Research Article

Biomedical Chromatography

Received: 15 December 2008, Revised: 17 December 2008, Accepted: 17 December 2008 Published online 7 April 2009 in Wiley Interscience

(www.interscience.wiley.com) DOI 10.1002/bmc.1204

Determination of risperidone and 9-Hydroxyrisperidone using HPLC, in plasma of children and adolescents with emotional and behavioural disorders

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ABSTRACT: A simple, rapid, selective, accurate and precise method is described for the determination of risperidone and its active metabolite, 9-hydroxyrisperidone, in plasma using a chemical derivative of risperidone (methyl-risperidone) as the internal standard. The sample workup involved a single-step extraction of 1 mL plasma, buffered to pH 10, with heptane–isoamyl alcohol (98:2 v/v), then evaporation of the heptane phase and reconstitution of the residue in mobile phase. HPLC separation was carried out at on C_{18} column using a mobile phase of 0.05 M dipotassium hydrogen orthophosphate (containing 0.3% v/v triethylamine) adjusted to pH 3.7 with orthophosphoric acid (700 mL), and acetonitrile (300 mL). Flow rate was 0.6 mL/min and the detection wavelength was 280 nm. Retention times were 2.6, 3.7 and 5.8 min for 9-hydroxy risperidone, risperidone and the internal standard, respectively. Linearity in spiked plasma was demonstrated from 2 to 100 ng/mL for both risperidone and 9-hydroxyrisperidone ($r \geq 0.999$). Total imprecision was less than 13% (determined as co-efficient of variation) and the inaccuracy was less than 12% at spiked concentrations of 5 and 80 ng/mL. The limit of detection, determined as three times the baseline noise, was 1.5 ng/mL. Clinical application of the assay was demonstrated for analysis of post-dose (0.55–4.0 mg/day) samples from 28 paediatric patients (aged 6.9–17.9 years) who were taking risperidone orally for behavioural and emotional disorders. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: HPLC; risperidone; 9-hydroxyrisperidone; children

Introduction

Risperidone (3-{2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl]-ethyl}-6,7,8,9-tetrahydro-2-methyl-4H-pyrido[1,2-a}pyrimidin-4-one) is a benzisoxazole derivative (Janssen *et al*., 1998). It is a second-generation antipsychotic developed for the treatment of schizophrenia (Leysen *et al*., 1988; Raggi *et al*., 2004). In children and adolescents, risperidone is used for a range of disorders, such as behavioural problems associated with developmental disorders, disruptive behaviour disorders and tic disorders in addition to psychotic disorders (Cheng-Shannon *et al*., 2004; Jensen *et al*., 2007).

Risperidone undergoes extensive metabolism by liver enzymes to 9-hydroxyrisperidone, which has a pharmacological activity similar to the parent (Leysen *et al*., 1994). Many researchers have examined the relationship between plasma concentrations of risperidone, 9-hydroxyrisperidone and observed clinical effects (Riedel *et al*., 2005; Heykants *et al*., 1994; Aravagiri *et al*., 2003; Olesen *et al*., 1998; Castberg and Spigset, 2005). It is well established that serum concentrations of risperidone and 9-hydroxyrisperidone differ widely among individuals due to liver enzyme polymorphisms (Riedel *et al*., 2005; Heykants *et al*., 1994; Aravagiri *et al*., 2003; Olesen *et al*., 1998). However, due to the similar pharmacological effects of both compounds, the total 'active moiety' within serum remains similar in all patients (Heykants *et al*., 1994). In 46 patients with schizophrenia, steady-state risperidone concentrations were almost negligible (<1 ng/mL) due to the extent of metabolism. However, 9-hydroxyrisperidone concentrations were on average 22-fold higher than risperidone (Aravagiri *et al*., 2003). The 9-hydroxyrisperidone concentrations were usually greater than 5 ng/mL, which suggests that the metabolite may be primarily responsible for the clinical effects of risperidone (Riedel *et al*., 2005). These workers also reported that responders to risperidone

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Contract/grant sponsor: Society of Hospital Pharmacists of Australia.

