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Determination of gacyclidine enantiomers in human plasma by gas chromatography–mass spectrometry using selected-ion monitoring

Guillaume Hoizey[a,*], Richard Vistelle[a], Denis Lamiable[b], Hervé Millart[b], Bertrand Gourdier[c], Pierre d'Arbigny[d]

[a]*Laboratoire de Pharmacologie et de Pharmacocinétique, U.F.R. de Pharmacie, 51 Rue Cognacq-Jay, 51096 Reims Cedex, France*
[b]*Laboratoire de Pharmacologie, Hôpital Maison Blanche, C.H.R. de Reims, 51092 Reims Cedex, France*
[c]*Laboratoire de Pharmacie Clinique, U.F.R. de Pharmacie, 51 Rue Cognacq-Jay, 51092 Reims Cedex, France*
[d]*BEAUFOUR IPSEN, 5 Avenue du Canada, 91966 Les Ulis Cedex, France*

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Abstract

A sensitive gas chromatographic assay using mass selective-detection has been developed for the simultaneous quantitation of the enantiomers of (±)-gacyclidine (a non competitive N-methyl-D-aspartate antagonist) in human plasma. Gacyclidine enantiomers and phencyclidine (PCP), the internal standard, were extracted using a single-step liquid–liquid extraction with hexane at pH 8.0. Each enantiomer was separated on a chiral gas chromatography capillary column and specifically detected by mass spectrometry (MS) in selected-ion monitoring (SIM) mode. Gacyclidine enantiomers and PCP were monitored using the fragment ions at m/z 206 and 200, respectively. No interference was observed from endogenous components. The limit of quantitation (LOQ) for each enantiomer of gacyclidine was 300 pg/ml by using plasma samples of 500 μl. The calibration curves were linear (r^2=0.998) over a range of 0.3125 to 20 ng/ml. The extraction efficiency was higher than 95% for both enantiomers. Intra- and inter-day bias were less than 10% at every standard curve concentration. Intra-day precision was less than 19% for (−)-gacyclidine and 15% for (+)-gacyclidine. Inter-day precision was below 15% for both enantiomers. The assay was validated for an enantioselective pharmacokinetic study in healthy male volunteers.

Keywords: Enantiomer separation; Gacyclidine

1. Introduction

Gacyclidine, a non competitive N-methyl-D-aspartate (NMDA) antagonist chemically related to the phencyclidine series [1], is currently developed for the treatment of spinal cord injuries. Gacyclidine HCl, the hydrochloride salt of *cis* (pip/Me) 1-[1-(2-thienyl)-2-methylcyclohexyl]piperidine, is a racemic mixture of two enantiomers (Fig. 1). It is thus important to determine the pharmacokinetic profiles of its individual enantiomers.

Up to now, no analytical method has been described to quantify the concentrations of gacyclidine enantiomers in plasma. Attempts to carry out these determinations in our laboratory revealed that enantiospecific high-performance liquid chromatography

*Corresponding author.

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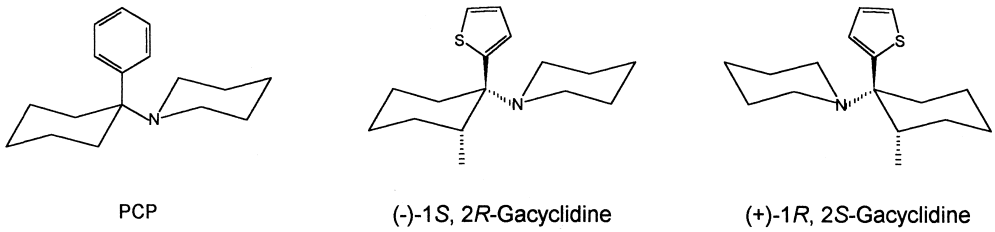

Fig. 1. Structures of PCP and gacyclidine enantiomers.

