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Determination of risperidone and 9-hydroxyrisperidone in plasma, urine and animal tissues by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method has been developed for the simultaneous determination of the new antipsychotic risperidone and its major metabolite 9-hydroxyrisperidone in plasma, urine and animal tissues. The alkalinized plasma samples were extracted with ethyl acetate and further purified prior to reversed-phase chromatography with ultraviolet detection at 280 nm. The method could also be applied to urine samples and animal tissue homogenates. Quantification limits were 2 ng/ml for plasma and urine and 10 ng/g for animal tissue. The method was applied to pharmacokinetic studies in experimental animals, human volunteers and patients.

INTRODUCTION

Risperidone is a new antipsychotic with both serotonin-S_2 and dopamine-D_2 antagonistic properties [1–3]. In humans and experimental animals it undergoes hydroxylation at the tetrahydropyrido-pyrimidine moiety, yielding 9-hydroxyrisperidone as the major metabolite, which has a pharmacological activity similar to that of the parent compound [4].

Radioimmunoassay methods for the determination of risperidone and the sum of risperidone and 9-hydroxyrisperidone have been developed earlier [5]. The methods proved to be very helpful for the sensitive determination of the unchanged drug after low doses in volunteers and for the monitoring of chronically treated patients.

For the determination of risperidone and its hydroxy metabolite we have developed a specific high-performance liquid chromatographic (HPLC) method. The method has mainly been used to study the pharmacokinetics of the compound in experimental animals and its urinary excretion in humans.

EXPERIMENTAL

Standards and reagents

Risperidone (R 64766) or 3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl]ethyl]-6,7,8,9-tetrahydro-2-methyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one, 9-hydroxyrisperidone (R 76477) and the internal standards (R 68808, IS-I and R 76307, IS-II) were obtained as reference compounds from the Janssen Chemical Research Department. Their molecular structures are given in Fig. 1.

Stock solutions, corresponding to 0.1 mg/ml of methanol, were prepared for all compounds. Standard solutions were obtained by serially di-

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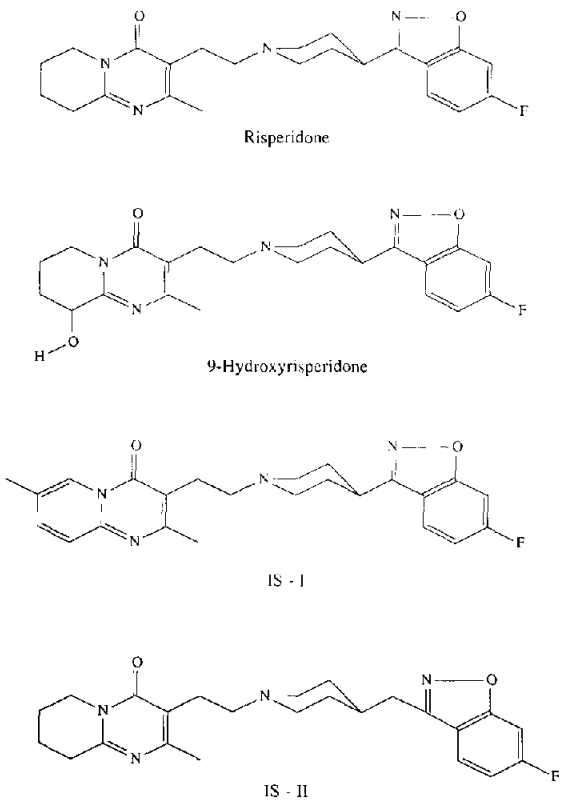

Fig. 1. Structures of risperidone, 9-hydroxyrisperidone and the internal standards (IS-I and IS-II).

luting the risperidone and 9-hydroxyrisperidone stock solutions with methanol to concentrations down to 0.020 μg/ml. The internal standard stock solution was diluted to a final concentration of 2 μg/ml. The stock solutions were stored in 20-ml glass screw-cap vials at −20°C for up to six months; the working standard solutions were stored in 10-ml glass test-tubes at 4°C for up to two months.