Contract/grant sponsor: Mater Children's Hospital Golden Casket Research Fund.

therapy and those who did not respond had similar concentrations of active drug moiety, which demonstrates that clinical response to risperidone may be due to a variety of factors apart from drug concentration. These large variations among studies and clinical response indicate a need for further investigations to determine appropriate therapeutic levels of risperidone between individuals.

Published high-performance liquid chromatography (HPLC) methods for risperidone and 9-hydroxyrisperidone in human plasma include UV (Woestenborghs *et al*., 1992; Olesen and Linnet, 1997; Nagasaki *et al*., 1999; Avenoso *et al*., 2000; Titier *et al*., 2002; Lerena *et al*., 2003), electrochemical detection (Aravagiri *et al*., 1993; LeMoing *et al*., 1993; Price and Hoffman, 1997; Aravagiri *et al*., 1998; Balant-Gorgia *et al*., 1999; Schatz and Saria, 2000) and tandem mass spectrometry (Aravagiri and Marder, 2000; Remmerie *et al*., 2003). Electrochemical detection allows more sensitive detection of risperidone and its metabolite, but lengthy maintenance is needed to preserve the integrity of the detector. As serum or plasma concentrations below 1 ng/mL have been shown to be due to extensive metabolism and generally no longer contribute to the clinical effect, the sensitivity available with electrochemical detection is unnecessary in clinical applications (Aravagiri *et al*., 2003). Mass spectrometry also provides greater sensitivity and specificity; however, the instrumentation and its maintenance are expensive and not available in many laboratories. Other methods require lengthy extraction procedures (Woestenborghs *et al*., 1992; Aravagiri *et al*., 1993; LeMoing *et al*., 1993), long run-times (Nagasaki *et al*., 1999; LeMoing *et al*., 1993; Price and Hoffman, 1997; Aravagiri *et al*., 1998; Balant-Gorgia *et al*., 1999; Schatz and Saria, 2000), inadequate lower limits of quantitation (Titier *et al*., 2002; Aravagiri *et al*., 1993) or poor choice of internal standard (Olesen *et al*., 1998; Nagasaki *et al*., 1999; Avenoso *et al*., 2000).

The aim of this study was to redress these deficiencies by developing a rapid, simple and improved method for the determination of risperidone and its active metabolite, 9-hydroxyrisperidone, in human plasma. Methyl-risperidone, a non-drug structural analogue of risperidone, was used as the internal standard (IS).

Experimental

Chemicals and Reagents

Risperidone, 9-hydroxyrisperidone and the IS (methyl-risperidone; R 068808) were provided by Janssen Cilag Pty Ltd (North Ryde, NSW, Australia; Fig. 1). All other reagents used were of HPLC or analytical grade. Water was of at least 18 MΩ quality from a Millipore water purification system (Millipore Australia Pty Ltd, North Ryde, NSW, Australia).

Preparation of Stock Solutions, Standards and Quality Control Samples

Stock solutions were prepared in methanol at a concentration of 1 mg/mL for risperidone, 9-hydroxyrisperidone and the IS. These standards were stable for at least 3 months when stored at −20°C. The stock solution of IS was diluted in Milli-Q water to 100 μg/mL to give an intermediate working solution. A working solution of the IS was prepared daily by diluting 80 μL of the intermediate working solution to 20 mL with 0.6 M sodium carbonate/bicarbonate buffer (pH 10) to yield an IS concentration of 400 ng/mL. The stock solutions of risperidone and 9-hydroxyrisperidone were diluted directly into plasma to prepare standards of 2, 10, 20, 40, 70 and 100 ng/mL, which were stored at −80°C. Independent seeded controls containing 80 ng/mL and 5 ng/mL of both risperidone and 9-hydroxyrisperidone were prepared likewise to determine inter-day and intra-day assay reproducibility.

