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Determination of risperidone and its major metabolite 9-hydroxyrisperidone in human plasma by reversed-phase liquid chromatography with ultraviolet detection

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Abstract

A simple and sensitive high-performance liquid chromatographic (HPLC) method with UV absorbance detection is described for the quantitation of risperidone and its major metabolite 9-hydroxyrisperidone in human plasma, using clozapine as internal standard. After sample alkalinization with 1 ml of NaOH (2 *M*) the test compounds were extracted from plasma using diisopropyl ether–isoamylalcohol (99:1, v/v). The organic phase was back-extracted with 150 μl potassium phosphate (0.1 *M*, pH 2.2) and 60 μl of the acid solution was injected into a C_{18} BDS Hypersil analytical column (3 μm, 100×4.6 mm I.D.). The mobile phase consisted of phosphate buffer (0.05 *M*, pH 3.7 with 25% H_3PO_4)–acetonitrile (70:30, v/v), and was delivered at a flow-rate of 1.0 ml/min. The peaks were detected using a UV detector set at 278 nm and the total time for a chromatographic separation was about 4 min. The method was validated for the concentration range 5–100 ng/ml. Mean recoveries were 98.0% for risperidone and 83.5% for 9-hydroxyrisperidone. Intra- and inter-day relative standard deviations were less than 11% for both compounds, while accuracy, expressed as percent error, ranged from 1.6 to 25%. The limit of quantitation was 2 ng/ml for both analytes. The method shows good specificity with respect to commonly prescribed psychotropic drugs, and it has successfully been applied for pharmacokinetic studies and therapeutic drug monitoring.

Keywords: Risperidone; 9-Hydroxyrisperidone

1. Introduction

Risperidone is an atypical antipsychotic which acts through selective antagonism of serotonin 5-HT2 and dopamine D2 receptors [1]. It is effective in the treatment of both positive and negative symptoms of schizophrenia, and it has a lower potential to cause extrapyramidal side effects compared to classical antipsychotics [2]. Recent evidence from open trials suggests that risperidone may be also beneficial in the treatment of schizoaffective and bipolar disorders [3,4].

Risperidone undergoes extensive 9-hydroxylation in the liver by cytochrome P450 isoenzymes (CYP), with *N*-dealkylation and 7-hydroxylation being less important pathways (Fig. 1) [5]. The formation of

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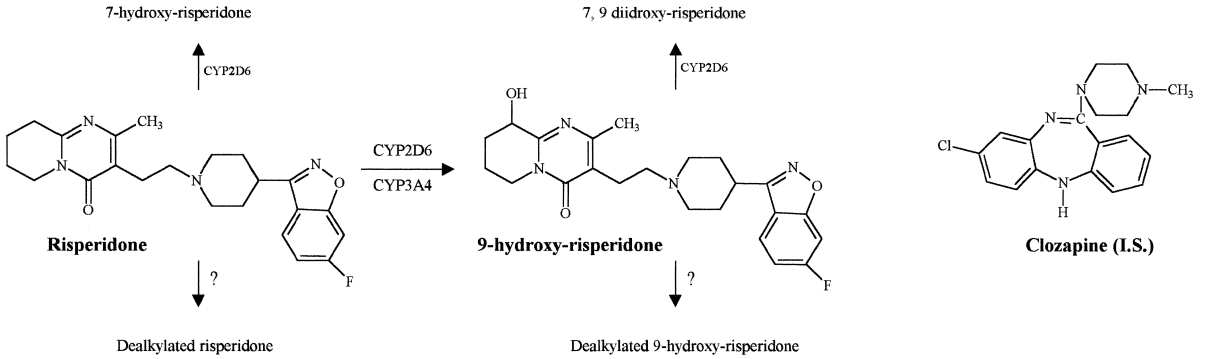

Fig. 1. Chemical structures of risperidone, 9-hydroxyrisperidone and clozapine (I.S.), and major pathway of risperidone metabolism.