(HPLC) methods are not sensitive enough to measure plasma concentrations of gacyclidine enantiomers lower than 10 ng/ml (data not shown). This was expected due to its molecular structure related to phencyclidine (PCP) (Fig. 1) for which gas chromatography–mass spectrometry (GC–MS) had sufficient sensitivity to allow measurements of its concentrations in body fluids [2].

The present paper describes the development and the validation of a sensitive and specific assay method for the simultaneous determination of gacyclidine enantiomers in human plasma by GC with mass-selective detection (MSD). The procedure uses a single-step liquid–liquid extraction and has a sensitivity high enough to allow measurement well below 10 ng/ml in plasma since the LOQ achieved is 0.3 ng/ml, requiring 0.5 ml of sample. It has been applied to a preliminary pharmacokinetic study in four healthy male volunteers, and the results show that the pharmacokinetics of gacyclidine in plasma does not seem to be stereoselective.

2. Experimental

2.1. Chemicals

Gacyclidine-HCl, *cis* (pip/Me) (1-[1-(2-thienyl)-2-methylcyclohexyl]piperidine hydrochloride), (+)-gacyclidine, (−)-gacyclidine and PCP (internal standard, I.S.), were supplied by Institut Beaufour Ipsen (Les Ulis, France). Hexane was purchased from SDS (Peypin, France). Methanol was purchased from Merck (Nogent-sur-Marne, France). All chemicals and reagents were of the highest grade available and were used without further purification.

2.2. Gas chromatography–mass spectrometry

A Fisons GC 8000 gas chromatograph equipped with a Fisons A200 S automatic sampler (Fisons Instruments, Arcueil, France) was used. Samples were introduced through a SGE 17 TCS 17 mm septum (SGE, Villeneuve St. Georges, France) into a split/splitless capillary injection port system using a 104×5 mm I.D. quartz glass splitless injection port liner. Sample injections onto the GC were performed in the splitless mode.

A chiral fused-silica capillary column (25 m×0.25 mm I.D., 0.25 μm film thickness) with a stationary phase of CP-Chirasil-Dex (Chrompack, Les Ulis, France) was used for analysis. Operating conditions for routine use were: injection port temperature: 250°C; initial oven temperature: 60°C for 1.0 min, increased to 120°C at a rate of 25°C/min, then increased to a final temperature of 165°C at 2°C/min and held constant for 12 min. Helium was used as carrier gas with a total inlet flow of 30 ml/min and septum purge of 5.0 ml/min. The column head pressure was 160 kPa which provided a 1.77 ml/min column flow.

For detection, a Fisons MD 800 MSD system (Fisons Instruments, Arcueil, France), was used in the electron impact (EI) ionization mode with 70 eV ionization energy and 250 μA emission current. The transfer line temperature was kept at 250°C. To improve the sensitivity of the detection, the MSD system was manually tuned to the molecular fragments of the mass-scale calibrant perfluorotributylamine (FC-43) of m/z 69, 219, 414 and 502.

Full-scale mass scanning (SCAN) was performed for qualitative purposes to determine the fragmentation pattern of gacyclidine and PCP. Gacyclidine enantiomers were quantitated, using an I.S. (PCP), in

the SIM mode by detecting the selected ion current of m/z 206 for both enantiomers, whereas PCP was monitored via its fragment ion at m/z 200. The dwell-time was set at 80 ms in both groups which provided 12.5 scan cycles/s for the compounds.

The detector output was digitized and data processed using a Masslab data system (Version 1.12, Fisons).

2.3. Human experiments

Four healthy male volunteers received a bolus intravenous (i.v.) injection of 0.05 mg/kg of gacyclidine. Blood samples were harvested before drug administration, and at 0.5, 1, 2, 3, 4, 6, 8, 12, 16, 24, 36, 48, 60, 72, 84, 96, 120, 144, 168, 192, 216, 240 and 360 h post-injection. Plasma samples were stored at −20°C until analysis.

