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Simultaneous determination of risperidone and 9-hydroxyrisperidone in plasma by liquid chromatography/electrospray tandem mass spectrometry

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A simple and highly sensitive liquid chromatographic/electrospray tandem mass spectrometric (LC/MS/MS) assay was developed for the simultaneous determination of risperidone (RSP) and its major circulating metabolite 9-hydroxyrisperidone (9-OH-RSP) in the plasma of humans and rats. A simple one-step solvent extraction with 15% methylene chloride in pentane was used to isolate the compounds from plasma. The compounds were eluted from a phenyl–hexyl column and detected with a Perkin-Elmer SCIEX API2000 triple-quadrupole mass spectrometer using positive ion atmospheric pressure electrospray ionization and multiple reaction monitoring. The assay was linear over the range 0.1–100 ng ml^{-1} when 0.5 ml of plasma was used in the extraction. The overall intra- (within-day) and inter- (between days) assay variations were <11%. The variations in the concentrations of two long-term quality control samples from pooled patient plasma samples analyzed over a period of 6 months were ∼10%. The analysis time for each sample was 4 min and more than 100 samples could be analyzed in one day by running the system overnight. The assay is simple, highly sensitive, selective, precise and fast. This method is being used for the therapeutic drug monitoring of schizophrenic patients treated with RSP and to study the pharmacokinetics and tissue distribution of RSP and 9-OH-RSP in rats.
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KEYWORDS: risperidone; 9-hydroxyrisperidone; liquid chromatography/electrospray tandem mass spectrometry; plasma; pharmacokinetics

INTRODUCTION

Risperidone (RSP), a benzisoxazole derivative, is one of the newer antipsychotic agents used in the treatment of schizophrenia and other psychotic disorders. It exhibits potent 5-HT_2 and moderate D_2 receptor blocking capacity.[1–5] RSP has been effective in the treatment of both positive and negative symptoms of schizophrenia with a low liability for causing EPS at therapeutically effective doses.[6–9] RSP is extensively metabolized to several metabolites, including 9-hydroxyrisperidone (9-OH-RSP). 9-OH-RSP is the major circulating metabolite in animals and in humans.[10,11] It appears to have similar receptor binding affinities to RSP.[11–13] Therefore, it is likely that the sum of RSP and 9-OH-RSP concentrations constitutes the total active moiety responsible for the pharmacological responses to RSP administration. In order to study the relationship among plasma concentrations of active moiety (RSP + 9-OH-RSP), dose of RSP and clinical outcome of the patients treated with RSP, the determination of plasma concentrations of both RSP and 9-OH-RSP may be useful.

Many high-performance liquid chromatographic (HPLC) methods for the determination of plasma RSP[14] and the simultaneous determination of plasma RSP and 9-OH-RSP[15–17] have already been reported. One of the methods[16] was reversed-phase HPLC with an ultraviolet diode-array detector and the other[17] was HPLC with electrochemical detection (ECD), both using a multistep liquid–liquid extraction procedure with a lower limit of determination of 2 ng ml^{-1}. A recently reported HPLC/ECD method[15] was more sensitive and could determine RSP and 9-OH-RSP at levels as low as 0.25 ng ml^{-1} of plasma when 1 ml of plasma was used for the analysis. We report here for the first time a simple, highly selective, rapid and more sensitive liquid chromatographic/tandem mass spectrometric (LC/MS/MS) method for the simultaneous determination of RSP and 9-OH-RSP in the plasma of humans and rats. This LC/MS/MS assay was developed in order to increase the sensitivity and selectivity and to decrease the sample run time as compared with

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the HPLC/ECD method.[15] This method has been used for the plasma concentration monitoring of RSP and 9-OH-RSP in schizophrenic patients receiving different doses of RSP and for studying the pharmacokinetics RSP and 9-OH-RSP in rats.

EXPERIMENTAL

Chemicals and materials

RSP (R 64766), 9-OH-RSP (R 76477) and the internal standard (INS, R 68808) were generously donated by Janssen Research Foundation (Beerse, Belgium). All solvents and chemicals were of HPLC grade procured from Fisher Scientific (Tustin, CA, USA) and used without further purification. Deionized, high-purity water was produced in the laboratory by reverse osmosis using an ROpure-Nanopure water purification system (Barnstead, MA, USA). All centrifugations were carried out using a refrigerated centrifuge (Centra GP 8R, IEC, Fisher Scientific) at 18 °C at 1725 *g*.