9-hydroxyrisperidone is predominantly catalysed by CYP2D6 [6], though recent in vitro data suggest the additional involvement of CYP3A4 [7]. As the pharmacological activity of 9-hydroxyrisperidone is considered to be similar to that of the parent compound, the sum of their plasma concentrations is often referred to as the ''active moiety'' [8]. Factors which are known to influence the plasma levels of the active moiety include age, impaired renal and hepatic function, CYP2D6 genotype and concomitant administration of other drugs [9–14]. Though no clear correlation between plasma concentrations and clinical effects has been documented [13–15], the monitoring of plasma risperidone and 9-hydroxyrisperidone may be of value in selected situations.

To date, few assays for the determination of risperidone and 9-hydroxyrisperidone in human plasma or serum have been reported, including a specific radioimmunoassay (RIA) [16] and high-performance liquid chromatography (HPLC) analysis with either ultraviolet [17,18] or electrochemical [19–22] detection after liquid–liquid extraction [17–19,21,22] or solid-phase extraction [20]. Some of the HPLC methods have low limits of detection and quantitation, but they are also time-consuming. In particular, they include a multi-step liquid–liquid extraction for sample cleaning, which involve long procedures and/or reduced recovery [17,19,21]. Some assays require long chromatographic runs [19,20,22].

In the present article we describe an innovative simple, rapid and sensitive HPLC method for the simultaneous determination of risperidone and 9-hydroxyrisperidone in plasma using a simple liquid–liquid extraction. The assay involves a short chromatographic run and fulfils the requirements for use in therapeutic drug monitoring. The method has been successfully applied in pharmacogenetic studies [11].

2. Experimental

2.1. Chemicals

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, and 9-hydroxyrisperidone were purchased from the Janssen Research Foundation (Beerse, Belgium). Clozapine was kindly donated by Sandoz (Basle, Switzerland). Potassium phosphate monobasic was obtained from Carlo Erba (Milan, Italy), triethylamine and sodium hydroxide from Sigma–Aldrich (Milan, Italy), isoamylalcohol, acetonitrile, diisopropyl ether and water from J.T. Baker (Deventer, The Netherlands). Water was deionized and purified by using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Drug solutions

Stock solutions of risperidone, 9-hydroxyrisperidone and internal standard (clozapine) were prepared by dissolving an appropriate amount of each compound in methanol to yield a concentration of 2 mg/ml. Working standard solutions of risperidone (10 μg/ml), 9-hydroxyrisperidone (10 μg/ml) and clozapine (20 μg/ml) were obtained by diluting the stock solutions with KH_2PO_4 (0.1 *M*, pH 2.2 with 25% orthophosphoric acid). Stock solutions

and working solutions were stable at −20°C for up to 4 months and at 4°C for up to 1 month, respectively. Drug-free plasma from healthy donors was used for the validation studies. Calibrators were prepared by spiking known amounts of working standard solutions in 1 ml blank plasma to yield the final concentrations of 5, 10, 30, 60, 100 ng/ml for each analyte. The concentration of the internal standard in each calibrator was 50 ng/ml. Standard curves were prepared daily and constructed by linear regression analysis of the analyte/internal standard peak-height ratio versus the respective concentration of the analyte in the calibrators.

2.3. Extraction procedure

Blood samples were collected in heparinized tubes and centrifuged immediately at 3000 *g* for 10 min. The plasma was stored at −20°C until analysis. A 10-μl volume of clozapine (20 μg/ml) as internal standard (I.S.) and 1 ml NaOH (2 *M*) were added to 1 ml of plasma. The tubes were vortex-mixed for 10 s and 4 ml of diisopropyl ether–isoamylalcohol (99:1, v/v) was added as extraction solvent. After 10 min shaking, the mixture was centrifuged at 3000 *g* for another 10 min at 4°C and the organic phase was transferred to tubes containing 150 μl of KH_2PO_4 (0.1 *M*, pH 2.2 with 25% H_3PO_4), mixed for 5 min at 250 cycles/min, and centrifuged at 3000 *g* for 2 min. The upper organic layer was carefully aspirated and 1 ml of diethyl ether was added. After gentle mixing, the ether phase was eliminated and a 60-μl aliquot of the remaining acid solution was injected into the HPLC system.