2.4. Sample preparation

To 500 μl of plasma in a glass centrifuge tube, 1.0 ng of I.S. (20 μl of 0.05 μg/ml standard solution of PCP in methanol) was added. After alkalinization to pH 8.0 (50 μl of Tris buffer) the mixture was extracted with 4 ml of hexane for 1 min on a vortex-mixer and centrifuged for 4 min at 3000 *g*. The upper organic layer was transferred into a conical tube and evaporated to dryness at +65°C under a stream of nitrogen gas. The residue was dissolved in 50 μl of methanol and an aliquot (2 μl) was injected into the GC–MS system.

2.5. Calibration curve

Stock standard solutions of gacyclidine (2 μg/ml) were prepared by dissolution of the drug in methanol and remained stable for at least one month when stored at −20°C and protected from light.

Stock solutions were further diluted to give a series of working racemic standards with concentrations of 1000, 500, 250, 125, 62.5 and 31.25 ng/ml. The standard curves of each gacyclidine enantiomer (concentrations of 0.3125, 0.625, 1.25, 2.5, 5, 10 and 20 ng/ml) were constructed by analyzing blank human plasma (500 μl) spiked with 10 μl of stock or working standard solutions of gacyclidine as previously described.

Gacyclidine enantiomers and the I.S. (PCP) were detected according to predetermined retention times. The quantitation of (+)-gacyclidine and (−)-gacyclidine concentrations was accomplished using an unweighted linear regression analysis by plotting the peak area ratios of each enantiomer versus internal standard.

2.6. Recovery, precision and accuracy

2.6.1. Recovery

The extraction efficiency of each gacyclidine enantiomer from stripped plasma was determined at concentrations of 20, 10, 2.5 and 0.625 ng/ml. The extraction efficiency (%) was calculated as follows:

$$\text{Efficiency} = \frac{\text{Peak area PCP extracted}}{\text{Peak area gacyclidine non-extracted}} \times \frac{\text{Peak area gacyclidine extracted}}{\text{Peak area PCP extracted}} \times 100$$

2.6.2. Precision and accuracy

2.6.2.1. *Intra-day*. Ten replicates of spiked human plasma samples at each calibration concentration were assayed against a single calibration curve. The intra-assay variability was determined as the coefficient of variation (C.V.).

2.6.2.2. *Inter-day*. Calibration curves of (+)-gacyclidine and (−)-gacyclidine were prepared daily in human plasma and assayed with the method previously described for seven days. Unweighted linear regression analysis were carried out and each calibration concentration was back-extrapolated using the regression parameters obtained (i.e., slope, intercept). The measured amounts were compared to the amounts of drug added. The inter-day precision was assessed by comparing the results of the seven measurements and determining the C.V. at each calibration level.

2.6.2.3. *Accuracy*. The accuracy (bias) was expressed as:

$$[(\text{measured amount}/\text{added amount}) \times 100] - 100$$

with (+)-bias and (−)-bias representing over- and underestimation, respectively.

3. Results and discussion

3.1. Extraction procedure

Several solvents (hexane, diethyl ether, chloroform and dichloromethane) were chosen to compare the effectiveness of extraction. The results showed that hexane is the most effective extraction solvent for gacyclidine and PCP. The hexane/plasma volume ratio used to extract the compounds of interest was chosen in order to avoid the formation of any emulsion when the mixture was vortexed. After extraction of gacyclidine at different pH ranged from 6 to 10, it was found that the high extraction value was obtained at pH 8.0, this value remaining unchanged for highest pH. Finally, a single step extraction is sufficient to ensure a satisfactory purification of the extractum.

3.2. Chromatograms and mass spectra

Typical SCAN and SIM chromatograms of gacyclidine enantiomers and PCP in human plasma are shown in Figs. 2 and 3, respectively. The peaks of the compounds of interest are well separated under the experimental conditions with no sign of interference from endogenous compounds.