Analytical conditions

The HPLC system consisted of a Perkin-Elmer Micro200 auto-injector with a 50 µl loop and a Micro200 pump both interfaced to a triple-quadrupole tandem mass spectrometer (API2000, Perkin-Elmer SCIEX, Foster City, CA, USA). The HPLC system, mass spectrometer and data acquisition were controlled by a Power Macintosh G3 microcomputer using MassChrom software (Perkin-Elmer SCIEX). The HPLC separations were carried out on a phenyl–hexyl column (5 µm particle size, 50 × 4.6 mm i.d.; Phenomenex, Torrance, CA, USA). The mobile phase consisted of 5% ammonium acetate (0.15 mM, pH not adjusted), 45% methanol and 50% acetonitrile, degassed by filtering through a 0.25 µm filter under vacuum. The compounds were eluted isocratically.

Stock solutions of RSP, 9-OH-RSP and INS were prepared in acetonitrile–water (1 : 1) by dissolving accurately weighed amounts of compounds (Cahn UltraMicro Balance; Thomas Scientific, Los Angeles, CA, USA). Calibration samples, with 10 concentration points ranging from 0.1 to 100 ng ml^{-1}, were prepared from the stock solution by serial dilution in blank plasma pooled from drug-free volunteers. Three quality control samples, QC-i, QC-ii and QC-iii, containing 30, 6 and 0.6 ng ml^{-1} of both RSP and 9-OH-RSP, respectively, were made to check the reliability of the calibration curve. Two plasma samples pooled from patients treated with RSP (Pt-QC-1 and Pt-QC-2) were used as long-term quality control samples to validate the spiked calibration curve prepared at various times.

Plasma samples

Rats were given a single bolus dose of 6 mg kg^{-1} of RSP by oral gavage. Four rats at each time point (pre-dose and at 0.25, 0.5, 1, 2, 5, 8, 12, 24, 36 and 48 h post-dose) were killed by decapitation. The trunk blood was collected in heparinized glass tubes and centrifuged at 4 °C for 10 min at 1725 *g*; plasmas were separated and stored at −70 °C until analysis.

Patients refractory to conventional antipsychotic treatment who met the DSM-IV criteria for schizophrenia[18] were admitted into a study to compare the efficacy of RSP with haloperidol. All patients gave informed consent for venipuncture and plasma concentration determination. The schizophrenic patients were treated with daily oral doses of 3, 4, 4.5 and 6 mg of RSP. Weekly blood samples were collected in the morning, ∼12 h after the last dose and just before the morning dose, by venipuncture in heparinized Vacutainer blood collection tubes (Becton and Dickinson, Rutherford, NJ, USA). The blood samples were immediately centrifuged for 15 min at 4 °C at 1725 *g*, and plasmas were separated and stored in glass vials at −70 °C until analysis.

Sample preparation

Both RSP and 9-OH-RSP were extracted from biological samples by a simple one-step liquid–liquid extraction as reported previously.[15] Briefly, to an aliquot of 0.5 ml of plasma sample (unknown, quality control (QC), calibration curve sample) taken in a borosilicate glass tube (15 ml capacity), 100 ng of INS (0.1 ml of 1 µg ml^{-1} solution) and 0.5 ml of a saturated solution of sodium carbonate were added (pH ≈ 10.5, not adjusted). The contents of tubes were mixed and extracted with 7 ml of 15% methylene chloride in pentane by shaking in a test-tube shaker. After centrifugation for 10 min at 18 °C, the supernatant organic layer was transferred into a borosilicate glass tube (10 ml capacity) and dried at 60 °C in a dry bath under a slow stream of nitrogen. The residue was reconstituted in 250 µl of mobile phase and an aliquot (30 µl) was injected into the HPLC system.

