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Modified gas chromatographic–mass spectrometric assay for the determination of gacyclidine enantiomers in human plasma

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

[a]*Laboratoire de Pharmacologie et de Pharmacocinétique, U.F.R. de Pharmacie, Université de Reims Champagne Ardenne, 51 rue Cognacq-Jay, 51096 Reims Cedex, France*

[b]*Laboratoire de Pharmacologie–Toxicologie, Hôpital Maison Blanche, C.H.R. de Reims, Reims, France*

[c]*Laboratoire de Pharmacie Clinique, U.F.R. de Pharmacie, Université de Reims Champagne Ardenne, Reims, France*

[d]*Beaufour Ipsen, Les Ulis, France*

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Abstract

A modified method for the determination of gacyclidine enantiomers in human plasma by GC–MS with selected-ion monitoring using the deuterated derivative of gacyclidine (d_3-gacyclidine) as internal standard was developed. Following a single-step liquid–liquid extraction with hexane, drug enantiomers were separated on a chiral fused-silica capillary column (CP-Chirasil-Dex; Chrompack). The fragment ion, m/z 266, was selected for monitoring d_3-gacyclidine (retention times of 35.2 and 35.6 min for the (+)- and (−)-enantiomer, respectively) whereas the fragment ion, m/z 263, was selected for quantitation of gacyclidine (retention times of 35.4 and 35.9 min for the (+)- and (−)-enantiomer, respectively). The limit of quantitation for each enantiomer was 0.3 ng/ml, using 1 ml of sample, with a relative standard deviation (RSD) <14% and a signal-to-noise ratio of 5. The extraction recovery of both gacyclidine enantiomers from human plasma was about 75%. The calibration curves were linear (r^2>0.996) over the working range of 0.312 to 20 ng/ml. Within- and between-day RSD were <9% at 5, 10 and 20 ng/ml, and <16% at 0.312, 0.625, 1.25 and 2.5 ng/ml. Intraday and interday bias were less than 11% for both enantiomers. The chromatographic behavior of d_3-gacyclidine remained satisfactory even after more than 500 injections. Applicability of this specific and stereoselective assay is demonstrated for a clinical pharmacokinetic study with racemic gacyclidine. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Gacyclidine

1. Introduction

Gacyclidine, *cis* (pip/Me) 1-[1-(2-thienyl)-2-methylcyclohexyl]piperidine (Fig. 1), is a noncompetitive *N*-methyl-D-aspartate antagonist currently developed for the treatment of the spinal cord injuries [1]. It is a racemic mixture of two enantiomers: (−)-1*S*, 2*R*-gacyclidine and (+)-1*R*, 2*S*-gacyclidine. Determination of the pharmacokinetic profiles of gacyclidine enantiomers in human plasma required the setup of a sensitive and selective chiral capillary gas chromatographic–mass spectrometric (GC–MS) assay [2]. The procedure consisted in a single-step liquid–liquid extraction with hexane by

*Corresponding author. Fax: +33-3-2605-3552.

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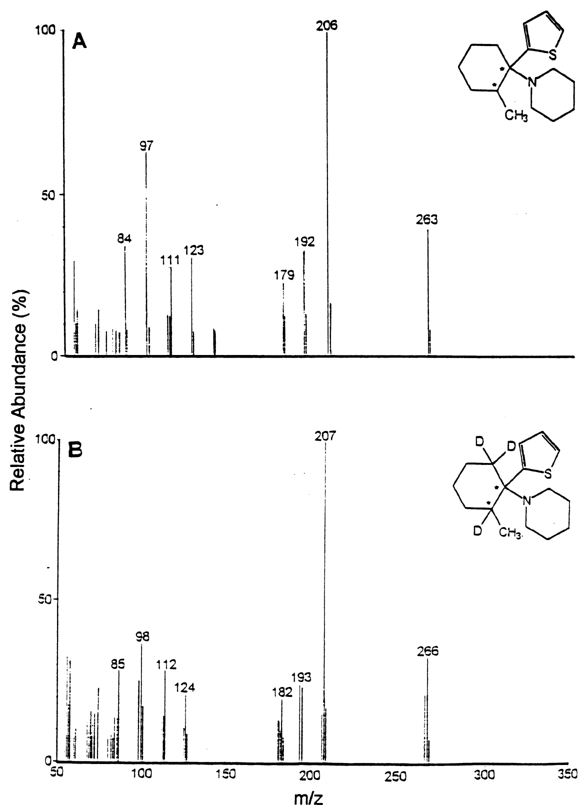