2.4. Apparatus

The chromatographic equipment was purchased from Shimadzu (Kyoto, Japan) and consisted of a Model LC-10ADvp dual reciprocating pump coupled to a manual injector (Rheodyne 7725i) with a 50-μl fixed loop, a Model SPD-10AV UV–Vis detector with variable-wavelength set at 278 nm and a Model SCL-10A System Controller. The apparatus was connected to a personal computer (Digital Venturis FX 5166s) with a CLASS-VP 4.1 chromatography data system software (Shimadzu).

2.5. Chromatographic conditions

Chromatographic separations were carried out on a C_{18} BDS-Hypersil analytical column (3 μm, 100× 4.6 mm I.D.). The mobile phase of potassium dihydrogenphosphate (0.05 *M*, pH 3.7 with 25% H_3PO_4)–acetonitrile (70:30, v/v) was delivered at a flow-rate of 1.0 ml/min. Triethylamine (0.3%) was added to suppress the ionization of solutes. The mobile phase was filtered using 0.45-μm filters (Alltech, Milan, Italy) in a Millipore solvent filtration apparatus (Millipore, Molsheim, France) and never recirculated. The run-time of the assay was 4 min and retention times for 9-hydroxyrisperidone, risperidone and clozapine (I.S.) were 2.0, 2.4 and 3.7 min, respectively.

2.6. Calculations

Concentration values were obtained by using a CLASS-V 4.1 chromatography data system software. The calibrators, together with a blank, were taken through the extraction and HPLC procedures described above, and calibration curves were constructed by plotting the analyte/internal standard peak-height ratio as a function of the concentration of the calibrators.

3. Results

3.1. Chromatography

A representative chromatogram of an unextracted working aqueous solution (0.1 *M* KH_2PO_4 pH 2.2 in 25% H_3PO_4) containing 9-hydroxyrisperidone, risperidone and clozapine (internal standard) is shown in Fig. 2A. The analytical column allowed satisfactory separation of about 500 samples.

The short retention times for both risperidone and 9-hydroxyrisperidone may be due to the lower pH of the mobile phase compared with that used in all other published methods (3.7 vs. 6.5–7.0 range). The retention time of the internal standard clozapine was highly dependent on the pH of the mobile phase and faster elution could be achieved by adjusting the pH value from 3.7 to 3.0, with retention times decreasing from 3.7 to 2.9 min without changes in sensitivi-