Acetonitrile (HPLC Far UV, Janssen Chimica, Geel, Belgium), methanol (Baker-analyzed, Baker, Deventer, Holland), *n*-heptane and ethyl acetate (Uvasol, Merck, Darmstadt, Germany) were of spectrophotometric grade. Diethylamine (Baker-analyzed, Baker) was of analytical grade. Isoamyl alcohol (3-methyl-1-butanol, Janssen Chimica) was redistilled in glass before use. The inorganic reagents were of analytical grade (Merck) and prepared in doubly distilled water.

Extraction procedures

To 1-ml aliquots of plasma or urine were added 200 μl of methanol (to match with standard curves), 100 μl of a suitable internal standard dilution, *e.g.* 2 μg/ml, and 1 ml of 0.05 *M* sodium borate decahydrate (borax). The samples were extracted twice with 4 ml of ethyl acetate using a rotary mixer (Cenco, Breda, Holland) at 10 rpm for 10 min. The organic layers were aspirated after centrifugation at 1000 g (Varifuge, Heraeus, Osterode, Germany) for 10 min, collected into 10-ml glass test-tubes and back-extracted with 3 ml of 0.05 *M* sulphuric acid. The aqueous layers were alkalinized with 150 μl of concentrated ammonia and finally re-extracted twice with 2.5 ml of heptane–isoamyl alcohol (90:10, v/v). The organic layers were combined into 5-ml glass centrifuge tubes and evaporated to dryness under nitrogen in a SC-3 sample concentrator (Techne, Cambridge, UK) at 60°C.

Animal tissues, ground in a Waring commercial blender, were homogenized (1:4, w/v) in distilled water using an Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, Germany). Aliquots of 1 ml of the homogenates were then pipetted into 10-ml glass test-tubes, spiked with 0.2 μg of internal standard, made alkaline by the addition of 1 ml of 0.10 *M* borax, and subsequently extracted using the procedure described above.

HPLC method

The extraction residues were redissolved in 80 μl of the HPLC solvent mixture, transferred to 0.2-ml conical polypropylene microvials, and 30-μl aliquots were injected into a Perkin-Elmer (Norwalk, CT, USA) Series 410 liquid chromatograph, equipped with a Perkin-Elmer Model ISS-100 autosampler and a Perkin-Elmer LC-235 diode-array detector operating at 280 nm. The separations were achieved on a reversed-phase column (15 cm × 2.1 mm I.D.), packed with 5 μm particle size ODS-Hypersil (Shandon, Cheshire, UK) by the balanced-density procedure by means of an air-driven fluid pump (Haskel, Burbank, CA, USA). The samples were eluted at ambient room temperature with water–acetonitrile–diethylamine (65:35:0.02) at a constant flow-rate of 0.8 ml/min.

Plotting of the chromatograms, area integrations and calculations were carried out by a Nelson Series 3000 chromatography data system (PE Nelson, Cupertino, CA, USA).

Calibration and calculation procedures

Using 100-μl aliquots of the standard solutions, 1-ml samples of blank plasma, urine or animal tissue homogenate were spiked with risperidone and 9-hydroxyrisperidone at concentrations ranging from 0.002 to 10 μg/ml, and with a constant internal standard (IS-I) concentration, *e.g.* 0.2 μg/ml. The calibration samples, together with a zero and a blank, were then taken through the extraction and HPLC procedures described above, and calibration curves were constructed by plotting the log-transformed peak-height or peak-area ratios (risperidone or 9-hydroxyrisperidone/internal standard) against the log-transformed risperidone or 9-hydroxyrisperidone standard concentrations. Final concentrations of unknowns were obtained by interpolation from these daily calibration curves.

RESULTS AND DISCUSSION

No chemical degradation occurred in the methanolic stock or standard solutions when stored as described. Although they are most probably usable for longer periods, the stock and standard solutions were not used for periods longer than six and two months, respectively.