Fig. 1. Chemical structures and electron impact mass spectra of gacyclidine (A) and d_3-gacyclidine (internal standard, B). (*) Denotes a chiral center.

using phencyclidine (PCP) as internal standard. Although PCP allowed an accurate quantitation of gacyclidine enantiomers, its disadvantage is the rapid appearance of bad peak shape. This requires the frequent replacement of the analytical column.

This disadvantage could be corrected by using a stable isotope labelled analogue of the compound to be measured as internal standard. Indeed, having similar physical and chemical properties as the unlabelled compound, it would seem to be an ideal internal standard [3,4]. Especially, the closer the retention times of the compound of interest and of its internal standard, the slighter the effect of variations in instrumental parameters. Since deuterium-labelled gacyclidine (d_3-gacyclidine) is now available (Fig. 1), we decided to modify the previous validated CG–MS assay by using it as internal standard. Plasma gacyclidine enantiomer concentrations were determined according to the proposed method in healthy male volunteers receiving racemic gacyclidine.

2. Experimental

2.1. Chemicals

Racemic gacyclidine [($\pm$)-gacyclidine, purity>99,0%] and its deuterated derivative (d_3-gacyclidine; purity>97,5%) were provided by Institut Beaufour Ipsen (Les Ulis, France). GC-grade hexane and methanol were purchased from SDS (Peypin, France) and Merck (Nogent-sur-Marne, France), respectively. Tris buffer (pH 8) was obtained from Dade-Behring (Paris, France).

2.2. Chromatographic conditions

A MD 800 mass selective detector for electron ionization coupled to a Model 8000 gas chromatograph (Fisons, Arcueil, France) was used. GC was performed on a fused-silica capillary column (25 m×0.25 mm I.D., 0.25 μm film thickness) with β-cyclodextrin as a chiral stationary phase: CP-Chirasil-Dex CB (Chrompack, Les Ulis, France). The carrier gas was helium (1.77 ml/min). Sample injections into the GC system were made in the splitless mode (splitless time: 1 min), with a septum vent flow-rate of 20 ml/min and septum purge flow-rate of 5 ml/min.

The operating conditions were: injection port temperature, 250°C; detector temperature, 260°C; column temperature programming, 60°C for 1 min, rising to 120°C at 25 C°/min, then increasing to 160°C at 2 C°/min and held at that temperature for 15 min. The run time was 40 min. The GC–MS was operated in electron impact mode with the ion energy set at 70 eV. The mass selective detector was monitored in the selected-ion monitoring (SIM) mode and set at m/z 263 and m/z 266 for the detection and quantitation of gacyclidine and d_3-gacyclidine enantiomers, respectively. The instrument was calibrated weekly using heptacosa (perfluoro-tributylamine). The detector output was digitized and data processed using a Masslab data system (Version 1.12, Fisons).

2.3. Volunteer study

Four healthy male volunteers received a bolus intravenous (i.v.) injection of 0.02 mg/kg of gacyclidine. Plasma samples were obtained from blood samples collected before dosing, and at 3, 10, 20, 30, 60, 120, 240, 360, 480, 620 and 1440 min after drug administration. Plasma samples were stored frozen at −20°C prior to analysis.