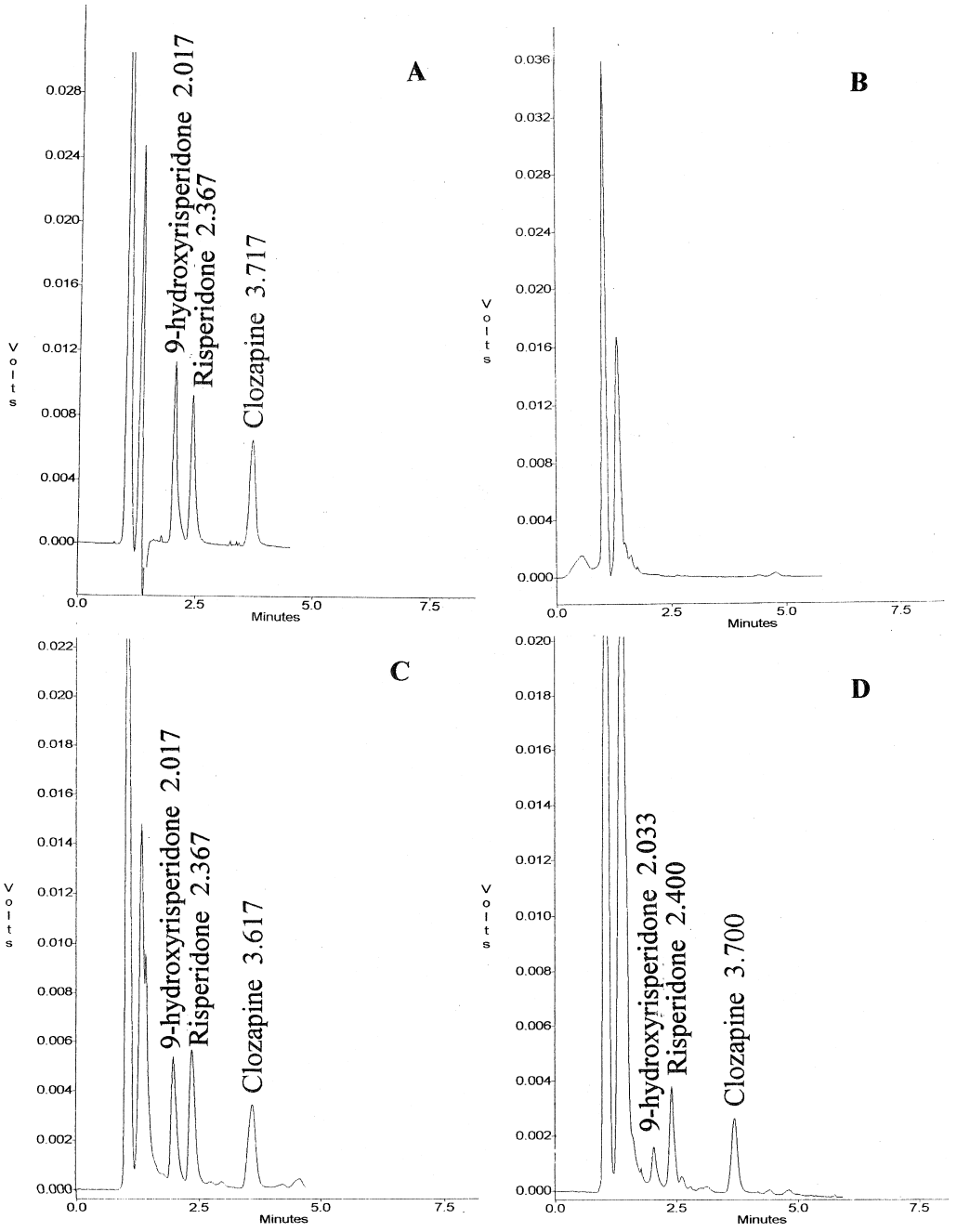

Fig. 2. Representative chromatograms of (A) pure standard of risperidone, 9-hydroxyrisperidone and clozapine in 0.1 *M* KH_2PO_4 injected without extraction; (B) blank plasma sample; (C) plasma spiked with 60 ng for risperidone and its metabolite and 50 ng for clozapine; (D) plasma from patient administered 6 mg of risperidone per day. Calculated concentrations were 48 ng/ml for risperidone and 14 ng/ml for 9-hydroxyrisperidone.

ty. Under the same conditions, the retention time of risperidone decreased slightly and that of 9-hydroxyrisperidone remained unchanged, but this resulted in poorer resolution of the two analytes. Increasing the pH from 3.7 to 4.4 led to a longer retention time for clozapine (4.2 min) without any effect on the retention time of risperidone and 9-hydroxyrisperidone. A similar effect of pH on clozapine retention time has previously been reported [25]. Lowering the pH to below 3 impaired the chromatographic resolution.

The chromatogram of an extracted blank plasma sample is shown in Fig. 2B, while the chromatogram of an extracted sample spiked with risperidone (60 ng/ml), 9-hydroxyrisperidone (60 ng/ml), and clozapine (50 ng/ml) is shown in Fig. 2C. All compounds were well separated from one another and from the solvent peaks. The chromatogram of an extracted plasma sample obtained from a patient receiving 6 mg/day of risperidone did not show additional peaks which could interfere with the quantitation of risperidone or 9-hydroxyrisperidone (Fig. 2D).