Extraction recovery

Absolute extraction recoveries of RSP, 9-OH-RSP and INS were determined by analyzing four 0.5 ml aliquots of spiked calibration curve plasma samples for RSP and 9-OH-RSP containing 100 ng of INS by the method described above. The absolute recoveries were calculated by comparing the peak areas obtained for spiked calibration curve plasma samples and peak areas obtained from direct injections of standard solutions containing known amounts of RSP, 9-OH-RSP and INS.

LC/MS/MS conditions

The compounds eluting from the phenyl–hexyl column were introduced into a Perkin-Elmer SCIEX API2000 triple-quadrupole tandem mass spectrometer for detection. The mass spectrometer was operated in the positive ion atmospheric pressure electrospray ionization mode using a TurboIonSpray ion source. The source temperature was kept at 300 °C. Nitrogen was used as the nebulizer gas (gas 1), auxiliary gas (gas 2), curtain gas and the gas for collisionally activated dissociation (CAD) in the collision cell. The gas flows were kept at the API2000 instrumental settings of 30, 20, 25 and 4, respectively. The detection and quantitation of compounds were performed in the multiple reaction monitoring (MRM) mode. The ion

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transitions monitored were *m/z* 411 → 191 for RSP, *m/z* 427 → 207 for 9-OH-RSP and *m/z* 421 → 201 for INS (see Fig. 1). These transition ions were selected based on the predominant fragmentation pathways of RSP, 9-OH-RSP and INS and their high intensity as observed in their product ion spectra (Fig. 1). The dwell time for each transition was set at 300 ms with an inter-channel pause time of 20 ms to provide optimum sampling of each peak of interest. The total scan time was 0.96 s. The CAD was ensured with nitrogen in the collision cell at $\sim 177 \times 10^{13}$ atoms cm^{-2}. The optimum collision energy was −34, −35 and −31 V for RSP, 9-OH-RSP and INS, respectively. The operations of mass spectrometer, LC system, mass calibration, data acquisition, data representation and post-acquisition quantitative analyses were carried out using a suite of software applications, MacDad, LCTune, Sample Control, Multiview and TurboQuan (Perkin-Elmer SCIEX).

Calibration curves

Calibration curves were constructed by plotting the peak-area ratio of RSP or 9-OH-RSP to INS against the respective concentration of RSP or 9-OH-RSP. The linear regression line was constructed covering the concentration range 0.1–100 ng ml^{-1} of RSP and 9-OH-RSP ($r^2 > 0.999$). The concentrations of unknown test samples were calculated from their peak-area ratios and the calibration curve and expressed in terms of ng ml^{-1}.

Pharmacokinetic analysis

Non-compartmental extravascular model pharmacokinetic parameters were estimated using WinNonlin microcomputer software (Pharsight, Mountain View, CA, USA). The statistical and graphical analyses were accomplished using commonly available commercial software packages (Microsoft Office, Microsoft; Prism, GraphPad Software, San Diego, CA, USA). The area under the concentration–time curves from the time of dosing to 48 h post-dose time point (AUC_{0-48}) was calculated by a linear trapezoidal method. The terminal half-life ($t_{1/2}$) was calculated using concentrations between 12 and 48 h post-dose. The area under the concentration–time curves from the time of dosing extrapolated to infinity ($AUC_{0-\infty}$) was calculated using the equation $AUC_{0-\infty} = AUC_{0-48} + C_{48} \times (t_{1/2}/\ln 2)$.

RESULTS AND DISCUSSION

Typical product ion mass spectra of RSP (A), 9-OH-RSP (B) and INS (C) are shown in Fig. 1. The flow-rates of the nebulizer gas, auxiliary gas, curtain gas and CAD gas were optimized to maximize the intensity of the product ions of RSP, 9-OH-RSP and INS. They were similar for these compounds. Then the collision energy for each ion transition was optimized to produce the highest intensity of the selected ion peak. The optimized collision energy parameters for ion transition for RSP *m/z* 411 → 191, for 9-OH-RSP *m/z* 427 → 207 and for INS *m/z* 421 → 201 were used in the MRM mode.

