Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 783 (2003) 461–472
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Validated method for the determination of risperidone and 9-hydroxyrisperidone in human plasma by liquid chromatography–tandem mass spectrometry

B.M.M. Remmerie[a], L.L.A. Sips[a], R. de Vries[a], J. de Jong[a], A.M. Schothuis[b], E.W.J. Hooijschuur[b], N.C. van de Merbel[b,*]

[a]*Department of Bioanalysis, Johnson and Johnson Pharmaceutical Research and Development, Turnhoutseweg 30, B-2340 Beerse, Belgium*
[b]*Pharma Bio-Research Group B.V., PO Box 200, 9470 AE Zuidlaren, The Netherlands*

Received 4 March 2002; accepted 17 September 2002

Abstract

Since the first entry of risperidone on to the market in the early 1990s, investigation of the pharmacokinetic behaviour of the compound for which the availability of a bioanalytical method was a conditio sine qua non, has received considerable attention. Most of the published methods, however, did not reach the level of sensitivity and selectivity which can be obtained today since the evolution of liquid chromatography–tandem mass spectrometry (LC–MS–MS) towards a routine technique in the bioanalytical laboratory. Therefore, we developed and validated a new LC–MS–MS method for the determination of risperidone and its active metabolite 9-hydroxyrisperidone in human plasma. This paper describes in detail the bioanalytical procedure and summarizes the validation results obtained. In addition, it focuses on the pitfalls one might encounter when developing similar assays. Despite the particular physicochemical characteristics of risperidone and 9-hydroxyrisperidone, the LC–MS–MS method enabled the quantification of both compounds down to 0.1 ng/ml. The method uses a sample preparation step by solid-phase extraction at pH 6 using a mixed-mode phase. In a short chromatographic run, separation of 9-hydroxyrisperidone from the minor metabolite 7-hydroxyrisperidone is achieved. Detection takes place by (turbo)ionspray tandem mass spectrometry in the positive ion mode. The validated concentration range is from 0.100 to 250 ng/ml, using 500 μl of sample, with accuracy (bias) and precision (coefficient of variation) being below 15%. Although new developments in equipment will allow us to further improve and speed up the method, the assay reported can be used as a routine method to support a wide range of pharmacokinetic studies.
© 2002 Elsevier Science B.V. All rights reserved.

Keywords: Risperidone; 9-Hydroxyrisperidone

1. Introduction

Risperidone is a benzisoxazole antipsychotic agent, used to treat schizophrenia and other psychoses. It exerts its effect by blocking serotonin ($5\text{-}HT_2$) and dopamine (D_2) receptors and causes a lower

*Corresponding author. Tel.: +31-592-303431; fax: +31-592-303223.
E-mail address: nvandemerbel@pbr.nl (N.C. van de Merbel).

1570-0232/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S1570-0232(02)00715-8

incidence of extrapyramidal side-effects than standard neuroleptic drugs. After oral administration, risperidone is rapidly and completely absorbed from the gastro-intestinal tract and mainly metabolised via hydroxylation and *N*-dealkylation [1,2]. The hydroxylation of risperidone is catalysed by the cytochrome P450 isoenzyme CYP2D6, which is subject to genetic polymorphism. Individuals are therefore either extensive (EM) or poor (PM) metabolizers of risperidone, the latter category constituting 5–10% of the Caucasian population and roughly 1% of the oriental population. Studies in animal models, however, showed the main metabolite 9-hydroxyrisperidone to be equipotent to risperidone both in vitro [1] and in vivo [3,4]. Risperidone and 9-hydroxyrisperidone thus constitute the clinically relevant active moiety, which does not depend on the metabolic status of the individuals.

For adequate support of clinical studies with risperidone, an analytical method is required for the determination of plasma levels of both risperidone itself and its active 9-hydroxy metabolite. Usually, oral doses of risperidone in the treatment of chronic schizophrenia are 2–6 mg per day. In EMs, this results in typical plasma levels of 1–10 ng/ml for risperidone and 10–70 ng/ml for 9-hydroxyrisperidone, while in PMs risperidone is much more abundant in plasma than its metabolite. Therefore, to quantify relevant plasma levels of both risperidone and 9-hydroxyrisperidone after low single doses of risperidone, an analytical method with a sub-ng/ml sensitivity for both analytes is required. In addition, it should be able to discriminate between 9-hydroxyrisperidone and the structurally very similar 7-hydroxyrisperidone and it should be selective towards the wide variety of psychotropic drugs (and their metabolites) that psychiatric patients commonly use.

Because of these quite stringent requirements, the analytical methodology for the determination of risperidone and its active metabolite has received considerable attention. Initially, two radioimmunoassays (RIAs) were used, an RIA after extraction for the determination of risperidone alone and a direct RIA for the determination of the sum of risperidone and 9-hydroxyrisperidone, after which the concentration of the metabolite can be calculated [5]. Although both sensitivity (0.1 ng/ml) and selectivity towards other drugs are good, the indirect determination of the important compound 9-hydroxyrisperidone is not really desirable and sample throughput is limited by the need to apply two methods for each sample. Moreover, the risperidone concentrations are slightly overestimated due to the cross-reactivity (ca. 2%) of the metabolite in the RIA after extraction, an effect which is most pronounced for low risperidone levels in the presence of high levels of the metabolite.

Since the early 1990s, therefore, a number of high-performance liquid chromatographic (HPLC) methods have been developed, enabling the simultaneous determination of both analytes. Those using conventional detection methods, however, are all inferior to RIA with regard to sensitivity. With UV detection, typically at 280 nm, detection limits are about 2 ng/ml after application of complicated multiple extraction schemes to remove interferences [6–8]. The application of electrochemical detection improves sensitivity to approximately 0.5 ng/ml, but also here sample preparation is critical and interference from co-medications is often unavoidable, probably as a result of the rather unselective detection potential of 0.8 V or above [9–12]. An additional drawback of all these methods is the need for at least 1 ml of plasma to obtain the reported detection limits.