3.2. Recovery and linearity

Recovery from plasma was calculated by comparing, in replicates of five, the peaks height of pure standards prepared in 0.1 *M* KH_2PO_4 (pH 2.2 with 25% H_3PO_4) and injected directly into the analytical column with those of extracted plasma samples containing the same amount of the test compounds. Recoveries were determined at five different concentrations ranging from 5 to 100 ng/ml (Table 1).

Calibration curves were linear over the concentrations range of 5–100 ng/ml. The linearity curves were defined by the following equations: $y=0.0182$ (± 0.00096)$x+0.0075$ (± 0.00066) ($r=0.998\pm 0.005$) for risperidone and $y=0.0174$ (± 0.0015)$x-0.0056$ (± 0.00035) ($r=0.999\pm 0.002$) for 9-hydroxyrisperidone, where y is the peak-height ratio of analyte to internal standard and x the drug concentration (Fig. 3). The slope and y-intercept values introduced in the equations represent the mean ($\pm$SD) of five different curves injected in the same day. The relative standard deviations (RSDs) of the two parameters obtained by analyzing three curves in five different days were 6.7 and 9.8% for risperidone and 5.3 and 10.7% for 9-hydroxyrisperidone, respectively.

Table 1
Extraction recovery of the three analytes from plasma

Analyte	Concentration added (ng/ml)	Recovery (%) (mean±SD, $n=5$)
Risperidone	5	95.6±4.1
	10	99.9±5.5
	30	96.5±5.1
	60	100.2±7.8
	100	97.8±4.8
9-Hydroxyrisperidone	5	91.8±6.7
	10	93.2±1.7
	30	82.0±1.8
	60	77.0±2.7
	100	73.6±1.9
Clozapine	50	90.2±1.5

3.3. Precision and accuracy

Intra- and inter-day precision and accuracy (Table 2) were evaluated by assaying blank plasma spiked with five different concentrations of risperidone and 9-hydroxyrisperidone. Intra- and inter-day precision were assessed by analyzing five samples at three concentrations (10, 60 and 100 ng/ml) and 10 samples at two concentrations (5 and 30 ng/ml) for 5 days, respectively. Accuracy was expressed as percent error [(measured concentration−spiked con-

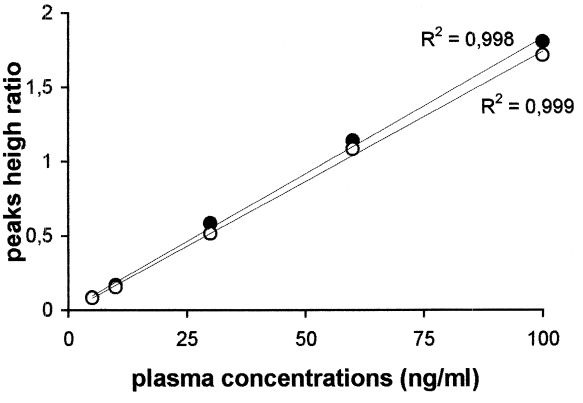

Fig. 3. Linear regression curves of (●) risperidone and (○) 9-hydroxyrisperidone, determined by plotting peak height ratio (analyte/internal standard) vs. spiked concentration in the calibrators.

Table 2
Precision and accuracy for the determination of risperidone and 9-hydroxyrisperidone in spiked plasma

Spiked value (ng/ml)	*n*	Measured value (ng/ml) (mean±SD)	Intra-day ($n=5$) (RSD, %)	Inter-day ($n=10$) (RSD, %)	Relative error (%)
Risperidone					
5	10	5.1±0.3		5.8	+2.0
10	5	10.3±1.1	10.6		+3.0
30	10	29.5±2.4		8.1	−1.6
60	5	57.2±1.9	3.3		−4.6
100	5	98.0±10.0	10.2		−2.0
9-Hydroxyrisperidone					
5	10	4.3±0.3		6.9	−14.0
10	5	8.8±0.3	3.4		−12.0
30	10	23.5±0.9		3.8	−21.6
60	5	47.0±2.1	4.4		−22.0
100	5	75.0±1.6	2.1		−25.0

centration)/spiked concentration]×100 (%), while precision was quantitated by calculating intra- and inter-day RSDs.