Typical LC/MS/MS total ion chromatograms of blank plasma (A) and plasma standard spiked with RSP, 9-OH-RSP and INS (B) on a phenyl–hexyl column are shown in Fig. 2. The extracted ion chromatograms of RSP, 9-OH-RSP and INS (Fig. 2(B1), (B2) and (B3), respectively) indicate that the compounds exhibited required retention

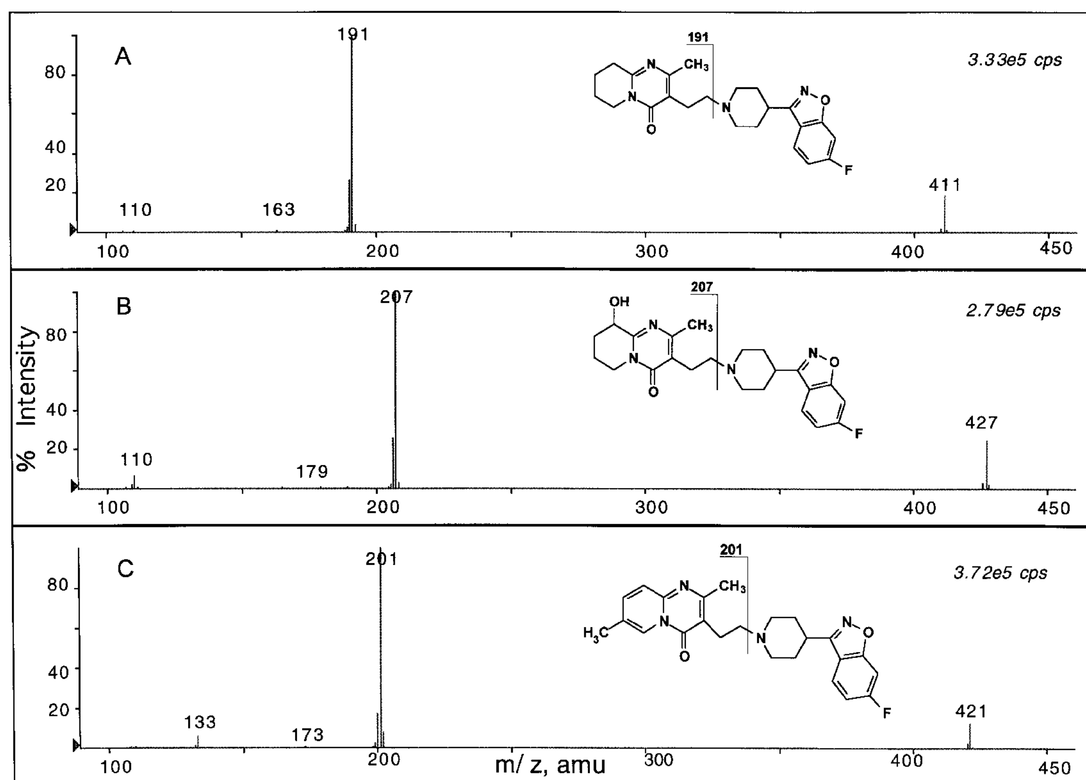

Figure 1. Product ion mass spectra of (A) RSP, (B) 9-OH-RSP, and (C) INS.