The extraction recovery of risperidone (pK_{a1} = 8.24, pK_{a2} = 3.11, log P = 3.04) and 9-hydroxyrisperidone (pK_{a1} = 8.3; log P = 2.32) from plasma, urine and animal tissue homogenates was calculated from comparison between the peak areas of directly injected standards and standards submitted to different extraction procedures. Ethyl acetate proved to be very efficient for the initial extraction of the analytes from the biological matrix, but a back-extraction step using heptane–isoamyl alcohol was necessary to purify the extracts and, thereby, improve the sensitivity of the method. For risperidone, the overall extraction recovery averaged 75% for plasma and urine and ranged from 65 to 70% for the investigated animal tissues. For 9-hydroxyrisperidone, the extraction recoveries averaged 70% for plasma and urine and 60% for animal tissues.

Adequate chromatographic separation was obtained using a 2.1 mm I.D. reversed-phase column and water–acetonitrile as an eluent, to which 0.02% diethylamine (pH 10) was added to suppress the ionization of the solutes. Representative chromatograms of plasma, urine and animal tissue extracts are shown in Figs. 2–4. The peaks have good shape, and no major interfering peaks co-eluted with 9-hydroxyrisperidone, risperidone or the internal standard (IS-I). Retention times averaged 1.9, 2.9 and 5.4 min, respectively. Fluctuations in these absolute retention times, which can be seen in the depicted chromatograms, are caused by the specific column conditions and, to a lesser extent, by partial evaporation of the diethylamine. They were, however, found to be acceptable because of the use of an internal standard and the within-run analysis of both study samples and standards. The specificity of the method was further assessed by the injection of risperidone metabolites under the described chromatographic conditions. 7-Hydroxyrisperidone, a minor plasma metabolite of risperidone, eluted prior to 9-hydroxyrisperidone (relative retention time = 0.76) and the "short" metabolites, resulting from oxidative N-dealkylation at the piperidine moiety, eluted with the solvent front. On the other hand, interference problems did occur in plasma samples from patients receiving co-medication of *e.g.* benzodiazepines. Oxazepam (t_R = 1.5 min), desmethyldiazepam (t_R = 2.9 min) and diazepam (t_R = 5.1 min) interfered with the HPLC assay of 9-hydroxyrisperidone, risperidone or the internal standard. These interference problems could be partially solved by increasing the capacity factors of the analytes by using a more polar elution mixture, *e.g.* water–acetonitrile–diethylamine (75:25:0.02). In that case, a less lipophilic internal standard, *viz.* the 4-methylpiperidinyl analogue (IS-II) is used to shorten the total analysis time. But still, in some cases, interference from some co-medication occurred. As this may cause severe misinterpretation of risperidone or 9-hydroxyris-