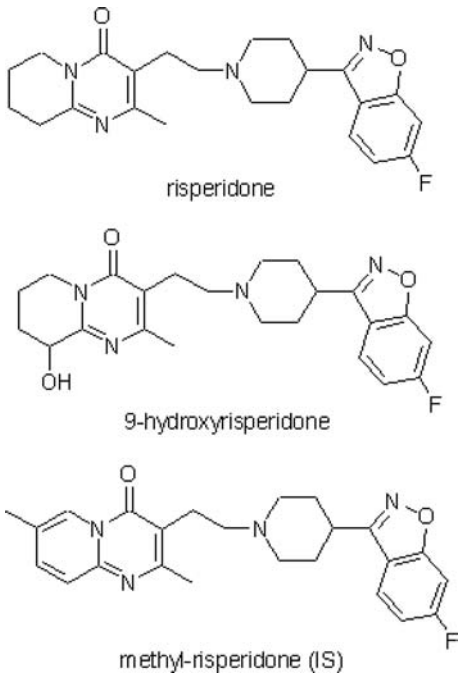

Figure 1. Structure of risperidone, 9-hydroxyrisperidone and methyl-risperidone.

Extraction Procedure

The sample preparation was a modification of a previously reported procedure (Olesen and Linnet, 1997). To a 12 mL Pyrex-glass culture tube (13 × 100 mm), 1 mL of plasma (collected in lithium heparin tubes) was added together with 0. 5 mL of 0.6 M sodium carbonate/bicarbonate buffer (pH 10) containing 400 ng/mL methyl-risperidone. Eight millilitres of heptane–isoamyl alcohol (98:2 v/v) were added and the tube was gently rotated for 15 min on a blood mixer. The aqueous layer was frozen at −80°C for ~30 min. The heptane layer was decanted into a second culture tube and evaporated to dryness under a gently stream of nitrogen at 60°C (~45 min). The residue was dissolved in 150 μl of mobile phase, of which 70 μl was injected into the HPLC. Standards and control samples were included in each batch.

Chromatographic Conditions

Chromatography was carried out on an 1100 series HPLC system (Agilent Technologies Australia Pty Ltd, Forest Hill, Vic, Australia) equipped with a model G1310A isocratic pump, model G1313A autosampler, model G1316A column oven and a model G1314A UV variable wavelength detector. HP ChemStation software was used to record and process the chromatograms. Separations were carried out at 20 ± 3°C on a Prevail C_{18} analytical column (5 μm particle size, 150 × 3.0 mm i.d. (Grace Davison Discovery Sciences, Baulkham Hills Business Centre, NSW, Australia). The mobile phase consisted of 0.05 M dipotassium hydrogen orthophosphate and 0.3% v/v triethylamine adjusted to pH 3.7 with orthophosphoric acid (700 mL) and acetonitrile (300 mL)