2.4. Standard and sample preparation

Stock solutions of (±)-gacyclidine (2 mg/ml) and d_3-gacyclidine (1 mg/ml) were prepared in methanol and remained stable at −20°C with protection from light for at least 6 months. Working solutions of (±)-gacyclidine (4 μg/ml) and d_3-gacyclidine (1 μg/ml) were prepared by dilution of the stock solutions with the same solvent. They were stable at +4°C for at least 1 month. (±)-Gacyclidine standards in the concentration range 62.5–2 000 ng/ml were prepared daily by dilution of the working solutions in methanol.

To 1.0-ml aliquots of plasma samples from healthy donors were added appropriate volumes of various standards and the working solution of gacyclidine to obtain calibration curves in the range 0.3125–20 ng gacyclidine per ml of plasma for each enantiomer and 20 μl of working internal standard solution (20 ng racemate per ml plasma final concentration). Each sample was mixed briefly following each addition. After alkalinisation to pH 8.0 (100 μl of Tris buffer), the mixture was extracted for 1 min with 6 ml of hexane on a Vortex Genie mixer. After centrifugation (3000 *g*; 5 min) to separate phases, the organic layer was transferred into conical glass tubes 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) injected directly into the GC–MS system. Analysis of samples from volunteers receiving the drug were prepared in the same way.

2.5. Quality control sample preparation

Quality control samples were prepared from a pool of human blank plasma spiked with racemic gacyclidine at 0.625, 1.25, 5 and 20 ng/ml. The plasma pool was divided into 1.0-ml aliquots and stored frozen at −20°C.

2.6. Quantitation

Quantitation was performed by the internal standard method with a calibration graph using peak-area ratios. Data were fitted by unweighted linear regression analysis.

2.7. Recovery and reproducibility

The extraction efficiency of gacyclidine enantiomers from stripped plasma was calculated at concentrations of 0.625, 2.5, and 20 ng/ml (n=5) according to the equation:

$$\frac{\text{Peak area } d_3\text{-gacyclidine extracted}}{\text{Peak area gacyclidine nonextracted}} \times \frac{\text{Peak area gacyclidine extracted}}{\text{Peak area } d_3\text{-gacyclidine extracted}} \times 100$$

Intra-day precision and accuracy (bias) were derived from the relative standard deviation (RSD) of replicate analysis ($n=10$) at each calibration concentration within-day. To assess the inter-day precision and accuracy (bias) four quality control samples (concentrations of gacyclidine enantiomers of 0.625, 1.25, 5, and 20 ng/ml) were analyzed on eight separate days.

3. Results and discussion

3.1. Internal standard and GC–MS conditions

The validation of the analytical method and the analysis of plasma samples of a clinical study lead us to analyze more than 500 samples. These investigations could be carried out by using the same analytical column due to the better chromatographic behavior of d_3-gacyclidine compared to PCP. However, the limitation to the use of d_3-gacyclidine as internal standard is the isotope effect on the base peak fragment ion of gacyclidine at m/z 206, especially at concentrations of gacyclidine enantiomers lower than 2.5 ng/ml. In contrast, this effect does not exist on the molecular ion (M+) of gacyclidine

at m/z 263 even at concentrations of gacyclidine enantiomers as low as the limit of detection of the analytical method (0.15 ng/ml; S/N: 3). The isotope effect can be thus avoided by selecting the following ions: m/z 263 for gacyclidine and m/z 266 for d_3-gacyclidine. However, there is a loss of response on the fragment ion m/z 263 due to its lower abundance than the base peak m/z 206. In order to minimize this phenomenon, the sample volume was increased to 1 ml. Similar GC conditions to those described in the previous assay ensured a satisfactory and reproducible resolution between enantiomers of gacyclidine and d_3-gacyclidine. The retention times of (+)- and (−)-d_3-gacyclidine enantiomers were 35.2±0.04 and 35.6±0.04 min, respectively (n=22). They were slightly shorter than those of (+)- and (−)-gacyclidine (35.4±0.05 and 35.9±0.06 min; n=22), and did not significantly vary as a function of time (coefficient of variation <0.15%). Complete mass spectra of gacyclidine and d_3-gacyclidine are depicted in Fig. 1. The typical GC–MS-SIM mode chromatogram presented in Fig. 2 shows that the peaks of interest were well-defined and well-separated.