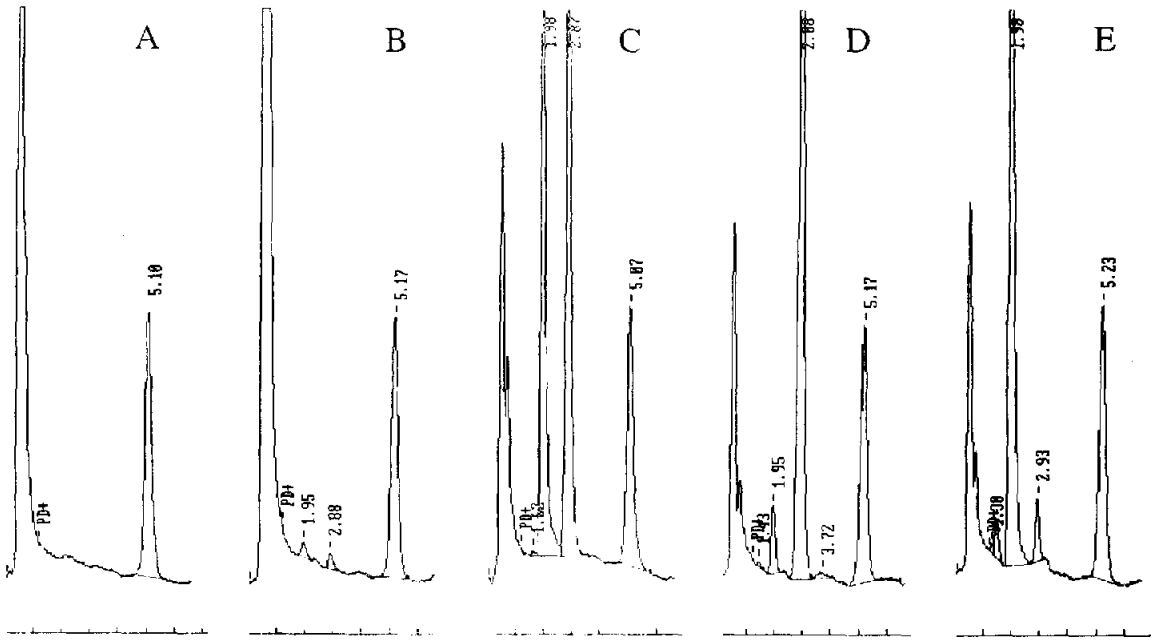

Fig. 2. Chromatograms of plasma extracts. (A) Control dog plasma spiked with 200 ng/ml internal standard IS-I (t_R = 5.10 min); (B) control dog plasma spiked with 5 ng/ml risperidone (t_R = 2.88 min), 5 ng/ml 9-hydroxyrisperidone (t_R = 1.95 min) and 200 ng/ml internal standard IS-I (t_R = 5.17 min); (C) control dog plasma spiked with 200 ng/ml risperidone (t_R = 2.87 min), 200 ng/ml 9-hydroxyrisperidone (t_R = 1.98 min) and 200 ng/ml internal standard IS-I (t_R = 5.07 min); (D) plasma from a dog, 10 min after a single oral administration of 0.63 mg/kg risperidone, containing 274 ng/ml risperidone (t_R = 2.88 min), 19.4 ng/ml 9-hydroxyrisperidone (t_R = 1.95 min) and 200 ng/ml internal standard IS-I (t_R = 5.17 min); (E) plasma from a dog, 4 h after a single oral administration of 0.63 mg/kg risperidone, containing 20.7 ng/ml risperidone (t_R = 2.93 min), 303 ng/ml 9-hydroxyrisperidone (t_R = 1.98 min) and 200 ng/ml internal standard IS-I (t_R = 5.23 min).