The mass spectra of gacyclidine and PCP are presented in Fig. 4. Gacyclidine underwent extensive fragmentation, showing the molecular ion $[M^+]$ at m/z 263 and the base peak fragment ion at m/z 206.

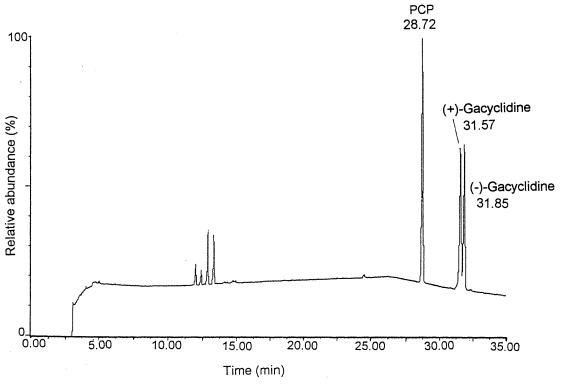

Fig. 2. Total ion chromatograms (SCAN mode) of blank human plasma and of 0.5 ml blank plasma spiked with 100 ng gacyclidine and 50 ng PCP (I.S.).

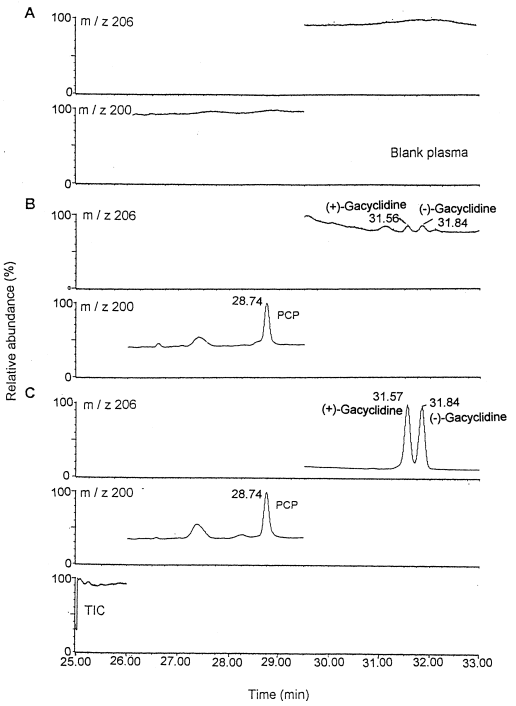

Fig. 3. Single ion chromatograms (SIM) of blank plasma (A), 0.5 ml blank plasma spiked with 0.62 ng/ml gacyclidine and 2 ng/ml (B), and 0.5 ml blank plasma spiked with 40.0 ng/ml gacyclidine and 2 ng/ml PCP (C).

Due to its high abundance, it was possible to selectively and individually monitor the ion m/z 206 for gacyclidine enantiomers. This method provides a very specific signal detection. PCP also underwent extensive fragmentation, showing the base peak fragment ion of m/z 200 and the molecular ion $[M^+]$ of m/z 242. PCP was selectively monitored by collecting the single ion of m/z 200. To enhance the selectivity of detection for gacyclidine enantiomers and PCP, the respective molecular ions $[M^+]$ were also monitored.

3.3. Stereoselective assay of gacyclidine enantiomers

The enantiomers of gacyclidine were well-separated on a CP-Chirasil-Dex column (Fig. 3) and the separation was reproducible over a study period. The mean retention times of both enantiomers measured repeatedly for one week ($n=36$) were 31.6 ± 0.06