Recently, Aravagiri and Marder demonstrated that the application of HPLC with tandem mass spectrometric (MS–MS) detection for the determination of risperidone and 9-hydroxyrisperidone in plasma is more sensitive (0.1 ng/ml) and requires less plasma (0.5 ml) than conventional methods [13]. In addition, the improved detection selectivity allows a more straightforward sample preparation and shorter chromatographic run times. The suitability of this method for therapeutic drug monitoring was supported by acceptable data for precision and accuracy, but no further validation results were presented. In order to ensure a reliable quantitation of risperidone and 9-hydroxyrisperidone for the support of pharmacokinetic studies, it is necessary to further explore the applicability and validity of LC–MS–MS. The present paper reports the development and validation of an LC–MS–MS method. Two approaches were followed: the first uses one internal standard, a structural analogue of risperidone, and the second uses two separate internal standards, which are

isotope-labelled forms of the analytes. The results of a thorough validation of this method are presented and a cross-comparison with the RIAs mentioned earlier is given. Finally, results for quality control samples obtained during application to pharmacokinetic samples illustrate the suitability of LC–MS–MS for routine bioanalysis.

2. Experimental

The analytical method employing one internal standard is described below as method A, while the method employing two internal standards is described as method B. The methods were validated at separate locations and were compared by means of a cross-comparison (see Section 2.5 below).

2.1. Chemicals

Risperidone, 9-hydroxyrisperidone, 7-hydroxyrisperidone and the internal standards R068809 (des-fluororisperidone, method A), R215640 ($^{2}H_{3}$-$^{13}C_{2}$-risperidone, method B) and R215639 ($^{2}H_{3}$-$^{13}C_{2}$-9-hydroxyrisperidone, method B), all depicted in Fig. 1, were provided by Johnson and Johnson Pharmaceutical Research and Development (Beerse, Belgium).

For method A, methanol and ammonium hydroxide (25%) were obtained from Malinckrodt Baker (Deventer, The Netherlands), acetonitrile and ammonium formate from Acros Organics (Geel, Belgium) and potassium dihydrogen phosphate, potassium hydroxide and formic acid from Merck (Darmstadt, Germany). For method B, methanol, acetonitrile, ammonium hydroxide (25%), formic acid and

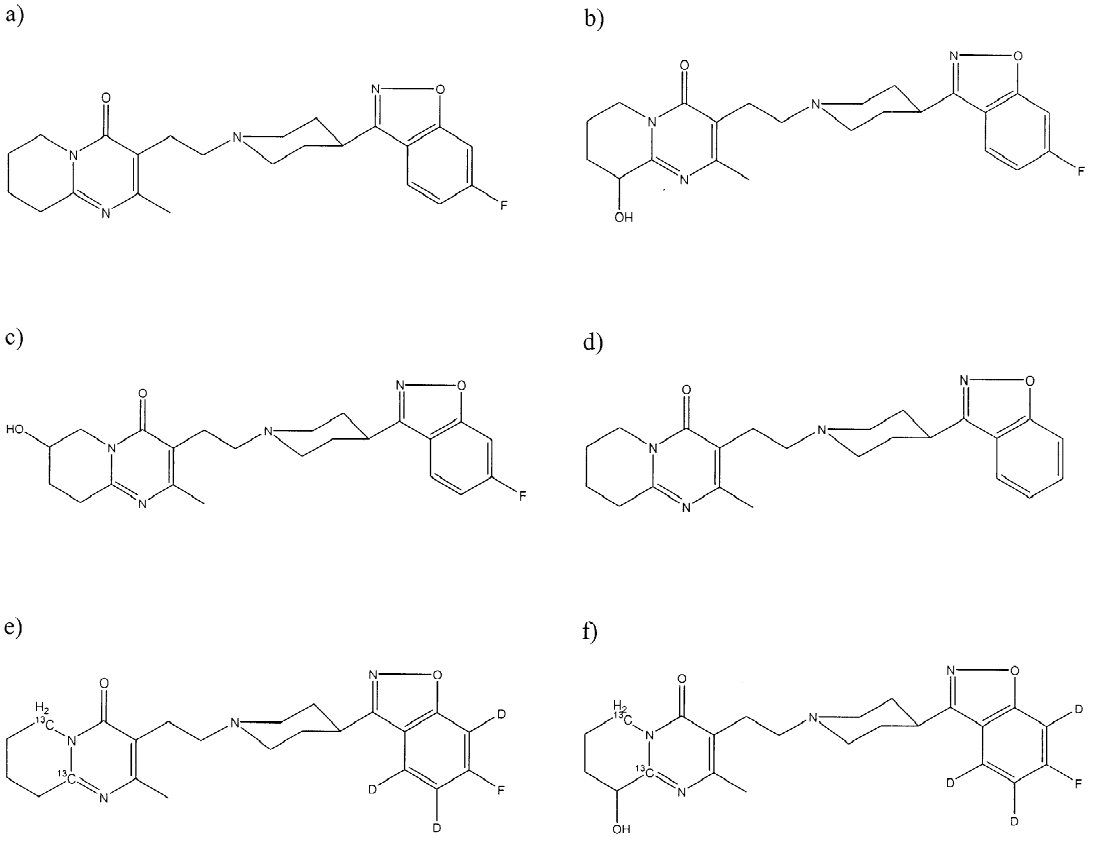

Fig. 1. Structures of (a) risperidone, (b) 9-hydroxyrisperidone, (c) 7-hydroxyrisperidone, (d) des-fluororisperidone, (e) $^{2}H_{3}$-$^{13}C_{2}$-risperidone, and (f) $^{2}H_{3}$-$^{13}C_{2}$-9-hydroxyrisperidone.