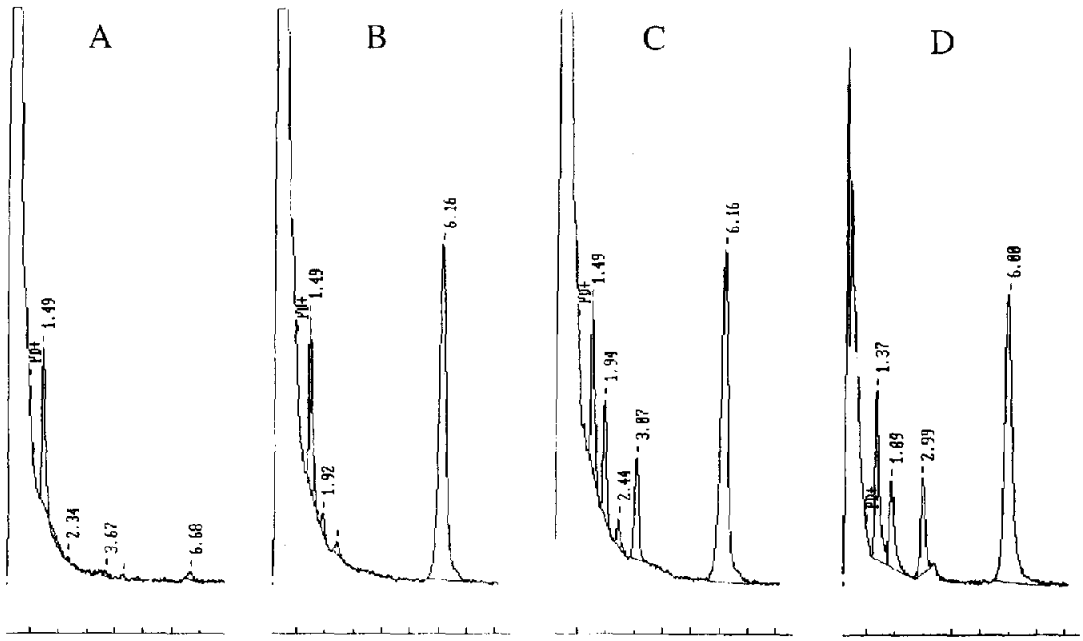

Fig. 3. Chromatograms of urine extracts. (A) Blank human urine; (B) control human urine spiked with 100 ng/ml internal standard IS-I (t_R = 6.16 min); (C) control human urine spiked with 10 ng/ml risperidone (t_R = 3.07 min), 10 ng/ml 9-hydroxyrisperidone (t_R = 1.94 min) and 100 ng/ml internal standard IS-I (t_R = 6.16 min); (D) urine from a volunteer, 2–4 h after a single oral administration of 1 mg of risperidone, containing 11.3 ng/ml risperidone (t_R = 2.99 min), 10.0 ng/ml 9-hydroxyrisperidone (t_R = 1.89 min) and 100 ng/ml internal standard IS-I (t_R = 6.00 min).

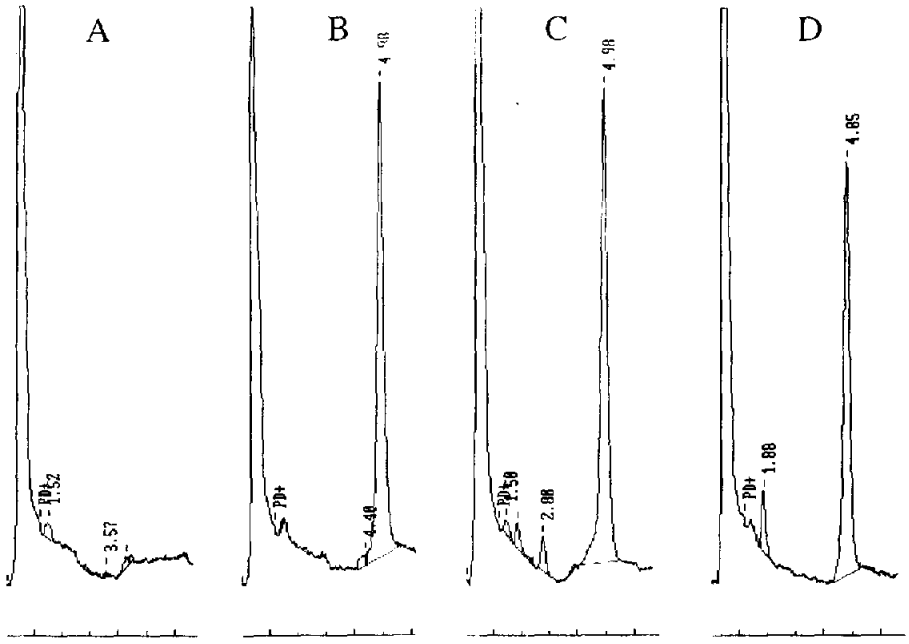

Fig. 4. Chromatograms of animal tissue extracts. (A) Blank dog muscle; (B) control dog muscle spiked with 1000 ng/g internal standard IS-I (t_R = 4.98 min); (C) control dog muscle spiked with 25 ng/g risperidone (t_R = 2.80 min), 25 ng/g 9-hydroxyrisperidone (t_R = 1.90 min) and 1000 ng/g internal standard IS-I (t_R = 4.98 min); (D) muscle from a dog, after chronic oral administration of 0.31 mg/kg risperidone per day during twelve months, containing 61.3 ng/g 9-hydroxyrisperidone (t_R = 1.88 min) and 1000 ng/g internal standard IS-I (t_R = 4.85 min); the risperidone concentration was below the detection limit.

peridone plasma levels in patients, radioimmunoassay, also because of its superior sensitivity, remains the method of choice for the monitoring of psychiatric patients.