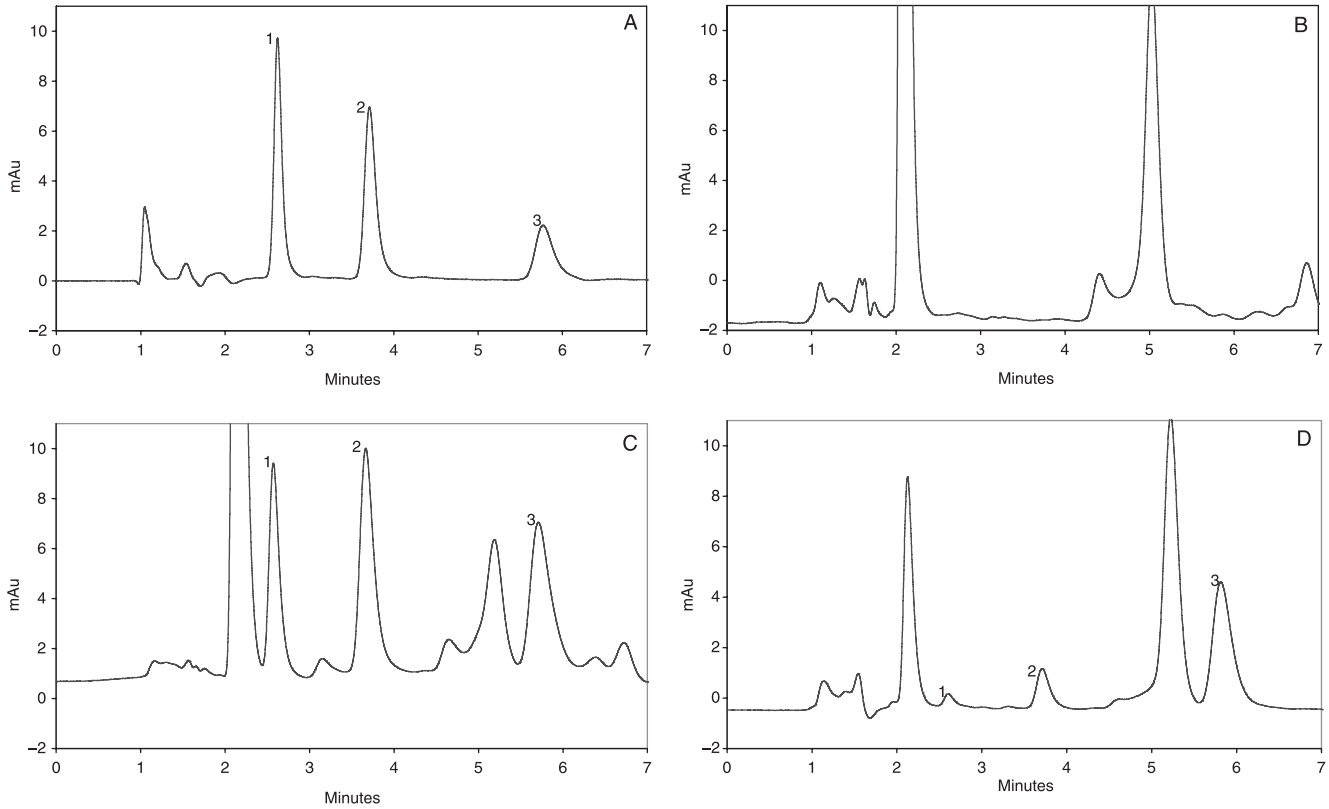

Figure 2. Representative chromatograms for: (A) mobile phase spiked with 400 ng/mL each of risperidone, 9-hydroxyrisperidone and internal standard (methyl-risperidone, IS); (B) extract of drug-free plasma; (C) extract of drug-free plasma spiked with 100 ng/mL risperidone and 100 ng/mL of 9-hydroxyrisperidone, and IS; (D) analysis of a plasma sample taken 2.25 h after the last dose in an 11-year-old male schizophrenic patient on 1.5 mg of risperidone per day; risperidone, 19.5 ng/mL; 9-hydroxyrisperidone, 6.6 ng/mL. Peaks: 1 = 9-hydroxyrisperidone, 2 = risperidone, 3 = IS.

and was filtered under negative pressure through a 0.45 μm organic filter membrane. The flow rate was 0.6 mL/min. The detector wavelength was set to 280 nm.

Assay Validation

Retention times of the compounds of interest were determined by injection of mobile phase spiked with 400 ng/mL of risperidone, 9-hydroxyrisperidone and the IS. To determine specificity, chromatograms were obtained for extracts of drug-free plasma (no IS in sodium carbonate/bicarbonate buffer during extraction), and of drug-free plasma spiked with 100 ng/mL of risperidone, and 9-hydroxyrisperidone (IS included in sodium carbonate/bicarbonate buffer).

The linearity was assessed by analysis of the six standards containing 2, 10, 20, 40, 70 and 100 ng/mL of risperidone and 9-hydroxyrisperidone prepared in human plasma. Standard curves were based on the ratio of the analyte to the internal standard peak areas vs analyte concentration. The line of best fit was determined using regression analysis with $1/x^2$ weighting, where x is the concentration of a standard.

Imprecision was determined by preparing human plasma spiked with 5 and 80 ng/mL of both analytes. These samples were subdivided and stored at −80°C until assayed in quadruplicate on three different occasions. The inter-day, intra-day and total coefficients of variation (CV%) were then determined simultaneously from the results of an analysis of variance using all the within-day and between-day data simultaneously (Krouwer and Rabinowitz, 1984).