acetic acid were purchased from Merck and ammonium formate from Fluka (Buchs, Switzerland). For both methods, HPLC grade water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Equipment

2.2.1. Method A

A Hewlett-Packard (Palo Alto, CA, USA) Model 1050 LC system was used to inject 20-μl aliquots of the processed samples on a 3-μm C_{18} BDS-Hypersil column (100×4.6 mm I.D.), obtained from Alltech (Deerfield, IL, USA), which was kept at ambient temperature. The mobile phase, a mixture of 0.01 *M* ammonium formate (adjusted to pH 4.0 with formic acid) as elution solvent A and pure acetonitrile as elution solvent B, was delivered at 0.8 ml/min. Isocratic elution of the analytes was achieved using a mixture of 67% A and 33% B for 3.2 min, after which a step gradient was applied with 15% A and 85% B until 4.7 min; finally, the column was re-equilibrated at 67% A/33% B until 7.5 min. The eluent was split in a ratio of 1:7 in order to introduce 0.1 ml/min into the mass spectrometer.

Quantitation was achieved by MS–MS detection in the positive ion mode, using a PE Sciex (Foster City, CA, USA) API 365 mass spectrometer, equipped with a Turboionspray™ interface at 300 °C. Detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring 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 and the *m/z* 393.2 precursor ion to the *m/z* 191.0 product ion for R068809.

2.2.2. Method B

Equipment and procedures were identical to those used with method A, with the following exceptions. As HPLC system a Waters (Milford, MA, USA) Separations Module Alliance 2690 was used. The chromatographic column was kept at 25 °C. Detection of the internal standards was performed by monitoring the *m/z* 415.2 to the *m/z* 193.0 transition for R215640 and the *m/z* 431.2 to the *m/z* 209.0 transition for R215639.

2.3. Standard solutions

Methanolic stock solutions at 100 μg/ml were prepared separately for risperidone and 9-hydroxyrisperidone; they were used to prepare combined calibration solutions at 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 125 ng of each analyte per 100 μl methanol. Separate stock solutions were prepared for the internal standards at 100 μg/ml in methanol; they were used to prepare an internal standard working solution at 5 ng of each compound per 100 μl methanol. All stock solutions were stored at −20 °C and all calibration and working solutions at +4 °C.

Calibration samples at 0.100, 0.200, 0.400, 1.00, 2.00, 4.00, 10.0, 20.0, 40.0, 100 and 250 ng/ml were prepared freshly every day by adding 100 μl of the appropriate calibration solution to 0.5-ml aliquots of analyte-free heparinized human plasma. Quality control samples were prepared in plasma at 0.100, 0.299, 10.0 and 201 ng/ml (method A) or 0.100, 0.250, 5.00 and 200 ng/ml (method B) and stored at −20 °C. Calibration and quality control samples were prepared from separate stock solutions.

2.4. Sample preparation

An aliquot of 0.5 ml of plasma was mixed with 3 ml of phosphate buffer (pH 6.0), 100 μl of internal standard working solution and 100 μl of methanol (100 μl of the appropriate calibration solution for calibration samples). The sample was transferred to a 10 cc/130 mg Bond Elut Certify solid-phase extraction (SPE) column, purchased from Varian (Walnut Creek, CA, USA), which had been conditioned with 3 ml of methanol, 3 ml of water and 1 ml of 0.1 *M* phosphate buffer (pH 6.0). After application of the sample, the SPE column was washed with 3 ml of water, 1 ml of acetic acid and 3 ml of methanol. The column was eluted with 3 ml of a mixture of methanol and 25% ammonium hydroxide (98:2, v/v) and the eluate was evaporated to dryness under nitrogen at 65 °C. The residue was re-dissolved in 300 μl of a mixture of 50% elution solvent A and 50% elution solvent B and 20 μl were injected into the LC–MS–MS system.

2.5. Validation experiments

Validation of the method was performed in accordance with the principles as described by Shah et al. [14]. A linear model was used to describe the relationship of analyte concentration and response (peak area ratio of risperidone or 9-hydroxyrisperidone over internal standard). For method A, log-transformed concentrations and responses were used; due to restrictions in availability of data reduction packages no log transformation was performed for method B but a weighting factor of $1/x^2$ was applied.

The *selectivity* towards endogenous plasma compounds and possible impurities of the internal standards was tested with six different lots of non-pooled, analyte-free human plasma by analysing blanks (non-spiked plasma), zeros (blanks plus the internal standards) and samples spiked at 0.100 ng/ml.

The *accuracy* and *precision* of the method were determined at four concentration levels by analysing quality control samples 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 actual 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 (C.V.) over the two results, and the overall precision as the C.V. over all ten results.

The extraction *recovery* was evaluated at 0.4, 10 and 250 ng/ml for the analytes and at 10 ng/ml for the internal standards. It was determined by comparing the peak areas, obtained from calibration samples after standard analysis, to the peak areas, obtained from extracts of blank plasma, spiked prior to evaporation to dryness.