Linear relationships ($r \geq 0.999$) were found when the peak-area or peak-height ratios of risperidone or 9-hydroxyrisperidone to the internal standard were plotted against the plasma concentrations on a log–log scale. Logarithmic transformation of the calibration data was used in order to achieve homoscedasticity, *i.e.* constant variance of the response variable over the large concentration range used [6]. Table I summarizes the mean regression parameters of the normal-

TABLE I

REGRESSION PARAMETERS FOR RISPERIDONE AND 9-HYDROXYRISPERIDONE STANDARD CURVES

The regression equation was $\log y = a \log x + b$, where y = peak-area or peak-height ratio and x = concentration ratio (risperidone or 9-hydroxyrisperidone/internal standard). Values are mean ± S.D. PAR: peak-area ratios; PHR: peak-height ratios.

Calculation method	Sample	*a*	*b*	*r*	*n*
Risperidone					
PAR	Plasma	1.0147 ± 0.0240	0.3082 ± 0.0336	0.9996 ± 0.0002	15
PHR	Urine	1.0170 ± 0.0211	0.5352 ± 0.0283	0.9997 ± 0.0002	8
PAR	Tissue	1.0014 ± 0.0154	0.3152 ± 0.0348	0.9994 ± 0.0004	7
9-Hydroxyrisperidone					
PAR	Plasma	1.0316 ± 0.0405	0.1837 ± 0.0525	0.9996 ± 0.0002	15
PHR	Urine	1.0397 ± 0.0432	0.4225 ± 0.0503	0.9995 ± 0.0003	8
PAR	Tissue	1.0532 ± 0.0276	0.1650 ± 0.0577	0.9990 ± 0.0005	7

ized standard curves obtained for both compounds. In order to compare standard curves with varying internal standard concentrations, concentration ratios rather than absolute concentrations are tabulated as the independent variable. In practice, separate series of standards were analysed together with each series of study samples, and these daily calibration curves were used for calibration and calculation purposes. Although peak-area ratios are used whenever possible, the peak-height ratio calibration method may offer some advantages in exceptional cases where accurate peak area integration is difficult, *e.g.* at very low concentrations or when interfering peaks show up.

The accuracy and precision of the method could be derived from the back-calculated standard concentrations and by replicate analyses of quality control plasma samples. The results for the back-calculated plasma standards are summarized in Table II. A varying number of replicates was obtained because, dependent on the application, variable concentration ranges were used in the different calibration curves. For risperidone, the mean coefficient of variation (C.V.) was 5.0% whereas the analytical recovery (accuracy) ranged from 97.9 to 103.8% of nominal. For 9-hydroxyrisperidone, a mean C.V. of 5.2% was found, and the analytical recovery ranged from 98.7 to 102.9%. Reliable results were thus obtained. even at the lower limit of detection, which appeared to be *ca.* 2 ng/ml for both compounds. Comparable results were obtained for the back-calculated urine and tissue homogenate

TABLE II

ACCURACY AND REPRODUCIBILITY OF THE HPLC METHOD FOR THE DETERMINATION OF RISPERIDONE AND 9-HYDROXYRISPERIDONE IN PLASMA SAMPLES

Concentration (ng/ml)		C.V. (%)	Accuracy (%)	*n*
Added	Found (mean ± S.D.)			
Risperidone				
2.0	2.1 ± 0.1	6.2	103.8	12
5.0	5.1 ± 0.3	6.5	101.3	25
10.0	10.0 ± 0.7	6.9	100.3	31
20.0	19.6 ± 1.0	4.9	97.9	23
50.0	49.8 ± 2.7	5.3	99.6	30
100	99.0 ± 5.0	5.1	99.0	33
200	199 ± 7	3.4	99.3	24
500	502 ± 15	3.0	100.3	31
1000	1021 ± 35	3.5	102.1	32
Mean/range		5.0	97.9–103.8	
9-Hydroxyrisperidone				
2.0	2.1 ± 0.1	5.5	102.9	3
5.0	5.0 ± 0.3	6.2	100.4	22
10.0	10.0 ± 0.6	5.8	100.5	26
20.0	19.8 ± 1.3	6.4	98.8	21
50.0	50.2 ± 3.9	7.7	100.4	29
100	99.5 ± 3.6	3.7	99.5	35
200	197 ± 8	4.0	98.7	20
500	496 ± 18	3.7	99.3	33
1000	1022 ± 37	3.7	102.2	34
Mean/range		5.2	98.7–102.9	