3.4. Limit of quantitation and limit of detection

The limit of detection (LOD), defined as the concentration corresponding to three times the level of baseline noise, was 1 ng/ml for both analytes. The lowest limits of quantitation (LOQs), expressed at the lowest concentrations at which percent deviation from the nominal concentration (accuracy) and RSD (precision) are less than 20%, were determined from the linearity tests and found to be 2 ng/ml for both analytes.

3.5. Specificity

Potential interference with determinations was investigated by assaying different blank plasma samples spiked with psychoactive drugs commonly co-administered with risperidone (Table 3). No significant chromatographic interference was found. Fig. 4 shows the chromatogram of a plasma sample containing some of the tested drugs (1 μg/ml), risperidone (0.1 μg/ml), 9-hydroxyrisperidone (0.1 μg/ml), and clozapine (0.05 μg/ml). All tricyclic antidepressants tested, mianserin, haloperidol, carbamazepine, some selective serotonin reuptake inhibitors (citalopram, paroxetine and fluvoxamine) were co-extracted by the procedure. Fluoxetine, sertraline, fenobarbital, lorazepam and the neuroleptics zuclopenthixol and fluphenazine were not extracted at all.

4. Discussion

Compared with other published methods, the HPLC assay described in this article is faster and simpler with regard to both extraction procedure and chromatographic analysis. The assay has been successfully applied to the evaluation of the steady-state plasma levels of risperidone and 9-hydroxyrisperidone in schizophrenic patients on monotherapy with risperidone, 4–8 mg/day [11]. Electrochemical detection has been preferred by other authors to achieve lower quantitation limits compared to those obtained with UV detection, especially for risperidone [19–22], but the use of electrochemical detectors in a routine laboratory setting is more demanding and requires greater care with respect to cleaning of glassware and apparatus. In any case, the sensitivity of our method, based on UV detection, is fully adequate to quantitate the plasma concentrations of risperidone and 9-hydroxyrisperidone after the administration of usual daily dosages (4–8 mg/day). Olesen and Linnet [18] also considered a quantitation limit of 2 ng/ml for risperidone and for 9-hydroxyrisperidone as adequate for the measure-

Table 3
Retention times (min) of extracted and unextracted compounds tested for interference

Extracted compounds	Unextracted compounds	Retention time (min)
Risperidone		2.05
9-Hydroxyrisperidone		2.4
Clozapine		3.6
Tricyclic antidepressants (TCAs)		
Clomipramine		5.0
Desipramine		7.9
Imipramine		8.6
Nortriptyline		9.3
Selective serotonin reuptake inhibitors (SSRIs)		
Citalopram		4.4
Fluvoxamine		7.5
Paroxetine		7.7
	Sertraline	15.0
	Fluoxetine	18.0
Atipycal antidepressants		
Mianserin		4.3
Benzodiazepines		
	Lorazepam	8.1
Neuroleptics		
	Fluphenazine	3.5
	Zuclopenthixol	5.1
Haloperidol		5.8
Antiepileptic drugs		
	Fenobarbital	3.1
Carbamazepine		5.5

ment of plasma concentrations in the therapeutic range.