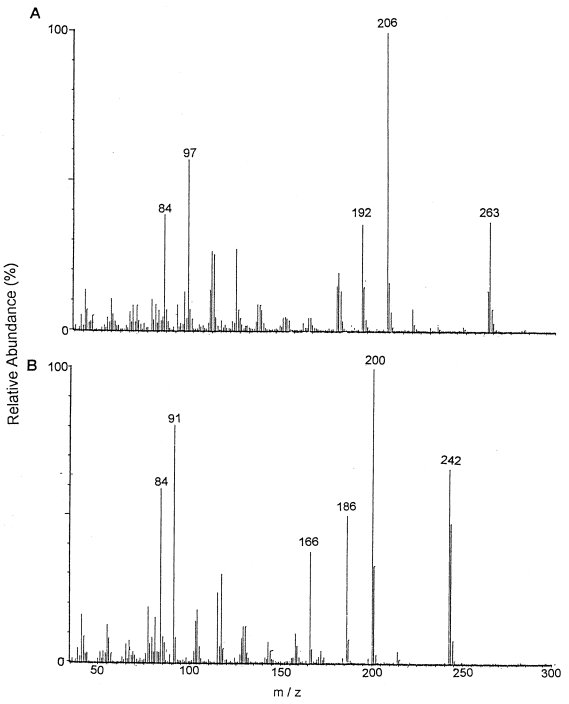

Fig. 4. Full scan EI mass spectra of gacyclidine (A) and PCP (B).

min for (+)-gacyclidine and 31.9±0.06 min for (−)-gacyclidine. The resolution factor between both enantiomers was 0.92±0.03 ($n=10$).

The choice of a suitable I.S. remains critical. Because no isotopically labeled gacyclidine was available for the experiment, and after having tested two different structurally related analogues: GK0, 1-[1-(2-thienyl)-cyclohexyl]piperidine (TCP) and GK12, the *trans* (pip/Me) gacyclidine diastereoisomer [3], PCP was selected as the most satisfactory reference compound: short retention time and complete separation from gacyclidine enantiomers. The retention time of GK12 was much longer (≈45 min) than that of gacyclidine enantiomers (data not shown) and GK0 and GK12 acquisitions provide an interference peak on the base fragment ion of m/z 206 of gacyclidine. As the chemical structure of PCP is close to that of gacyclidine, it is a good I.S. for quantitation of gacyclidine enantiomers. However, time-dependent changes in chromatographic behavior of PCP frequently requires the use of a new analytical column after no more than 200 injections. In contrast, the chromatographic behavior of gacyclidine enantiomers remains satisfactory over long periods of time (>500 injections). This handicap, which is both labor intensive and time consuming, could be improved using an isotopically labeled gacyclidine as internal standard. Since the deuterium-labelled gacyclidine racemate (d3-gacyclidine) is now available, this alternative will be tested as soon as we may use it, and a particular attention will be paid to the isotope effect which can be very important at low concentrations.

3.4. Limit of quantitation

Satisfactory sensitivity can be achieved with GC–MS since the limit of quantitation (LOQ) was found to be 300 pg/ml (signal-to-noise ratio, $S/N=6$) for each enantiomer. This LOQ was about 30 times lower than that (10 ng/ml) obtained previously with an enantiospecific HPLC method with UV detection set up in our laboratory during preliminary assays.

3.5. Calibration curve and linearity

The calibration curves of gacyclidine enantiomers in human plasma showed good linearity over the working concentration range (0.3125 to 20 ng/ml). Using unweighted linear regression analysis, the calibration curves of (+)-gacyclidine were best described by the equation: $y=0.19x-0.026$, and that of (−)-gacyclidine by: $y=0.20x-0.029$ with good coefficients of determination ($r^2=0.998$) for both enantiomers.

3.6. Recovery

The recovery values of (+)-gacyclidine and (−)-gacyclidine (mean±S.D.) from human plasma are summarized in Table 1. Results show that the extraction efficiency of each enantiomer of

Table 1
Recovery of gacyclidine enantiomers from human plasma

Concentration (ng/ml)	n	(+)-Gacyclidine (%)	(−)-Gacyclidine (%)
20.000	5	105.4±11.2	103.5±13.6
10.000	4	92.2±9.7	95.3±9.5
2.500	5	106.5±10.5	105.6±10.4
0.625	5	94.6±24.6	91.1±22.9

gacyclidine from human plasma was constant over the whole concentration range studied, thus allowing reliable quantitation of gacyclidine enantiomers.