The *stability* of the analytes and internal standard R068809 was assessed under various conditions and, unless otherwise indicated, using method A. Stability in stock solution (methanol) was determined with HPLC–UV at 100 μg/ml for the analytes as well as the internal standard, after storage for 6 months at −20 °C, 1 month at +4 °C and 3 days at ambient temperature. Peak areas found after injection of the stored stock solutions were compared to the peak areas found after injection of freshly prepared stock solutions. Stability of risperidone and 9-hydroxyrisperidone in heparinized human plasma was evaluated at three concentrations (0.289, 9.64 and 96.4 ng/ml for risperidone and 0.288, 9.62 and 96.2 ng/ml for 9-hydroxyrisperidone). The concentrations after storage for 9 months at −20 °C, 72 h at ambient temperature and after four complete freeze–thaw cycles were determined against a freshly prepared calibration curve. Likewise, the stability of both analytes in human whole blood was determined after storage for 72 h at +4 °C, 24 h at ambient temperature and 2 h at +37 °C at the following concentrations: 3.13 and 95.0 ng/ml for risperidone and 3.14 and 95.4 ng/ml for 9-hydroxyrisperidone. Finally, the stability of the analytes in processed plasma at ambient temperature was evaluated at 0.250, 10.0 and 200 ng/ml for a storage interval of 6 days. The same parameter was assessed at +4 °C for 4 days, using method B and at 0.250 and 200 ng/ml.

Method *robustness* was evaluated by determining the retention times of risperidone, 9-hydroxyrisperidone and 7-hydroxyrisperidone and calculating the chromatographic resolution of the hydroxylated metabolites under slightly different chromatographic conditions. The ease of method transfer was investigated using a set of spiked plasma samples, which were prepared and analysed at one laboratory and analysed at a second laboratory by different operators using different equipment.

The influence of *matrix effects* on the performance of the method was evaluated by using a calibration curve, prepared in a different lot of plasma, in each analytical batch and calculating the concentrations of the validation samples against these different calibration curves.

3. Results and discussion

3.1. Sample preparation

In the methods published previously [7–13], sample preparation is typically achieved by adjusting the sample pH to >10, thus converting the analytes (pK_a ~8) into their neutral forms, and extracting them into an organic solvent. However, in order to obtain a

reasonable extraction recovery for 9-hydroxyrisperidone, a polar extraction solvent such as ethyl acetate is necessary, which leads to co-extraction of many basic endogenous plasma components of comparable polarity. When using HPLC with UV detection, these endogenous compounds show up in the chromatograms, but also with MS–MS they might interfere with detection by (irreproducibly) suppressing analyte ionization.

In order to improve method robustness, a more selective sample preparation approach was required. SPE with a mixed-mode phase, containing both ion-exchange and reversed-phase functionalities, was applied at pH 6 in order to effect retention of the, positively charged, analytes based on both cation-exchange and hydrophobic interactions. Similar and almost complete extraction recoveries were found for both risperidone and its more polar 9-hydroxy metabolite, indicating that analyte extraction most probably is mainly due to ion-exchange effects. This means that the selectivity of sample preparation is complementary to the (reversed-phase) selectivity of the subsequent chromatographic separation, which is advantageous for the robustness of the total analytical procedure.

3.2. Chromatography

Although 7-hydroxyrisperidone is a minor metabolite, its concentrations in plasma after risperidone administration are by no means negligible (up to 6% of 9-hydroxyrisperidone). Therefore, since 9- and 7-hydroxyrisperidone produce identical precursor and product ions and thus cannot be discriminated by MS–MS, it is essential that these compounds be chromatographically separated prior to their detection. This item has been overlooked in previously reported methods and may have led to a minor overestimation of 9-hydroxyrisperidone plasma levels. Therefore, chromatographic conditions had to be chosen in such a way that sufficient resolution of the hydroxy-metabolites was achieved, while at the same time the chromatographic run time was kept to a minimum in order to maximize sample throughput. It was found that, with an eluent composition of 33% acetonitrile and 67% buffer and an ordinary octadecyl stationary phase, baseline resolution of the metabolites could be realized within 4 min (Fig. 3d). As the most logical approach, reversed-phase chromatography under conditions at which the analytes are mostly uncharged, i.e. at an eluent pH of 9.0, was tried at first. However, with large series of samples, this led to unacceptable carry-over, apparently originating from adsorption of the analytes to various parts of the LC system and their continuous release into the eluent, causing a small but steady elevation of the detection background. This carry-over effect was much less pronounced at an eluent pH of 4.0, at which the analytes are charged and thus more soluble. Since the peak form of the analytes was essentially similar at both pH values (which also indicates that ion-exchange effects are not involved in analyte retention), it was decided to use an eluent of pH 4.0. In order to overcome any carry-over effect also under these conditions, a step gradient was incorporated at the end of each chromatographic run. This extended the run time to 7.5 min, but did improve method robustness and also removed possible non-polar co-medications from the system.

3.3. Detection

As reported previously [13], the molecular ions of risperidone (m/z 411.2) and 9-hydroxyrisperidone (m/z 427.2) are fragmented almost exclusively by cleavage of the ethyl-piperidinyl bond to produce intense product ion signals at m/z 191.0 and m/z 207.0, respectively. It was found that the three internal standards used in this study behave in a similar manner, as is illustrated for the isotope-labelled internal standards in Fig. 2. Here, it should be noted that these internal standards, although referred to as the $^{2}H_{3}$-$^{13}C_{2}$-forms of the analytes, in fact are a mixture of the $^{2}H_{3}$-$^{13}C_{2}$- and $^{2}H_{2}$-$^{13}C_{2}$-forms, with the latter species being most abundant. Therefore, the m/z 415.2 and m/z 431.2 ions, being four rather than five mass units higher than the corresponding analyte ions, were selected in the first quadrupole. For R068809, the desfluoro-form of risperidone, the predominant transition was that of the molecular ion (m/z 393.2) to the product ion at m/z 191.0.