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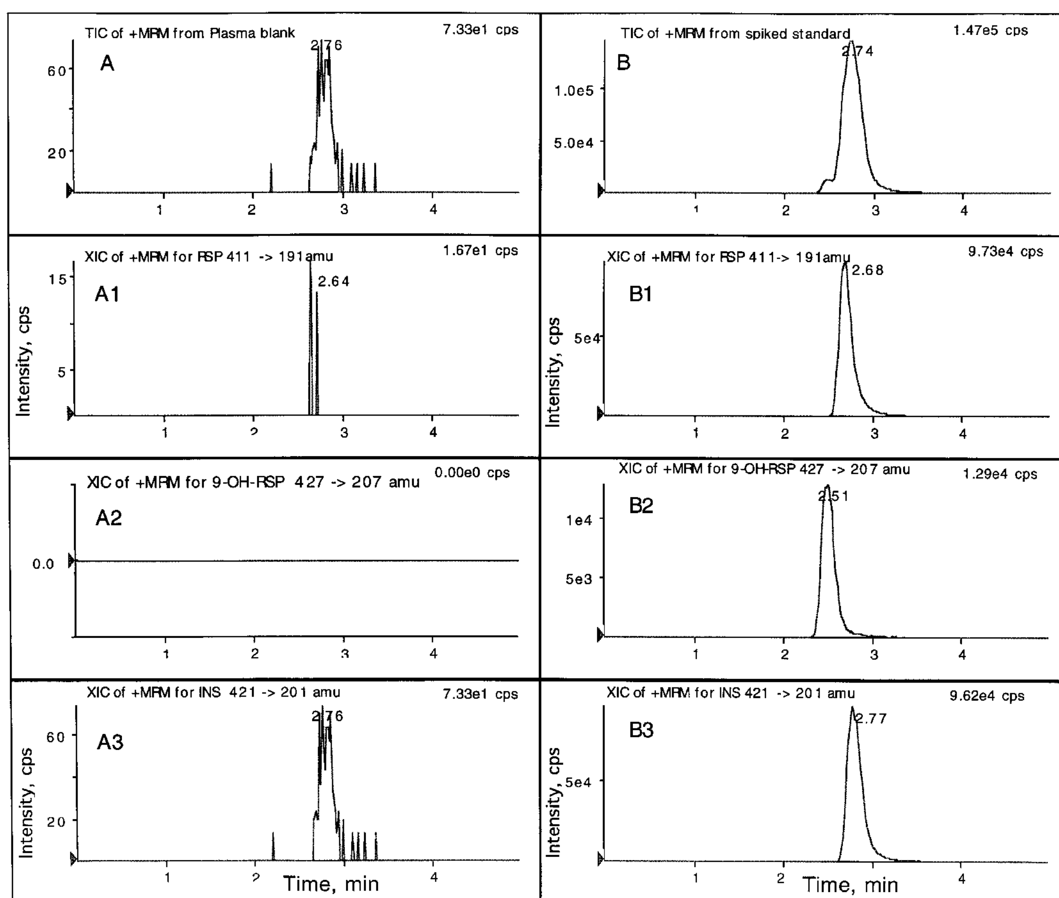

Figure 2. LC/MS/MS MRM total ion chromatograms (TIC) of (A) blank plasma and (B) solution mixture containing RSP, 9-OH-RSP and INS (2 ng each). The extracted ion chromatograms (XIC) of RSP, 9-OH-RSP and INS from TIC of blank plasma (A1, A2 and A3) and the solution mixture (B1, B2 and B3), respectively.

on a relatively short phenyl–hexyl column (5 cm). All compounds were eluted within 4 min. The compounds were not chromatographically fully resolved, as it was not essential to resolve them completely for accurate quantitation. In fact, the concentration values of RSP and 9-OH-RSP in spiked samples and patient quality control samples were similar when analyzed with and without using the column and showed a high overall linear correlation ($r^2 = 0.997$ and 0.994, slope = 1.092 and 1.021 for RSP and 9-OH-RSP, respectively; $p < 0.0001$). However, the phenyl–hexyl column was employed to retain the compounds sufficiently to obtain sharper peaks. The blank plasma extract did not show any interfering compounds [Fig. 2(A1), (A2) and (A3)]. The total ion (C) and extracted ion chromatograms of RSP (C1), 9-OH-RSP (C2) and INS (C3) in a plasma sample from a patient treated with RSP are given in Fig. 3.

Assay precision and accuracy

The precision and accuracy of the LC/MS/MS method were evaluated by the determination of intra- (within day) and inter- (between-days) assay variations. They were assessed as the coefficient of variation (CV) of the concentrations of the quality control samples determined by the assay. The intra-assay variance was determined by analyzing four 0.5 ml aliquots of each of three spiked QC plasma samples containing 30, 6 and 0.6 ng ml^{-1} of RSP and 9-OH-RSP and four 0.5 ml aliquots of each of two Pt-QC samples. These concentrations were determined from the calibration curve samples analyzed on the same day. The inter-assay variation was determined by analyzing 0.5 ml aliquots of spiked QC plasma and the Pt-QC plasma samples on 10 different days and determining their concentration from the calibration curve samples analyzed on each of the respective days. The calibration curves were linear and had highly reproducible correlation coefficients (0.999–0.9999), slopes (0.0008–0.0128) and intercepts (−0.0001 to 0.0005). The intra- and inter-assay variations for RSP and 9-OH-RSP are given in Table 1. For both RSP and 9-OH-RSP determinations, the intra- and inter-assay variations were <11%. The overall extraction recovery using the extraction procedure described here was 84, 89 and 76% for RSP, 9-OH-RSP and INS, respectively.