Inaccuracy was measured by calculating the difference between the mean assayed concentration and the spiked concentration according to the equation:

$$\text{Accuracy (\%)} = [(\text{mean assayed concentration}/\text{weighed-in concentration}) \times 100]\%$$

The limit of detection (LOD) was determined as three times the average baseline noise.

Clinical Application

To establish the practical application of this method in the clinic, plasma was assayed from blood drawn between 40 min and 24 h post-dose (range 0.25–4.0 mg/day) in 28 patients (six female) aged 6.9–17.9 years, who were being prescribed risperidone tablets (Risperdal®, Janssen-Cilag Pty Ltd) for a variety of emotional and behavioural disorders.

Results

The retention times were 2.6, 3.7 and 5.8 min for 9-hydroxyrisperidone, risperidone and methyl-risperidone, respectively. There was adequate baseline separation from the peaks of interest and endogenous peaks [Fig. 2(A–D)]. Replicate (n = 5) calibrations ranging from 2 to 100 ng/mL showed satisfactory linearity for both risperidone and 9-hydroxyrisperidone ($r \geq 0.999$) (see Fig. 2).

Total coefficients of variation (%) for both risperidone and 9-hydroxyrisperidone were less than 13% at spiked concentrations

Table 1. Accuracy and imprecision of risperidone and 9-hydroxyrisperidone in plasma, determined from the assay of 4 seeded controls on each of 3 days (n = 12)

Concentration (ng/mL)	Imprecision[a]			Inaccuracy[b]
	Within-day	Between-day	Total	
Risperidone				
5	7.84	6.25	10.0	+12.0
80	3.26	3.30	6.63	+5.0
9-Hydroxyrisperidone				
5	9.33	8.29	12.5	+6.0
80	9.69	2.80	10.1	+10.0

[a] Expressed as CV%. [b] Expressed as %.

Table 2. Plasma concentrations of risperidone (R) and 9-hydroxyrisperidoine (OH-R) in children and adolescents

ID	Sex	Age (years)	Dose[a] (mg)	Postdose[b] (h)	Concentration		Comedication[c]
					R (ng/mL)	R-OH (ng/mL)	
1	M	13.9	1.5	2.17	<5.0	7.8	dex, srt, sln
2	F	13.7	1.0	15.25	8.0	9.7	fvx
3	M	10.4	2.0	3.25	32.1	8.7	dex, cln, irn
3	M	11.8	1.5	2.25	19.5	6.6	dex, cln, irn, omg
4	F	12.2	2.0	1.33	<5.0	14.6	amt
5	F	16.5	0.25	20.5	<5.0	<5.0	srt, ltd, pct
6	M	8.6	2.0	9.17	<5.0	28.4	cln, vlp, omz, flx
7	M	14.1	4.0	2.67	<5.0	14.4	mph, omg, vlr
8	M	13.1	4.0	2.33	12.4	18.8	mph, omg, vlr
9	M	14.4	2.0	8.0	<5.0	9.7	dex, omg, brh, bvt
10	M	12.2	2.0	8.08	<5.0	11.9	mph, cal
11	M	6.9	2.0	3.33	8.6	33.6	mph
12	M	12.3	0.5	7.5	<5.0	<5.0	dex, amt
13	M	13.7	0.5	10.0	5.1	<5.0	atx, srt
14	F	15.7	0.5	15.33	12.8	<5.0	fvx
15	F	13.9	3.0	12.5	<5.0	18.2	olz, phs
16	M	7.0	1.5	1.58	11.2	15.3	
17	M	8.0	2.0	0.67	<5.0	11.1	mph, cln
18	M	16.7	2.0	24.0	<5.0	14.7	
19	M	9.0	0.5	15.5	<5.0	7.3	dex
20	M	14.7	0.5	14.83	<5.0	5.4	tmz
21	M	13.0	0.5	21.25	<5.0	<5.0	omg, slb
22	M	15.1	1.5	5.67	<5.0	7.3	mph
23	M	16.3	3.0	9.0	<5.0	18.0	mph, cln, sal, crg
24	F	15.8	2.0	4.92	<5.0	13.8	mph, hom
25	M	10.8	1.5	0.25	<5.0	13.8	mph, amt
26	M	15.3	2.0	4.25	5.1	19.8	atx, srt, pct, cdn, ibf
27	M	9.0	1.0	1.25	<5.0	6.7	flt
28	M	7.9	1.0	7.42	5.9	<5.0	dex, cbz, vlp, esz, cln, clh, lmt
29	M	17.9	1.0	1.0	<5.0	9.7	