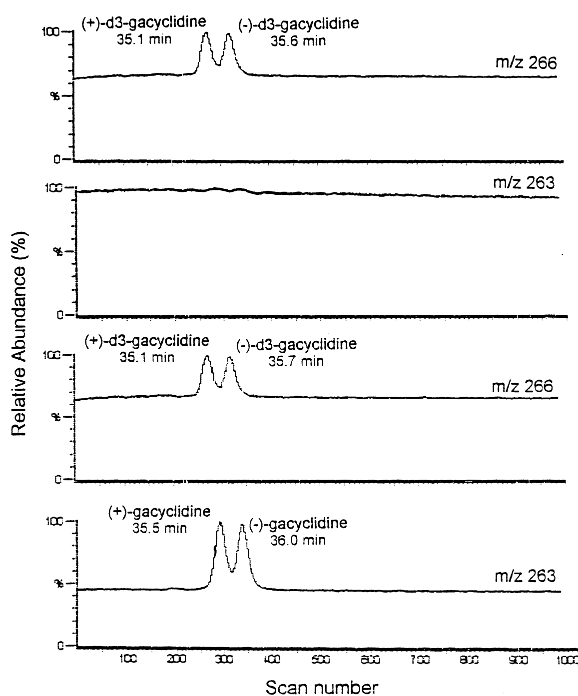

Fig. 2. Representative selected-ion chromatograms (SIM mode) obtained during enantioselective assay of gacyclidine and d_3-gacyclidine (internal standard). (Top) blank control plasma; (bottom) plasma sample (1 ml) spiked with gacyclidine (2.5 ng/ml) and d_3-gacyclidine (20 ng/ml).

3.2. Specificity and selectivity

Good specificity was achieved by mass spectrometry when selectively and individually monitoring the ions m/z 263 and m/z 266 for gacyclidine and d_3-gacyclidine enantiomers, respectively. No interferences from endogenous substances were detected at the retention time of the compounds of interest, gacyclidine enantiomers and the internal standard. (Fig. 2).

3.3. Calibration curve and sensitivity

The linearity was satisfactory in the range 0.3125–20 ng/ml for both gacyclidine enantiomers (r^2=0.9974±0.003; n=10). The limit of detection of gacyclidine enantiomers was 0.15 ng/ml (S/N: 3). Applying the common requirement of the RSD (<20%), the limit of quantitation was 0.30 ng/ml (S/N: 5/1).

3.4. Recovery, precision and accuracy

The extraction efficiency of gacyclidine enantiomers was about 75% within the range of concentrations studied (Table 1). Increasing the volume of hexane to 8 ml and/or a second extraction step did not significantly improve the recovery (data not shown). Within and between-day precision and accuracy are summarized in Table 2. The RSD and the deviations from the nominal values are all satisfac-

Table 1
Recovery of gacyclidine enantiomers from human plasma (mean±SD; n=5)

Concentration (ng/ml)	(+)-Gacyclidine (%)	(−)-Gacyclidine (%)
20.000	77.4±8.8	78.2±5.6
2.500	71.1±3.4	71.7±2.0
0.625	74.3±6.5	73.7±11.8

Table 2
Accuracy (bias) and precision of the gacyclidine enantiomers assay in human plasma