Pharmacokinetic analysis

The plasma concentration–time curves for RSP and 9-OH-RSP after a single oral dose of 6 mg kg^{-1} of RSP in rats are shown in Fig. 4. The non-compartmental extra-vascular model pharmacokinetic parameters determined from the concentration–time profiles of RSP and 9-OH-RSP in rats are given in Table 2. The plasma concentration of both compounds decreased in a multi-phase

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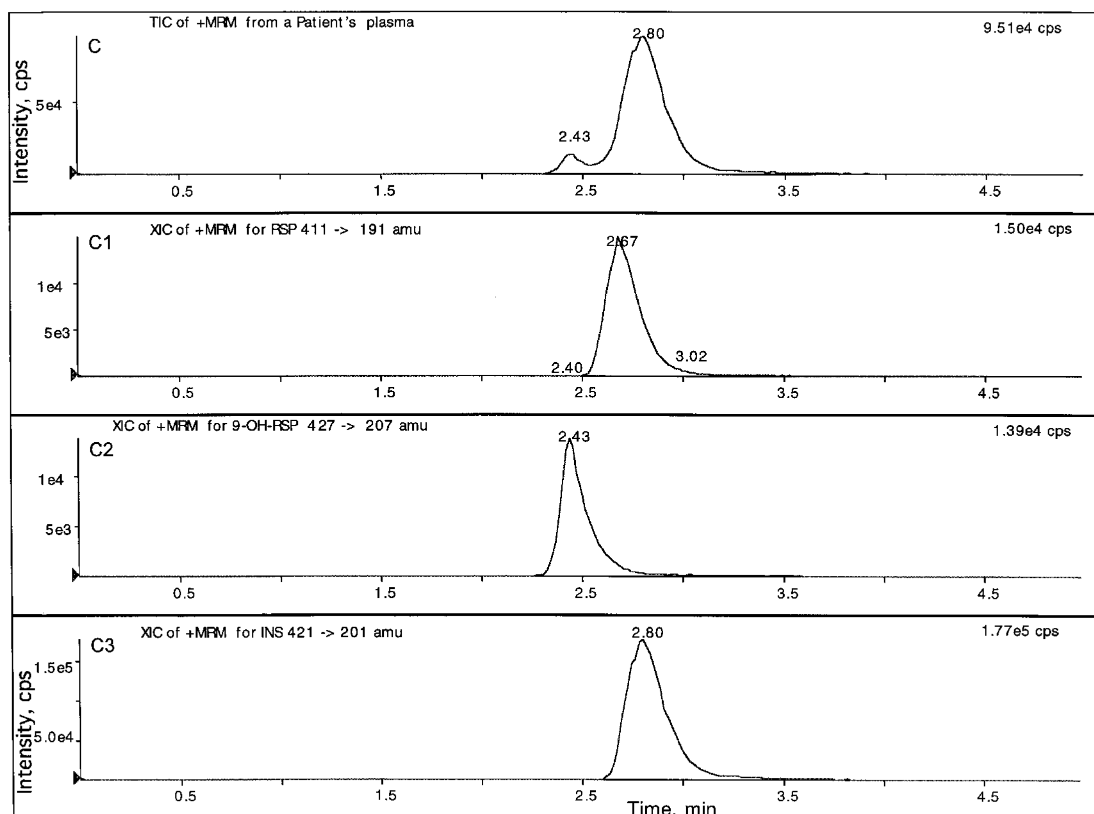

Figure 3. (C)LC/MS/MS MRM total ion chromatogram (TIC) of plasma from a patient treated with 8 mg day^{-1} extracted with 100 ng of INS. The extracted ion chromatograms (XIC) of (C1) RSP, (C2) 9-OH-RSP and (C3) INS, respectively, from TIC of plasma extract (C).