[a] Daily oral dose. [b] Time after dose. [c] Comedication code: amt, amitriptyline; atx, atomexetine; brh, brahmi (bacopa); bvt, b vitamins; cal, calcium compound; cbz, carbamazepine; cdn, codeine; clh, chloral hydrate; cln, clonidine; crg, cromoglycate; dex, dexamphetamine; esz, esomeprazole; flt, fluoxetine; flx, flixotide; fvx, fluvoxamine; hom, homeopathy; ibf, ibuprofen; irn, iron supplements; ltd, loratidine; lmt, lamotrigine; mph, methylphenidate; olz, olanzapine; omg, omega-3-fatty acids; omz, omeprazole; pct, paracetamol; phs, phosphate salts; sln, selenium compound; slb, salbutamol; srt, sertarline; tmz, temazepam; vlp, valproate; vlr, valerian/hops.

of 5 and 80 ng/mL, indicating acceptable imprecision (Table 1). Inaccuracy was less than 12% at a spiked concentration of 5 ng/mL for both risperidone and 9-hydroxyrisperidone. At 80 ng/mL, the inaccuracy was less than 10% for both compounds.

The limit of detection, determined as three times the baseline noise, was 1.5 ng/mL. The lower limit of quantification (LLOQ), as determined by inaccuracy and imprecision data, was set *a priori* to 5 ng/mL.

Table 2 shows risperidone and 9-hydroxyrisperidone concentrations in plasma samples obtained during administration of risperidone tablets to 29 children and adolescents with significant emotional and behavioural disorders. There was no apparent