Ionization and fragmentation were found to be highly efficient and, as a result, a substantial detection response was found at the lower limit of quantitation (0.100 ng/ml) using only 500 μl of

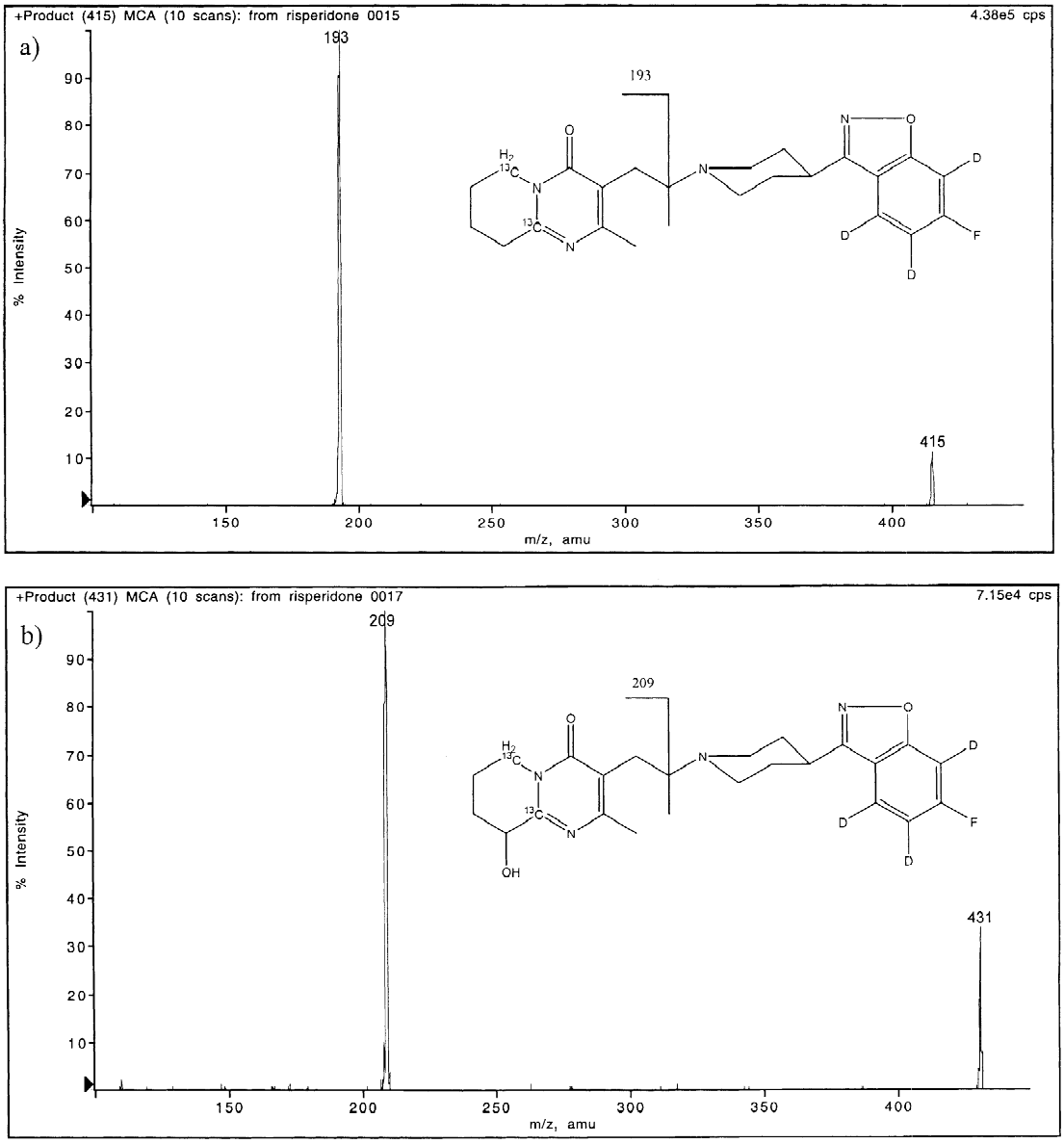

Fig. 2. Product ion mass spectra of (a) $^{2}H_{3}$-$^{13}C_{2}$-risperidone, and (b) $^{2}H_{3}$-$^{13}C_{2}$-9-hydroxyrisperidone.

sample and injecting just 20 μl out of 300 μl of sample extract (Fig. 3c).

3.4. Validation results

In all cases, back-calculated concentrations in the calibration curves were within 15% of the nominal values, which is in agreement with international guidelines [14] and indicates that the linear model acceptably describes the relationship between concentration and response.

The *selectivity* of the method towards endogenous plasma compounds and possible impurities of the internal standards was found to be fully satisfactory. In none of the tested lots of analyte-free plasma was any unacceptable interference seen. This is illustrated

