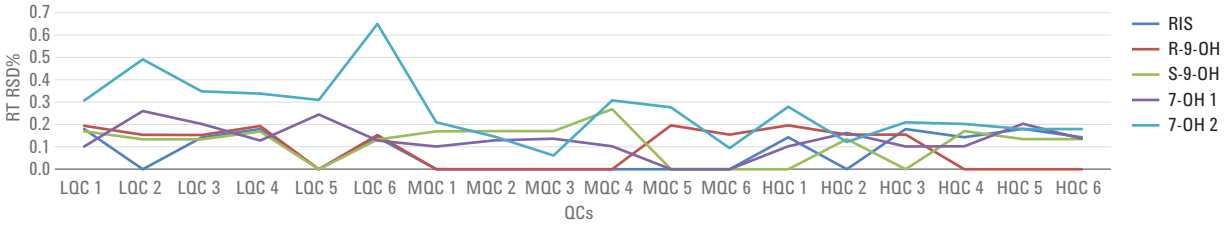

Figure 7A. RT RSD values for six different preparations of three levels of QC samples.

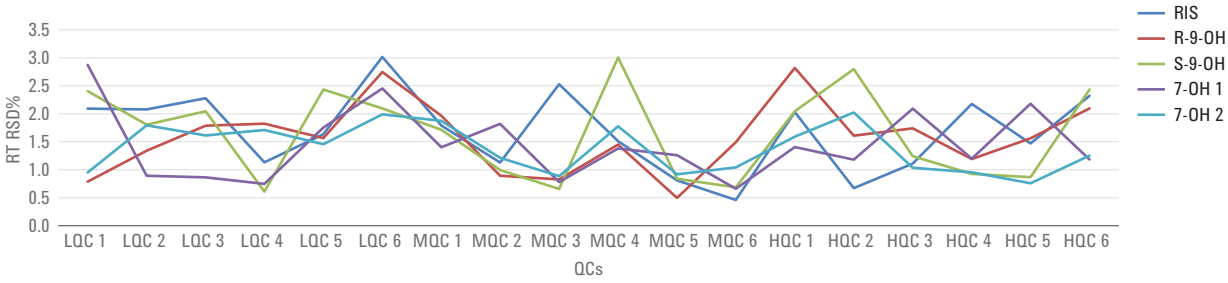

Figure 7B. Area RSD values for six different preparations of three QC samples.

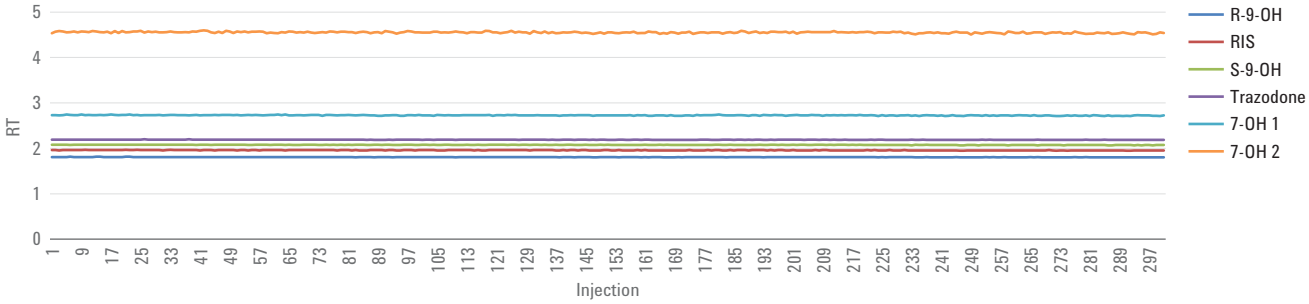

Figure 8A. Retention time reproducibility of risperidone, internal standard, and hydroxy metabolites over 301 injections.