The procedure used to extract risperidone and 9-hydroxyrisperidone was fast and efficient and ensured better recoveries (Table 1) than many previously published methods [17–19,21]. Price and Hoffman [20] reported a higher recovery for 9-hydroxyrisperidone (93.6%), but a lower recovery for risperidone (94.6%), while the reverse was found by Aravagiri et al. [22], who reported recoveries of 89.7% for 9-hydroxyrisperidone and 94.5% for risperidone. In our assessment of different extraction solvents, diisopropyl ether–isoamylalcohol (99:1, v/v) provided the best recoveries for the two analytes and for the internal standard clozapine. Increasing the percentage of the more polar solvent isoamylalcohol led to a higher recovery for 9-hydroxyrisperidone, but to a poorer recovery of the internal standard. Although the recovery of 9-hydroxyrisperidone falls at higher concentrations (Table 1), probably because the saturation point is being approached, at risperidone doses of approximately 6 mg/day, which are considered the usual therapeutic doses for most patients, mean plasma concentrations of this metabolite have been reported to be 40 ± 25 ng/ml [22], 34 ± 17 ng/ml [23] and 39 ± 18 ng/ml [24] which are lower than the higher concentrations of our standard curve. The influence of extraction mixture polarity on clozapine extraction from alkalinized plasma has already been described [25]. Using diethyl ether during the last step of the extraction procedure, we could obtain a cleaner sample with less baseline noise during the chromatographic analysis.

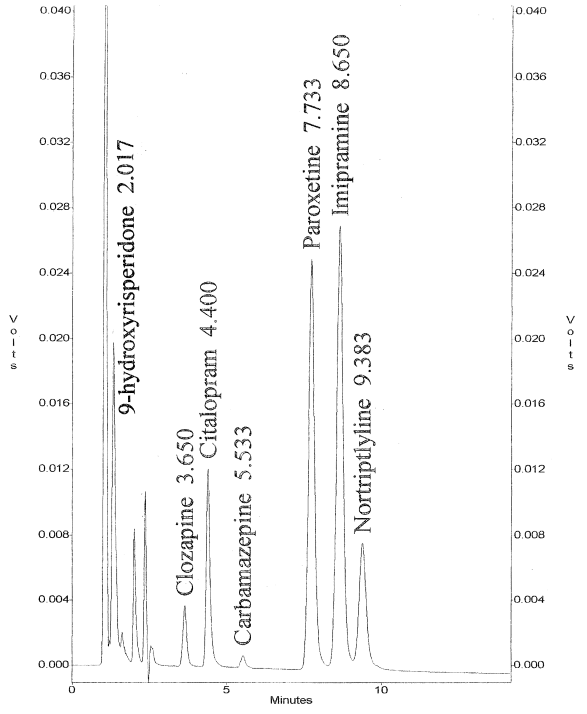

Fig. 4. Chromatogram after extraction of a plasma sample spiked with 1 μg/ml of different compounds tested for potential interference, 100 ng/ml risperidone, 100 ng/ml 9-hydroxyrisperidone and 50 ng/ml clozapine.

For the chromatographic separation two C_{18} columns were tested, including an Ultremex (3 μm, 100×4.6 mm) and a Hypersil BDS (3 μm, 100×4.6 mm) column. The latter was found to ensure a better resolution an a shorter separation time (4 min vs. 6 min), and it was therefore selected for the validation studies. Clozapine was selected as an internal standard because of its favorable extraction properties, short retention time and elution in an area of the chromatogram which is free from interfering peaks. Methyl-risperidone was also tested as a potential internal standard. Although it is chemically related to risperidone, this compound showed a longer retention time (5.7 min) and was susceptible to interference by co-eluting peaks.

Since patients treated with risperidone may receive additional medication, the potential for interference with other commonly used psychotropic drugs was evaluated, and none of the agents tested was found to interfere. Obviously, since our assay utilizes clozapine as internal standard, it cannot be applied to the determination of risperidone and 9-hydroxyrisperidone in patients receiving risperidone and clozapine co-medication. The latter drug association, however, is rarely used in clinical practice.

In conclusion, the assay described above has several advantages, being fast, precise and specific. Although the use of electrochemical detector may improve the limit of quantitation by a factor of 5 [19], the quantitation limit obtained with the use of UV detection was found to be adequate for therapeutic drug monitoring purposes.