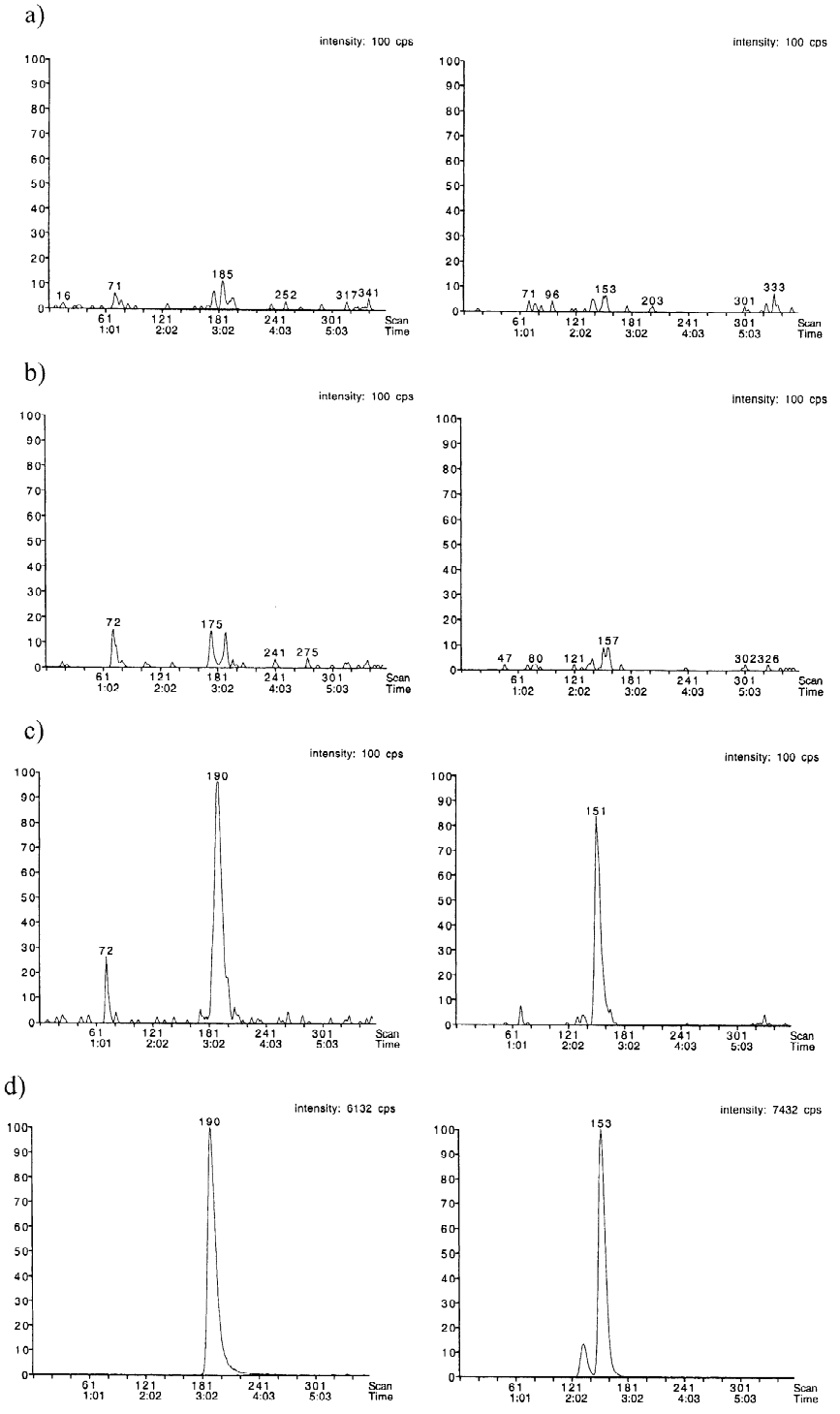

Fig. 3. Chromatograms showing the mass transition of risperidone (left panels) and 9-hydroxyrisperidone (right panels) in (a) blank plasma, (b) blank plasma containing the internal standards, (c) plasma spiked at 0.100 ng/ml with the analytes, and (d) plasma taken from a volunteer 3 h after oral administration of 2 mg risperidone; small peak in right panel is 7-hydroxyrisperidone.

for method B in Fig. 3a and b, which show a blank and a zero chromatogram, respectively. As can be concluded by comparing these to the chromatograms at 0.100 ng/ml in Fig. 3c, quantitation down to this level is not affected by any interference from either the sample matrix or the chemicals used.

The *accuracy* and *precision* of both methods (Table 1) were well within the limits established for pharmacokinetic analysis [14] across the entire concentration range. Both accuracy and precision seem to be slightly better for method B, especially at the lower concentrations, which could very well be related to the improved compensation of experimental variations during sample preparation and detection, which is provided by the isotope-labelled internal standards.

The extraction *recovery* was consistent over the calibration range and essentially complete for both analytes. For risperidone, the mean recovery was 97.2% with method A and 91.2% with method B; for 9-hydroxyrisperidone, the values were 95.7 and 89.9%, respectively. The internal standard R068809 yielded a mean recovery of 96.2%, i.e. comparable to the results of the analytes. The isotope-labelled internal standards R215640 and R215639, however, showed lower recoveries of 83.9 and 73.2%, respectively. It should be noted that it is not uncommon in LC–MS–MS that the absolute detection responses (peak areas), which were used for the calculation of the recoveries, vary from sample to sample. Since the relative results (peak area ratios), which are used for the calculation of analyte concentrations, invariably were acceptable, it can be concluded that the isotope-labelled internal standards provide a proper correction for these variations.

The *stability* results, which are summarized in Table 2, show that the analytes can be considered stable under the various conditions investigated, since the concentrations decreased by no more than 5% relative to the reference for any of the tests. Whole blood samples containing risperidone and 9-hydroxyrisperidone may therefore be kept for up to 2 h at +37 °C, 24 h at ambient temperature and 72 h at +4 °C, before plasma is prepared. In addition, plasma samples may be stored up to 9 months at −20 °C and up to 72 h at ambient temperature prior to processing. Once processed, samples can be stored at +4 °C for 4 days and at ambient temperature for 6 days before they are analysed. Finally, methanolic stock solutions 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 ambient temperature, either protected or unprotected from light. The internal standard R068809 in methanol was also investigated and found to be stable for the same periods of time at the different conditions as the analytes. Since R215540 and R215539 are isotope-labelled forms of the analytes, their physical–chemical properties can

Table 1
Summary of accuracy and precision results

Method	Analyte	Nominal concentration (ng/ml)	Overall accuracy (%)	Overall precision (%C.V.)
A	Risperidone	0.100	86.9	10.2
		0.299	88.9	5.8
		10.0	99.1	2.6
		201	98.8	2.5
	9-Hydroxyrisperidone	0.100	86.8	7.2
		0.299	89.9	6.9
		10.0	102.1	2.8
		201	94.1	1.7
B	Risperidone	0.100	97.2	8.3
		0.250	89.3	3.6
		5.00	91.2	3.1
		200	94.9	2.6
	9-Hydroxyrisperidone	0.100	98.0	7.7
		0.250	90.9	5.3
		5.00	92.7	3.3
		200	97.1	2.9