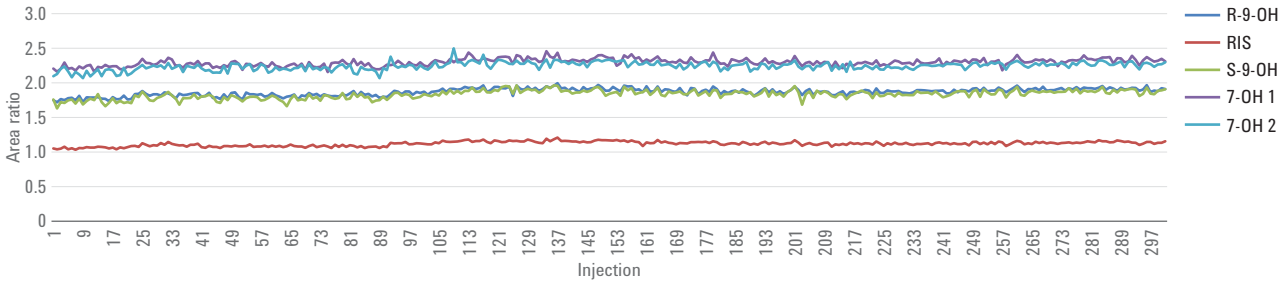

Figure 8B. Area ratio reproducibility of risperidone and hydroxy metabolites over 301 injections.

In-vitro sample results

A chiral SFC method was successfully used for the analysis of risperidone metabolites. RLMs were incubated with 10 µM risperidone, and the formation of metabolites over five time points (0, 5, 10, 20, 30, and 60 minutes) was monitored. It was observed that S-(+)-9-hydroxyrisperidone is the major enantiomer formed in rat liver microsomes (Figure 9).

To investigate the CYP enzymes responsible for the formation of 9-hydroxy metabolites, a brief reaction phenotyping experiment was performed with 1 µm risperidone incubated with CYP3A4, CYP3A5, and CYP2D6 for 60 minutes. CYP2D6 was found to be the major enzyme involved in the formation of 9-hydroxy metabolites (Figure 10). CYP3A4 and CYP3A5 did not show significant involvement in risperidone metabolism.

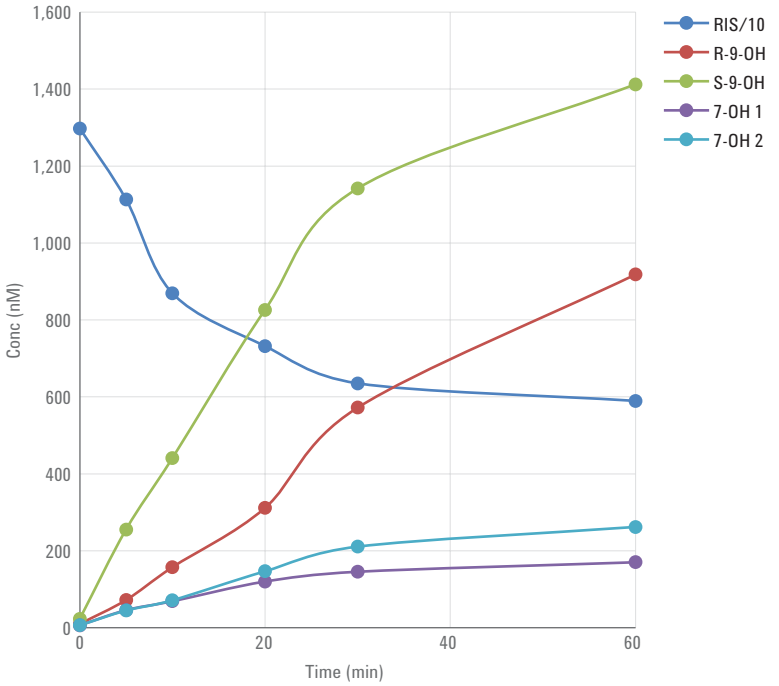

Figure 9. Representation of risperidone metabolism and formation of metabolites over 1 hour.