3.7. Intra-day precision, inter-day precision and accuracy

Intra-day precision, inter-day precision and accuracy are presented in Tables 2 and 3, respectively. Bias did not exceed 10% over the entire calibration range. Although at the concentration of 0.3125 ng/ml of (+)-gacyclidine, the C.V. reaches 19%, the reproducibility of the present method appears to be reliable and satisfactory within the range 0.3125 to 20 ng/ml.

3.8. Determination of gacyclidine enantiomers in human plasma

Table 4 shows the time course of gacyclidine enantiomers in plasma following a 0.05 mg/kg intravenous dose of racemic gacyclidine in four healthy volunteers. In all subjects, the presence of gacyclidine in plasma is due to that of its both enantiomers. During the length of the experiment, plasma concentrations of each enantiomer were very close to each other, with that of (−)-gacyclidine plasma concentrations being very slightly higher than that of (+)-gacyclidine. In two out of the four volunteers, the results show that after a rapid decay, concentrations of gacyclidine enantiomers were below the LOQ as soon as 12 and 24 h after the drug administration. In the two other subjects, the plasma concentrations of gacyclidine enantiomers are characterized by a rapid decay within 4 to 6 h after the end of injection, followed by persistent (15 days) low plasma concentrations (<2.0 ng/ml) with marked fluctuations. The fluctuation of plasma concentrations during the terminal phase does not allow an accurate curve-fit. Thus, the terminal half-life and other pharmacokinetic parameters such as total clearance and volume of distribution cannot be estimated.

In conclusion, a sensitive and selective GC assay using MSD was developed for the quantitation of gacyclidine enantiomers in human plasma. Sample preparation involves a single liquid–liquid extraction

Table 2
Intra-day accuracy and precision data for the stereoselective assay of gacyclidine enantiomers in human plasma

Spiked concentration (ng/ml)		Concentration found (mean±S.D.) (ng/ml)[a]	Bias[b] (%)	C.V.[c] (%)
0.3125	(+)-Gacyclidine	0.31±0.06	−1.7	18.9
	(−)-Gacyclidine	0.30±0.04	−4.9	14.9
0.6250	(+)-Gacyclidine	0.64±0.07	3.0	11.2
	(−)-Gacyclidine	0.61±0.07	−2.7	11.7
1.2500	(+)-Gacyclidine	1.21±0.13	−3.2	11.1
	(−)-Gacyclidine	1.22±0.17	−2.0	13.8
2.5000	(+)-Gacyclidine	2.52±0.22	0.9	8.6
	(−)-Gacyclidine	2.44±0.22	−2.2	9.1
5.0000	(+)-Gacyclidine	5.24±0.46	5.0	8.8
	(−)-Gacyclidine	4.84±0.34	−3.1	7.0
10.0000	(+)-Gacyclidine	10.02±0.38	0.2	3.8
	(−)-Gacyclidine	10.23±0.43	2.3	4.2
20.0000	(+)-Gacyclidine	19.74±0.94	−1.3	4.8
	(−)-Gacyclidine	20.28±0.64	1.4	3.2

[a] $n=10$.
[b] Expressed as [(mean calculated concentration/spiked concentration)×100]−100.
[c] Coefficient of variation.