Table 2
Summary of stability results

Matrix	Temperature (°C)	Period	Mean deviation from $t=0$ (%)	
			Risperidone	9-Hydroxyrisperidone
Whole blood	+4	72 h	+4.5	+4.1
	Ambient	24 h	−2.4	−2.4
	+37	2 h	−3.1	−2.7
Plasma	−20	9 Months	−1.9	−2.4
	Ambient	72 h	−2.7	−3.9
	Freeze–thaw	Four cycles	−0.4	+2.2
Methanol	−20	6 Months	−0.7	+2.3
	+4	1 Month	+5.8	+13.2
	Ambient (dark)	3 Days	+6.0	+2.2
	Ambient (light)	3 Days	+3.8	+3.0
Processed plasma	Ambient	6 Days	−4.2	+0.6
	+4	4 Days	−0.2	−0.5

be assumed to be similar and no stability assessment was therefore undertaken.

In order to evaluate method *robustness*, the chromatographic resolution between 9-hydroxyrisperidone and 7-hydroxyrisperidone was determined under conditions that slightly differed with respect to eluent composition (modifier content, pH and buffer molarity) and column temperature. As is shown in Table 3 for method B, in all cases the resolution was well above 1.0, which indicates that slight differences in chromatographic conditions do not unacceptably affect the performance of the method. In addition, it was found that the method can be easily transferred to a different laboratory, another indication of method robustness. The accuracy with which plasma levels, spiked at a different laboratory, could be determined varied between 97.9 and 103.2% for risperidone and between 91.3 and 100.7% for 9-hydroxyrisperidone.

In LC–MS–MS analysis, the accuracy of analyte quantitation may very well be influenced by *matrix effects*, i.e. the differing composition of individual plasma samples, because the ionization of analytes is susceptible to interference from matrix components. Since the concentrations in the validation samples were calculated against a calibration curve, which was prepared in a different lot of plasma in each analytical batch, the accuracy and precision results in Table 1 show that application of either of the methods gives reliable results for both risperidone

Table 3
Chromatographic resolution between 7- and 9-hydroxyrisperidone during robustness testing

Proportion A:B (%)	Buffer pH	Buffer molarity (m*M*)	Column temperature (°C)	Mean R_S ($n=2$)
67:33	4.0	10	23	1.42
67:33	4.0	10	25	1.40
67:33	4.0	10	27	1.50
65:35	4.0	10	25	1.37
69:31	4.0	10	25	1.57
67:33	3.8	10	25	1.50
67:33	4.2	10	25	1.50
67:33	4.0	8	25	1.55
67:33	4.0	12	25	1.54

and 9-hydroxyrisperidone, regardless of the composition of the plasma sample.

3.5. Comparison of methods A and B

Although the LC–MS–MS method performs acceptably both with the single- and with the multiple-internal standard approach, it was decided to employ the method with two internal standards for routine analyses. Since isotope-labelled internal standards provide the best correction for variations in experimental conditions and for variations in sample composition, this approach will give the most reliable results.

3.6. Cross-comparison with RIAs

A total of 50 samples taken from subjects dosed with risperidone, after being analysed by RIA [5], were re-analysed with LC–MS–MS method A. The results for the sum of risperidone and 9-hydroxyrisperidone, as found by the direct RIA, correlated very well with the results found for the sum of the analytes by LC–MS–MS: the mean accuracy was 100%. The results for risperidone alone, however, as quantified by the RIA after extraction showed an average overestimation of 23% as compared to the results obtained by LC–MS–MS (Fig. 4), an effect which is most pronounced at low risperidone concentrations. As mentioned earlier in this paper, this is most probably due to the cross-reactivity of co-extracted 9-hydroxyrisperidone. However, since the sum of risperidone and 9-hydroxyrisperidone represents the clinically relevant active moiety, this small overestimation at low risperidone plasma concentrations does not affect the validity of the conclusions derived from these data.

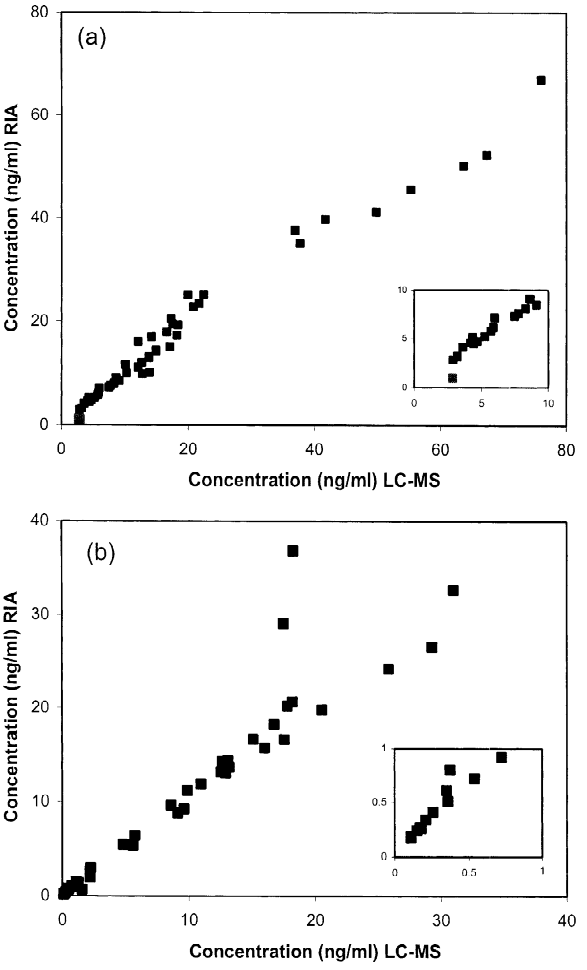

Fig. 4. Correlation plots showing the plasma concentrations as obtained by RIA and LC–MS–MS for (a) the sum of risperidone and 9-hydroxyrisperidone and (b) for risperidone alone. Insets show detailed results for lowest concentrations.