Added (ng/ml)	Gacyclidine enantiomer	Found (ng/ml)	SD (ng/ml)	RSD[a] (%)	Bias[b] (%)
Within-day ($n=10$)					
0.3125	(+)	0.32	0.04	12.06	2.72
	(−)	0.28	0.04	13.24	−10.72
0.6250	(+)	0.66	0.05	8.26	6.32
	(−)	0.64	0.05	7.94	2.56
1.2500	(+)	1.19	0.15	12.64	−4.96
	(−)	1.24	0.15	11.81	−0.80
2.5000	(+)	2.49	0.19	7.51	−0.56
	(−)	2.51	0.19	7.41	0.44
5.0000	(+)	5.15	0.43	8.36	2.92
	(−)	5.09	0.33	6.53	1.80
10.0000	(+)	9.29	0.43	4.64	−7.15
	(−)	9.39	0.50	5.33	−6.12
20.0000	(+)	19.76	1.17	5.94	−1.21
	(−)	20.74	0.92	4.46	3.69
Between-day ($n=8$)					
0.6250	(+)	0.63	0.08	12.78	1.17
	(−)	0.61	0.09	14.34	−1.77
1.2500	(+)	1.30	0.11	8.58	3.77
	(−)	1.24	0.13	10.76	−0.46
5.0000	(+)	5.10	0.30	5.90	1.95
	(−)	5.21	0.33	6.39	4.26
20.000	(+)	19.83	0.33	1.65	−0.83
	(−)	19.71	0.24	1.24	−1.44

[a] Relative standard deviation.
[b] Expressed as [(mean calculated concentration/spiked concentration)×100]−100.

tory (<15%), and in agreement with the acceptance criteria reported by Shah et al. [5] and Bressolle et al. [6].

3.5. Analyses of samples from clinical studies

More than 240 human plasma samples from a clinical study were analyzed using the stereoselective assay described above. As an example, the mean plasma concentration versus time profiles of gacyclidine enantiomers obtained from four healthy male volunteers who received a single i.v. bolus injection of (±)-gacyclidine (0.02 mg/kg) are shown in Fig. 3. The concentrations of gacyclidine enantiomers were similar within subjects with no statistical differences between concentrations of both isomers at all collection times. Concentrations of gacyclidine enantiomers measured 3 min after drug injection were 15.6±7.2 and 13.8±6.3 ng/ml for (+)- and (−)-gacyclidine, respectively. In all subjects there was a rapid initial distribution phase followed by a slow terminal phase. Concentrations of gacyclidine

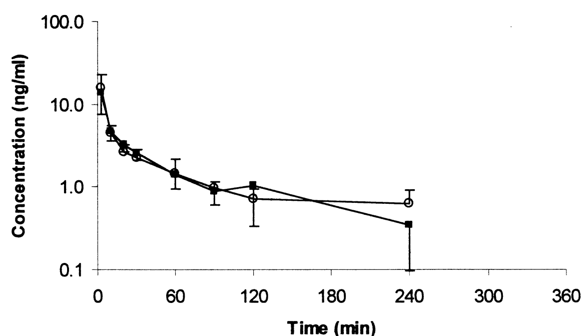

Fig. 3. Mean plasma concentration–time profiles (mean±SD) of (+)-gacyclidine (○) and (−)-gacyclidine (■) following a 0.02 mg/kg intravenous bolus dose of racemic gacyclidine in healthy male volunteers ($n=4$).

enantiomers measured in plasma samples 4 h post-injection were 0.63±0.30 and 0.35±0.25 ng/ml for (+)- and (−)-gacyclidine, respectively and were not statistically different. Detailed pharmacokinetic data for all subjects enrolled in the clinical study will be reported in a separate article.

In conclusion, a specific and sensitive modified GC–MS-SIM assay was developed for the simultaneous quantitation of gacyclidine enantiomers in human plasma. Deuterated gacyclidine as internal standard, instead of PCP, allows the use of the same analytical column even after more than 500 injections. The assay demonstrates good reproducibility and was successfully applied to the determination of the pharmacokinetic profile of gacyclidine enantiomers in healthy volunteers after intravenous injection of the racemic drug.

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