Table 1. Intra- (within-day) and inter- (between days) assay variations for the determination of RSP and 9-OH-RSP in plasma by the LC/MS/MS method

Sample	Concentration determined by LC/MS/MS (ng ml^{-1}) RSP	9-OH-RSP	CV (%) RSP	9-OH-RSP
Intra- (within-day) assay variation[a]	—			
Spiked plasma QC[b]:				
QC-i	29.1 ± 0.42	28.34 ± 0.79	1.5	2.8
QC-ii	5.9 ± 0.12	6.45 ± 0.3	2.0	4.5
QC-iii	0.68 ± 0.06	0.7 ± 0.02	9.2	3.4
Patient Plasma QC[c]:				
Pt-QC-1	13.82 ± 0.65	25.49 ± 2.3	4.7	2.3
Pt-QC-2	0.99 ± 0.06	39.58 ± 2.08	5.7	5.3
Inter- (between-days) assay variation[d]	—			
Spiked plasma QC:				
QC-i	27.29 ± 2.0	29.0 ± 2.51	7.3	8.7
QC-ii	6.06 ± 0.57	6.23 ± 0.57	9.3	9.1
QC-iii	0.64 ± 0.04	0.61 ± 0.06	6.1	9.6
Patient Plasma QC:				
Pt-QC-1	12.78 ± 1.2	21.57 ± 2.37	9.4	10.9
Pt-QC- 2	1.06 ± 0.01	38.3 ± 4.06	9.6	10.6

[a] Intra- (within-day) assay variations were calculated as coefficient of variation (CV, SD/mean) from the determined concentrations of four aliquots of each of three spiked quality control (QC-i, QC-ii and QC-iii) plasma samples and patient plasma samples assayed in a single day.
[b] Quality control samples QC-i, QC-ii and QC-iii (30, 6 and 0.6 ng ml^{-1} of RSP and 9-OH-RSP, respectively) were prepared by spiking pooled blank plasma from drug-free volunteers with RSP and 9-OH-RSP.
[c] Patient plasma QC (Pt-QC-1and Pt-QC-2) were pooled plasma samples collected from patients treated with RSP and used as a long-term QC to validate calibration curves prepared at various times.
[d] Inter- (between-days) assay variations were calculated as coefficient of variation (CV) from the determined concentrations of QC samples assayed on 10 different days.

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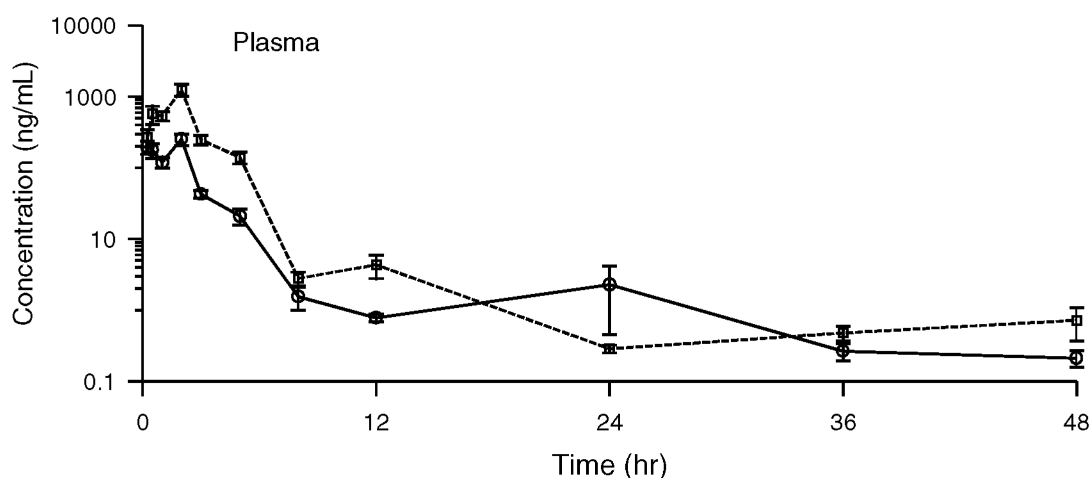

Figure 4. Plasma concentration (mean ± SD) Vs. time curve for RSP (O, solid line) and 9-OH-RSP (dashed line) in rats after a single oral dose of 6 mg^{-1} kg of RSP.