TABLE III

QUALITY CONTROL SAMPLES: STATISTICAL EVALUATION OF THE ANALYSIS RESULTS FOR RISPERIDONE AND 9-HYDROXYRISPERIDONE

Analyte	Concentration (ng/ml)	Within-assay precision		Between-assay precision			Overall precision		
		C.V. (%)	n	C.V. (%)	Relative error (%)	n	C.V. (%)	Relative error (%)	n
Risperidone	6.5	5.7	27	9.3	4.5	32	10.1	4.3	59
	81.1	3.3	34	5.4	2.3	39	6.2	2.3	73
9-Hydroxyrisperidone	12.7	5.5	5	14.3	−6.1	6	13.8	−7.8	11
	159	5.8	5	7.3	4.8	6	9.4	5.7	11

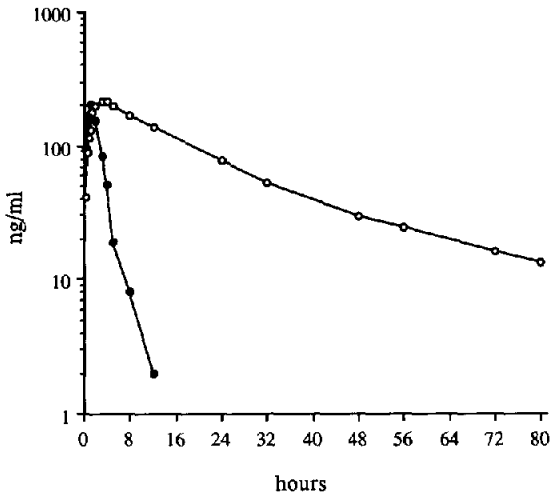

Fig. 5. Mean plasma concentrations (n = 4) of risperidone (●) and 9-hydroxyrisperidone (○) in beagle dogs following a single oral administration of risperidone at 0.31 mg/kg.

standards. The mean C.V.s for risperidone and 9-hydroxyrisperidone were 4.9 and 6.0% in urine samples and 5.7 and 6.4% in animal tissues. Detection limits were 2 ng/ml in urine and 10 ng/g in tissue.

Analysis results for the independently prepared quality control samples are summarized in Table III. For risperidone, the mean overall C.V. was 10.1% at 6.5 ng/ml, improving to 6.2% at 81.1 ng/ml. Mean relative errors were +4.3 and +2.3%, respectively. For 9-hydroxyrisperidone, the mean overall C.V.s were 13.8 and 9.4% at 12.7 and 159 ng/ml, respectively. Relative errors averaged −7.8 and +5.7%, respectively. Within-assay C.V.s were all below 6%.

The stability of risperidone and 9-hydroxyrisperidone in deep-frozen plasma could be studied from the quality control charts set up at the concentrations mentioned above. No deterioration occurred [slope not different from zero ($p > 0.05$) after regression analysis of the individual concentrations *versus* time] when the compounds were stored at −20°C for sixteen months.

The method described has been used extensively to study the pharmacokinetics of risperidone and 9-hydroxyrisperidone in experimental animals, healthy volunteers and patients. As an example, Fig. 5 shows the mean plasma concentration–time curves for both analytes after a single oral dose of 0.31 mg/kg risperidone to four beagle dogs. Elimination half-lives were *ca.* 1.4 h for risperidone and 21 h for the 9-hydroxy metabolite.

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