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Selective method for plasma quantitation of the stereoisomers of a new aminotetralin by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A high-performance liquid chromatographic method is described for the quantitation in plasma of the four stereoisomers of a new aminotetralin, (*SRR*, *RSS*)(*SRS*, *RSR*)-5,6-dimethoxy-2-[3′-(*p*-hydroxyphenyl)-3′-hydroxy-2′-propyl]aminotetralin (CHF 1255, internal code). After liquid–liquid extraction of the drug, separation was obtained after chiral derivatization with *R*-(+)-α-methylbenzyl isocyanate. The selective derivatization of the amino group was obtained by controlling the pH of the reaction medium at 7.5. The reaction was quantitative after a period of 16 h. The structures of the urea derivatives were confirmed by proton nuclear magnetic resonance spectroscopy and high-performance liquid chromatography with mass spectrometric detection. The use of an electrochemical detector, operating in the oxidative mode, allows the quantitation in plasma of all four urea derivatives at the nanogram level. The method was demonstrated to be precise, reproducible and applicable to pharmacokinetics studies after administration of the two epimeric racemates.

INTRODUCTION

5,6-Dimethoxy-2-[3′-(*p*-hydroxyphenyl)-3′-hydroxy-2′-propyl]aminotetralin (I) is a new compound under investigation for the treatment of heart failure [1]. It has three chiral centres and could theoretically exist as a mixture of four pairs of racemates. As the key synthetic intermediate is (±)-*erythro*-*p*-hydroxy-norephedrine, only the two epimeric racemates related to it are obtained (±A and ±B, Fig. 1).

The pharmacokinetics and metabolism of drugs containing chiral centres often reveal large differences in the behaviour of enantiomers in both animals and humans [2–5]. It is therefore essential to perform individual assays of the enantiomers of a drug in biological fluids with a selective and validated method.

High-performance liquid chromatographic (HPLC) assays using chiral stationary phases have recently been introduced, but HPLC methods using optically active derivatization reagents are still the most common procedures in pharmacokinetic studies [6–8].

Separation of all the isomers of I by HPLC can be achieved after chiral derivatization with *R*-(+)-α-methylbenzyl isocyanate (MBIC). This reagent is relatively stable and shows no isomerization during storage [9]; furthermore, it is frequently used for the separation of enantiomers in biological fluids [10,11]. As already reported [11], the secondary amino and the phenolic hydroxy

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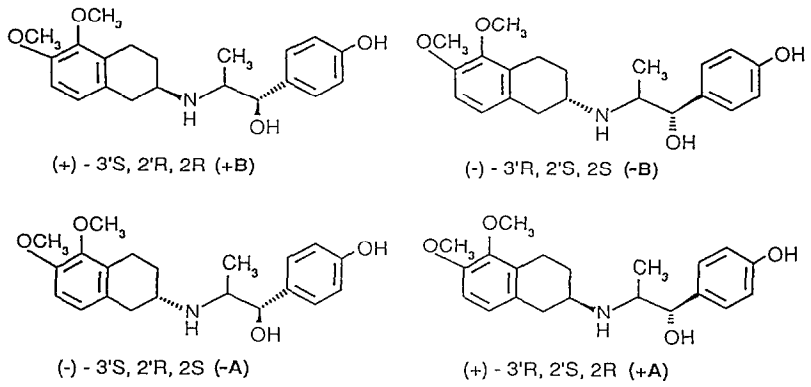

Fig. 1. Structures of the stereoisomers of I.

groups can simultaneously react with MBIC forming a mixture of the mono-MBIC derivative (urea derivative) and di-MBIC derivatives (urea and carbamate derivatives), whereas the secondary hydroxyl group of β-blockers does not react under the reported reaction conditions.

The aim of this study was to obtain a selective and quantitative derivatization of only the amino group of I, by optimizing the reaction conditions. The structures of MBIC derivatives were investigated by HPLC with mass spectrometry (MS) and ^{1}H NMR spectroscopy. Moreover, we have applied this derivatization procedure to assay the drug extracted from plasma samples. The separation of the four derivatized isomers of I was performed by reversed-phase HPLC with a coulometric electrochemical detector. The method was fully validated.

EXPERIMENTAL

Chemicals and reagents

The four isomers of compound I (+A, –A, +B, –B; Fig. 1), the internal standard (I.S.) ((*S*)-(–)-5,6-dimethoxy-2-[2′-(*p*-hydroxyphenyl)-1′-ethyl]aminotetralin) and the external standard (E.S.) ((*R*,*S*)-(5,6-dimethoxy-2-[3′-(*p*-hydroxyphenyl)-2′-propyl]aminotetralin) were synthesized in our laboratories. The two epimeric racemates, ± A and ± B, of the batch of I used in this trial were in the ratio 54:46 (calculated using chemically pure ± A and ± B racemates).