Table 2. Mean ± SD values of non-compartmental extravascular input pharmacokinetic parameters of RSP and 9-OH-RSP in plasma of rats after the administration of a single oral dose of 6 mg kg^{-1} of RSP

Pharmacokinetic parameters[a]	RSP	9-OH-RSP
C_{max} (ng ml^{-1})	288.7 ± 56.5	1308.5 ± 418.4
T_{max} (h)	1.6 ± 0.8	1.6 ± 0.8
Terminal $t_{1/2}$ (h)	14.8 ± 9.1	13.7 ± 2.8
AUC_{0-48}, (ng h ml^{-1})	618.6 ± 88.6	2709.1 ± 417.8
AUC_{0-8} (ng h ml^{-1})	624.2 ± 87.4	2725.2 ± 434.4
MRT_{last} (h)	3.1 ± 1.0	2.5 ± 0.2

[a] C_{max} = peak concentration attained after a single oral dose; T_{max} = time to reach C_{max}; AUC_{0-48} = area under the concentration–time curve for 48 h after oral dose using trapezoidal method; $AUC_{0-\infty} = AUC_{0-48} + C_{48} \times t_{1/2}/\ln 2$; MRT_{last} = mean residence time; $t_{1/2}$ = elimination half-life. The terminal $t_{1/2}$ was calculated using concentrations at 12–48 h post-dose; $n = 4$ rats at each time point.

fashion, first decreasing rapidly until 8 h and then slowly afterwards. The terminal elimination half-life ($t_{1/2}$) was calculated using concentrations at the last four time points between 12 and 48 h. The terminal elimination half-life was similar for RSP and its metabolite 9-OH-RSP, 14.8 and 13.7 h, respectively. The AUC_{0-48} and $AUC_{0-\infty}$ of 9-OH-RSP were four times larger than the respective AUCs for the parent compound, indicating that RSP was extensively metabolized to 9-OH-RSP.

Therapeutic drug monitoring in schizophrenic patients

Plasma concentrations of RSP and 9-OH-RSP were determined as part of an ongoing study in which plasma levels were routinely monitored for compliance and to adjust the RSP dose to achieve the optimum clinical outcome. The plasma concentrations of RSP and 9-OH-RSP in schizophrenic patients treated with various doses of RSP are given in Table 3. The plasma levels of RSP and 9-OH-RSP varied widely within and among patients. The

Table 3. Mean ± SD plasma concentrations of RSP and 9-OH-RSP in patients treated with daily oral doses of RSP determined by the LC/MS/MS method.

Dose mg day^{-1}	No. of patients	Plasma concentration (ng ml^{-1}) RSP	9-OH-RSP
3	4	2.6 ± 1.6	14.4 ± 8.2
4	2	3.2 ± 2.2	11.7 ± 5.3
4.5	3	1.3 ± 1.2	21.0 ± 10.0
6	4	2.6 ± 2.3	28.2 ± 15.4

data revealed that 9-OH-RSP metabolite was present at 8–20 times higher concentrations in plasma than that of RSP. These results were generally similar to our previously reported results.[15] The higher concentrations of 9-OH-RSP and its receptor binding potency similar to RSP reveal that the determination of 9-OH-RSP along with RSP will be useful in therapeutic drug monitoring of schizophrenic patients treated with RSP to achieve the optimum clinical outcome.

In conclusion, an LC/MS/MS method has been developed and validated for the simultaneous determination of RSP and 9-OH-RSP in plasma samples. The method is simple, selective and highly sensitive with a lower limit of determination of 0.1 ng ml^{-1} of RSP and 9-OH-RSP (~5 pg on-column) when 0.5 ml of plasma is used for the analysis. It takes only 4 min for the analysis of each sample, compared with 25 min with HPLC/ECD,[15] and is more sensitive than previously reported HPLC methods. The method is currently being used in the therapeutic drug monitoring of schizophrenic patients treated with RSP and in pharmacokinetic studies in animals.

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