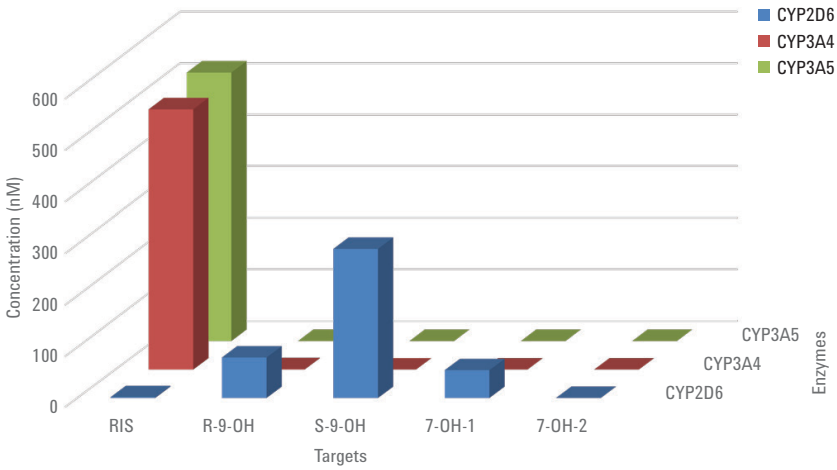

Figure 10. Risperidone metabolism after 60 minutes in the presence of three different enzymes. The CYP2D6 enzyme is involved in the formation of hydroxy metabolites, whereas CYP3A4 and CYP3A5 did not show significant involvement in risperidone metabolism.

Conclusions

A sensitive and selective SFC/MS/MS method was developed for the fast quantification of risperidone and its chiral hydroxy metabolites. The SFC-Triple Quadrupole method was tested for *in vitro* quantitation of risperidone and enantiomers of 9- and 7-hydroxyrisperidones down to a concentration of 0.9 and 0.5 nM, respectively. Sample preparation was performed by simple protein precipitation with methanol containing Trazodone as ISTD. Chromatographic separation was achieved in less than 6 minutes using an Agilent 1260 Infinity SFC system with a CHIRALPAK AD-3 column. The selectivity, accuracy, precision, and linearity range were evaluated to ensure method reproducibility. Consistent reproducibility over 300 repeated injections confirmed the method robustness.

This approach successfully demonstrated the separation of risperidone, an achiral parent, and its four chiral enantiomeric hydroxy metabolites in *in vitro* metabolism incubation samples. This method further helped to understand the CYP enzyme responsible for the formation of these chiral metabolites.

The results in this study meet the typical bioanalytical method development guidelines for selectivity, accuracy, linearity, and sensitivity, suggesting that SFC-MS/MS can be a good choice to develop bioanalytical methods, with the additional benefits of speed and green chromatography.

Acknowledgements

We acknowledge Timothy Olah, Sandhya Mandlekar, and Murali Subramanian from Biocon BMS R&D Center, Syngene International Ltd., Bangalore, India for their support of this collaboration. We also acknowledge Rakshit Tanna and Anoop from Syngene International for his assistance in the preparation of *in vitro* samples.

References

1. Mannens, G.; *et al.* Absorption, metabolism and excretion of risperidone in humans. *Drug Metabolism and Disposition*, *21(6)*, 1134-1141.

2. Meuldermans, W.; *et al.* The metabolism and excretion of risperidone after oral administration in rats and dogs. *Drug Metabolism and Disposition*, *22(1)* 129-138.

3. Čabovska, B.; Cox, S. L.; Vinks, A. A. Determination of risperidone and enantiomers of 9-hydroxyrisperidone in plasma by LC-MS/MS, *J. Chromatog. B Analyt. Technol. Biomed. Life Sci.* **2009**.

4. De Meuldera, M.; *et al.* Validated LC-MS/MS methods for the determination of risperidone and the enantiomers of 9-hydroxyrisperidone in human plasma and urine, *J. Chromatog. B* **2008**, *870*, 8-16.

www.agilent.com/chem

For Research Use Only. Not for Use in Diagnostic Procedures.

This information is subject to change without notice.

Published in the USA, January 1, 2017
5991-7708EN

Agilent Technologies