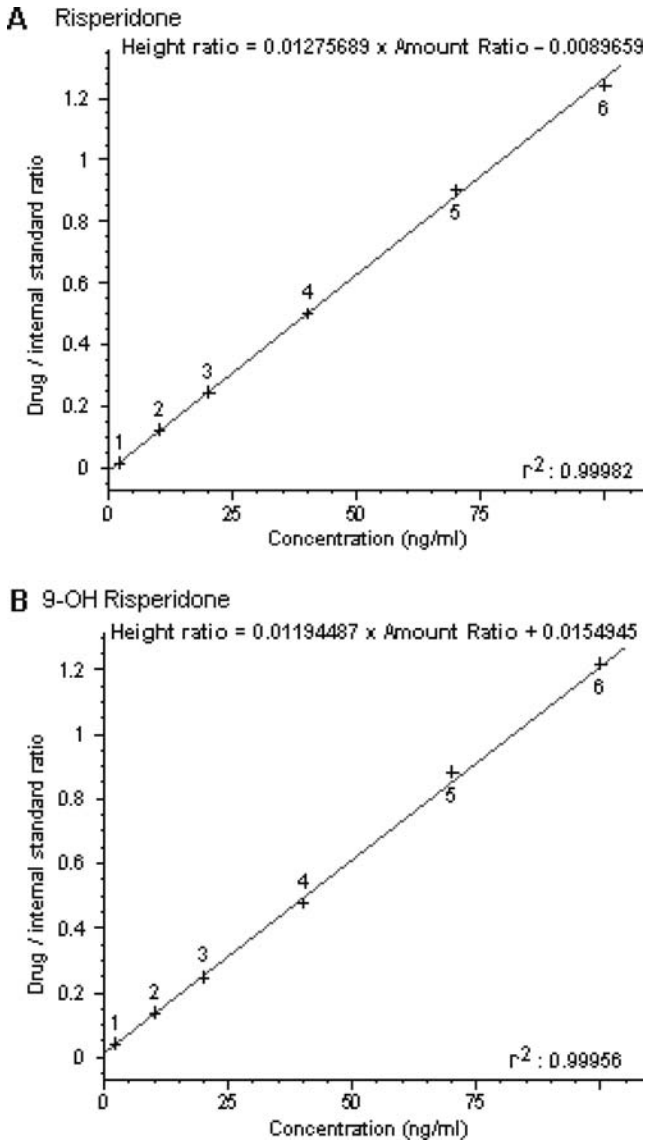

Figure 3. Calibration plots of peak height ratio vs concentration for: (A) risperidone; (B) 9-hydroxyrisperidone.

interference from co-prescribed medication, including a number of other antipsychotics, psychostimulants and antidepressant agents.

Discussion and Conclusions

The HPLC method presently described for the determination of plasma risperidone and 9-hydroxyrisperidone offers considerable improvements over previously published methods. Most existing methods had much longer run times than the 7 min in the current method. Although two papers claimed very short run-times of 4–5 min (Avenoso *et al*., 2000; Titier *et al*., 2002), we were unable to reproduce these methods because of poor resolution of 9-hydroxyrisperidone from the solvent front and early eluting peaks. Our sample workup involved a single-step liquid–liquid extraction, which compares favourably from a cost perspective with methods that use solid-phase extraction (Nagasaki *et al*., 1999; Price and Hoffman, 1997; Schatz and Saria, 2000), or multi-step liquid–liquid extraction (Woestenborghs *et al*., 1992; Aravagiri *et al*., 1993; LeMoing *et al*., 1993). Solid-phase extraction is more expensive than liquid–liquid extraction due to the cost of the extraction cartridges, while multiple extractions are often lengthy, labour-intensive and have reduced sensitivity because of losses at each step.

The present method used methyl-risperidone, an analogue of risperidone, as the internal standard. Some published methods also used other common psychotropic agents such as haloperidol, clozapine or trazodone as the internal standard (Olesen and Linnet, 1997; Nagasaki *et al*., 1999; Avenoso *et al*., 2000). This presents two problems in that these compounds are not structurally related to risperidone and, more importantly, they cannot be used if patients are co-prescribed these agents with risperidone. Since methyl-risperidone is not used clinically, it has a marked advantage in this regard together with similar solubility, chromatographic behaviour and UV spectral characteristics to risperidone.

The present method is currently being applied to a population pharmacokinetic and pharmacodynamic study of paediatric patients receiving ongoing treatment with oral risperidone within a large metropolitan children's hospital. As reported in adult schizophrenic patients (Arvagiri *et al*., 2003), there was marked pharmacokinetic variability in our paediatric patients' data as seen by the widely differing concentrations measured in samples drawn at the same time after dosing in patients of similar ages on similar dosing regimens. In 22/28 samples, the 9-hydroxyrisperidone concentrations exceeded the corresponding risperidone concentrations at each sampling time. This finding is consistent with published data which show that, for normal metabolizers of risperidone, the half-life of the metabolite is longer than for the parent drug (Vermeulen *et al*., 2007). While this variability may have biopharmaceutical or pharmacogenetic causes, there is the possibility of poor medication compliance contributing to variability, which makes this assay suitable for risperidone plasma concentration monitoring as recently recommended (Hiemke, 2008).

In conclusion, the HPLC method described for determination of risperidone and its active metabolite 9-hydroxyrisperidone in human plasma is rapid, selective, accurate and precise. In the clinic, this method facilitates rapid turnaround from sampling to result reporting for compliance monitoring of risperidone therapy in outpatients, but also for investigations of clinical efficacy and adverse effects.

Acknowledgements

We thank Janssen-Cilag Pty Ltd (North Ryde, NSW, Australia) for supplying risperidone, methyl-risperidone and 9-hydroxyrisperidone reference materials. This research was generously supported by research grants from the Society of Hospital Pharmacists of Australia, and the Mater Children's Hospital Golden Casket Research Fund. A.D. is supported by a Research Fellowship provided by the Australian National Health and Medical Research Council.

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