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References

[1] J.E. Leysen, W. Gommeren, A. Eens, D. de Chaffoy de Courcelles, J.C. Stoof, P.A.J. Janssen, J. Pharmacol. Exp. Ther. 247 (1988) 661.
[2] G. Chouinard, W. Arnott, Can. J. Psychiatry 38 (Suppl. 3) (1993) S89.
[3] P.E. Kech Jr., S.L. McElroy, S.M. Strakowski, J. Clin. Psychiatry 57 (Suppl. 9) (1996) 41.
[4] M. Tohen, C.A. Zarate Jr., J. Clin. Psychiatry 59 (Suppl. 1) (1998) 38.
[5] G. Mannens, M.L. Huang, W. Meuldermans, J. Hendrickx, R. Woestenborghs, J. Heykants, Drug Metab. Dispos. 21 (1993) 1134.
[6] M.L. Huang, A. van Peer, R. Woestenborghs, R. De Coster, J. Heykants, A.A.I. Jansen, Z. Zylicz, H.W. Visscher, J.H.G. Jonkman, Clin. Pharmacol. Ther. 54 (1993) 257.
[7] J. Fang, M. Bourin, G.B. Baker, Naunyn-Schmiederberg's Arch. Pharmacol. 359 (1999) 147.
[8] L.E.C. Van Beijsterveldt, R.J.F. Geerts, J.E. Leysen, A.A. Megens, H.M. Van den Eynde, W.E. Meuldermans, J.J. Heykants, Psychopharmacology 114 (1994) 53.
[9] E. Snoeck, A. Van Peer, M. Sack, M. Horton, G. Mannens, R. Woestenborghs, R. Meibach, J. Heykant, Psychopharmacology 122 (1995) 223.
[10] D.W. Hoffman, M.S.T. Winer, S.J. Bartels, Pharmacotherapy 15 (1995) 382.
[11] M.G. Scordo, E. Spina, G. Facciolà, A. Avenoso, I. Johansson, M.L. Dahl, Psychopharmacology 147 (1999) 300.
[12] L. Ereshefsky, J. Clin. Psychiatry 57 (Suppl. 11) (1996) 12.

[13] O.V. Olesen, R.W. Licht, E. Thomsen, T. Bruun, J.E. Viftrup, K. Linnet, Ther. Drug Monit. 20 (1998) 380.
[14] G. Bondolfi, H. Doufour, M. Patris, J.P. May, U. Billeter, C.B. Eap, P. Baumann, Am. J. Psychiatry 155 (1998) 499.
[15] H.S. Lee, C.H. Tan, Y.M. Khoo, Br. J. Clin. Pharmacol. 47 (1999) 459.
[16] R. Woestenborghs, I. Geuens, D. Van Roosbroeck, L. Cornelissen, F. Van Rompaey, F. Knaeps, J. Heykants, Janssen Preclinical Research Report R 64766/24, Janssen, Beerse, August 1990.
[17] R. Woestenborghs, W. Lorreyne, F. Van Rompaey, J. Heykants, J. Chromatogr. 583 (1992) 223.
[18] O.V. Olesen, K. Linnet, J. Chromatogr. 698 (1997) 209.
[19] J.P. Le Moing, S. Edouard, J.C. Levron, J. Chromatogr. 614 (1993) 333.
[20] M.C. Price, D.W. Hoffman, Ther. Drug Monit. 19 (1997) 333.
[21] A.E. Balant-Giorgia, M. Gex-Fabry, C. Genet, L.P. Balant, Ther. Drug. Monit. 21 (1999) 105.
[22] M. Aravagiri, S.R. Morder, D. Wirshing, W.C. Wirshing, Pharmacopsychiatry 31 (1998) 102.
[23] J.K. Darby, D.J. Pasta, L. Elfand, L. Dabiri, L. Clark, J. Herbert, J. Clin. Psychopharmacol. 17 (1997) 478.
[24] H.S. Lee, C.H. Tan, Y.M. Khoo, K.T. Chee, K.E. Wong, S.A. Chong, R. Mahendran, H.L. Yap, B.L. Low, C.H. Choo, A. Chan, Br. J. Clin. Pharmacol. 47 (1999) 459.
[25] A. Avenoso, G. Facciolà, G.M. Campo, A. Fazio, E. Spina, J. Chromatogr. B 714 (1998) 299.