Table 3
Inter-day accuracy and precision data for the stereoselective assay of gacyclidine enantiomers in human plasma

Spiked concentration (ng/ml)		Concentration found (mean±S.D.) (ng/ml)[a]	Bias[b] (%)	C.V.[c] (%)
0.3125	(+)-Gacyclidine	0.29±0.03	−5.9	10.9
	(−)-Gacyclidine	0.33±0.02	4.3	5.4
0.6250	(+)-Gacyclidine	0.68±0.06	9.0	9.3
	(−)-Gacyclidine	0.65±0.03	4.2	5.3
1.2500	(+)-Gacyclidine	1.25±0.16	0.1	12.6
	(−)-Gacyclidine	1.27±0.19	1.7	15.1
2.5000	(+)-Gacyclidine	2.28±0.23	−8.9	10.2
	(−)-Gacyclidine	2.34±0.27	−6.2	11.6
5.0000	(+)-Gacyclidine	5.06±0.43	1.2	8.5
	(−)-Gacyclidine	5.05±0.42	0.9	8.3
10.0000	(+)-Gacyclidine	10.27±0.28	2.7	2.8
	(−)-Gacyclidine	10.26±0.29	2.6	2.8
20.0000	(+)-Gacyclidine	19.95±0.07	−0.3	0.4
	(−)-Gacyclidine	19.96±0.08	−0.2	0.4

[a] $n=7$. [b] Expressed as [(mean calculated concentration/spiked concentration)×100]−100. [c] Coefficient of variation.

Table 4
Individual plasma concentrations of gacyclidine enantiomers (ng/ml) after i.v. administration of a single dose of 0.05 mg/kg of gacyclidine

Time (h)	Subject 1		Subject 2		Subject 3		Subject 4	
	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)
0	<0.30	<0.30	<0.30	<0.30	<0.30	<0.30	<0.30	<0.30
0.5	5.01	6.24	2.74	2.97	6.72	7.33	34.90	37.20
1	5.33	6.32	2.23	2.45	3.14	3.50	6.03	6.76
2	1.61	2.09	1.49	1.61	2.29	2.49	2.10	2.44
3	1.64	2.24	1.00	1.09	2.06	2.21	1.46	1.69
4	1.44	1.95	0.76	0.82	1.32	1.42	0.96	1.08
6	0.99	1.34	0.58	0.66	2.89	2.96	0.86	1.00
8	–	–	0.51	0.61	0.72	0.76	0.52	0.60
12	0.75	0.86	0.55	0.60	0.41	0.46	<0.30	<0.30
16	1.03	1.09	0.58	0.61	<0.30	<0.30	<0.30	<0.30
24	0.48	0.41	0.52	0.55	<0.30	<0.30	<0.30	<0.30
36	0.69	0.73	0.42	0.43	<0.30	<0.30	<0.30	<0.30
48	0.39	0.44	0.42	0.46	<0.30	<0.30	<0.30	<0.30
60	0.68	1.08	0.43	0.46	<0.30	<0.30	<0.30	<0.30
72	0.56	0.82	0.38	0.40	<0.30	<0.30	<0.30	<0.30
84	0.39	0.51	0.38	0.40	<0.30	<0.30	<0.30	<0.30
96	0.88	1.19	0.31	0.35	<0.30	<0.30	<0.30	<0.30
120	0.30	0.36	0.66	0.70	<0.30	<0.30	<0.30	<0.30
144	0.59	0.74	0.42	0.45	<0.30	<0.30	<0.30	<0.30
168	<0.30	<0.30	0.56	0.63	<0.30	<0.30	<0.30	<0.30
192	0.49	0.61	0.38	0.40	<0.30	<0.30	<0.30	<0.30
216	<0.30	0.34	0.36	0.41	<0.30	<0.30	<0.30	<0.30
240	0.30	0.35	0.30	0.33	<0.30	<0.30	<0.30	<0.30
360	0.41	0.50	0.33	0.36	<0.30	<0.30	<0.30	<0.30

with hexane which provides clean chromatograms. This procedure is fast and allows a recovery of about 95% for both enantiomers. This assay, which demonstrates a good reproducibility and a low limit of quantitation, has been successfully applied to the determination of the pharmacokinetics of gacyclidine enantiomers in four healthy male volunteers after i.v. bolus administration of the racemic drug.

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