3.7. Application to pharmacokinetic studies

To date, more than 20 000 samples from clinical trials with risperidone have been analysed with method B. The average accuracy (expressed as %bias from nominal value) and precision (expressed as %C.V.) found for spiked quality control samples, typically are in the order of 10% for the 0.250 ng/ml level, 7% for the 10.0 ng/ml level and 5% for the 200 ng/ml level, for both analytes. Because of the relatively short chromatographic run time and straightforward sample preparation procedure, a sample throughput of 120 per day is routinely achieved. Fig. 5 shows typical plasma concentration versus time profiles for risperidone, 9-hydroxyrisperidone and the sum of both compounds (active moiety) following a single oral dose of 2 mg to an extensive and a poor metabolizer of CYP 2D6. It clearly indicates that the method also allows the determination of the pharmacokinetics of the analytes after low doses of risperidone.

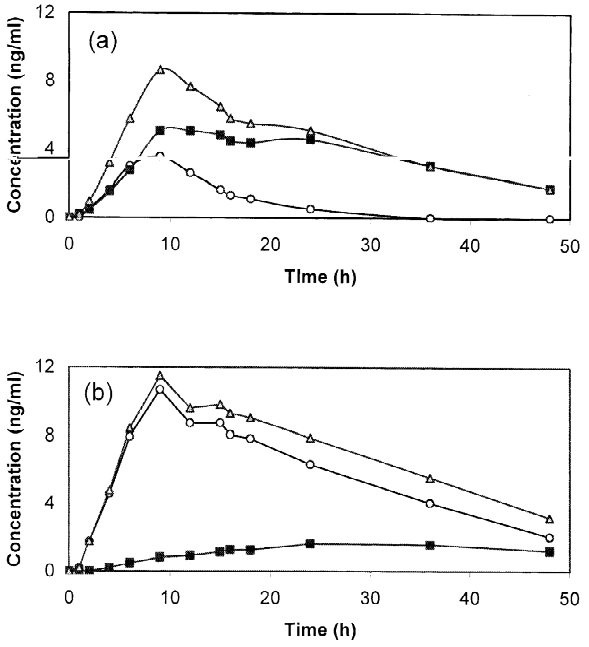

Fig. 5. Plasma concentration–time curves for (○) risperidone, (■) 9-hydroxyrisperidone and (△) active moiety following a dose of 2 mg (controlled release) to an (a) extensive metabolizer and (b) a poor metabolizer for CYP 2D6.

4. Conclusion

An LC–MS–MS method has been developed and validated for risperidone and its active metabolite 9-hydroxyrisperidone in human plasma. Because of its robust nature, its high sensitivity and selectivity, in combination with its favourable accuracy and precision, the method is very suitable for routine analysis to support clinical trials with risperidone. The application of isotope-labelled internal standards for each of the analytes provides optimum compensation for experimental variability and is preferred over the use of one structural analogue as internal standard.

Acknowledgements

The authors would like to thank Cor Janssen and co-workers for the synthesis of the stable isotope labelled internal standards.

References

[1] M.-L. Huang, A. Van Peer, R. Woestenborghs, R. De Coster, J. Heykants, A. Jansen, A. Zylicz, H. Visscher, J. Jonkman, Clin. Pharmacol. Ther. 54 (1993) 252.
[2] G. Mannens, M.-L. Huang, W. Meuldermans, J. Hendrickx, R. Woestenborghs, J. Heykants, Drug Metab. Dispos. 21 (1994) 1134.
[3] L. Van Beijsterveldt, R. Geerts, J. Leysen, A. Megens, H. Van den Eynde, W. Meuldermans, J. Heykants, Psychopharmacology 114 (1994) 53.
[4] A. Megens, F. Awouters, Drug Dev. Res. 33 (1994) 399.
[5] R. Woestenborghs, I. Geuens, H. Lenoir, C. Janssen, J. Heykants, in: E. Reid, I.D. Wilson (Eds.), Methodological Surveys in Biochemistry and Analysis, Vol. 20, Royal Society of Chemistry, Cambridge, 1990, p. 241.
[6] R. Woestenborghs, W. Lorreyne, F. van Rompaey, J. Heykants, J. Chromatogr. 583 (1992) 223.
[7] O.V. Olesen, K. Linnet, J. Chromatogr. B 698 (1997) 209.
[8] A. Avenoso, G. Facciolà, M. Salemi, E. Spina, J. Chromatogr. B 746 (2000) 173.
[9] M. Aravagiri, S.R. Marder, Th. van Putten, K.K. Midha, J. Pharm. Sci. 82 (1993) 447.
[10] J.P. Le Moing, S. Edouard, J.C. Levron, J. Chromatogr. 614 (1993) 333.
[11] M.C. Price, D.W. Hoffman, Ther. Drug Monit. 19 (1997) 333.
[12] A.E. Balant-Gorgia, M. Gex-Fabry, C. Genet, L.P. Balant, Ther. Drug Monit. 21 (1999) 105.
[13] M. Aravagiri, S.R. Marder, J. Mass Spectrom. 35 (2000) 718.
[14] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, J. Pharm. Sci. 81 (1992) 309.