MBIC (purity >98%) was purchased from Fluka (Buchs, Switzerland). Sodium acetate, hydrochloric acid and boric acid were of Suprapur grade and were obtained from Merck (Darmstadt, Germany). Diethyl ether (RS grade, 447671) was purchased from Carlo Erba (Milan, Italy). Sulphatase (with β-glucuronidase activity) type H-5 (S-3009, from *Helix pomatia*) came from Sigma (St. Louis, MO, USA). All the other solvents and reagents were of analytical grade.

Standard solutions

Stock solutions of I and the I.S. were prepared by dissolving the compounds in distilled water. Working standard solutions (0.5, 1.0, 2.0, 4.0, 7.0 and 10.0 μg/ml I containing 1.0 μg/ml I.S.) were prepared from these stock standards.

Instrumentation and chromatographic conditions

The HPLC system included a Kontron (Zurich, Switzerland) Model 420 pump equipped with an SSI pulse damper and a Waters (Bedford, MA, USA) Wisp 712 automatic sampler. The detector system consisted of an ESA (Bedford, MA, USA) Model 5100 electrochemical detector equipped with a Model 5020 guard cell, placed in line before the injector, and a Model 5011 analytical cell operating in an oxidative screen mode. Data acquisition and processing were performed with a Waters Maxima 820 software program.

Reversed-phase HPLC separation was carried out at room temperature using a guard column (25 mm × 2.4 mm I.D.) packed with Bondapak C_{18}/Corasil 37–50 μm (Waters) in series with a

Spherisorb ODS 2 column (250 mm × 4.6 mm I.D., particle size 5 μm). The mobile phase was acetonitrile–50 m*M* sodium acetate (46:54, v/v). The pH was adjusted to 7.5 with glacial acetic acid. The mixture was filtered through a 0.2-μm filter and then deaerated for 15 min, using a sonicator, before being pumped through the column. The flow-rate was 1.2 ml/min. The applied potential of the screen electrode (D1) was set at 0.45 V, and that of the sample electrode (D2) at 0.70 V. The guard cell potential was set at 0.9 V. The gain on the sample electrode was set at 10 × 10, and the response time was 4 s.

Quantitative analysis of underivatized racemates of I was carried out for the determination of extraction yield from plasma (see above). In this case a Phenomenex C_{18} column (300 mm × 3.9 mm I.D., particle size 10 μm) was used, and the mobile phase was acetonitrile–sodium dihydrogenphosphate (pH 3.2) (25:75, v/v) containing 0.005 *M* heptanesulphonic acid at a flow-rate of 1.5 ml/min. The detector potentials were 0.5 V (D1), 0.7 V (D2) and 0.9 V (guard cell). This method was previously validated (data not reported).

The HPLC–MS analyses were carried out on a Hewlett-Packard 5988 quadrupole mass spectrometer via a thermospray interface with the filament current on (0.2 A). The vaporizer tip temperature was maintained at 140°C and the source temperature at 180°C. The analyser pressure was kept at *ca.* $1.5 \cdot 10^{-5}$ Torr, with a mobile phase flow-rate of 0.5 ml/min (water–acetonitrile, 50:50, v/v). ^{1}H NMR spectra were recorded at 300.13 MHz on a Bruker CXP 300 instrument in [$^2H_4$]methanol. The assignment of the protons was achieved through homonuclear decoupling experiments.

Sample preparation

Extraction of free compound I. Plasma aliquots (1 ml) were placed in screw-cap tubes and 100 ng of the I.S. (100 μl of 1 μg/ml solution) were added. The samples were alkalinized with 1.5 ml of 0.5 *M* borate buffer (pH 9.5) and extracted twice with 5 ml of diethyl ether. The tubes were mechanically shaken for 15 min and centrifuged for 10 min at 690 *g*. Each organic phase was transferred into another tube, and 1.5 ml of 0.01 *M* HCl were added. The samples were shaken and centrifuged as previously and, finally, the organic phases were discarded. The acidic phases were added to 2.5 ml of 0.5 *M* borate buffer (pH 9.5) and extracted with 7 ml of 90:10 diethyl ether–methanol. Organic phases were evaporated to dryness under a gentle stream of air, with the tubes in a water-bath (37°C).

Extraction of total (free + conjugated) compound I. Each plasma sample (0.5 ml) was added to 0.15 ml of 1 *M* acetate buffer (pH 4.7) and 50 μl of 80 mg/ml sulphatase. Samples were kept in a bath at 37°C for 16 h. The I.S. (100 μl of 1 μg/ml solution) and 2 ml of 0.5 *M* borate buffer (pH 9.5) were added to the samples. The extraction procedure was the same as described for free compound I.

Recovery

The extraction yield from plasma was determined at three different concentrations ($n = 5$ each).

Two experiments were carried out. In the first, blank plasma samples (1 ml) were extracted as described (Fig. 2). After evaporation of the organic phase, 100 μl of water solution containing I (corresponding to 50, 200, 400 ng/ml of plasma), the I.S. (100 ng) and the external standard (E.S.) (100 ng) were added. The solutions were evaporated to dryness, dissolved in the HPLC mobile phase (200 μl) and injected (30 μl) using the appropriate chromatographic conditions previously described for the analysis of the racemates.

In the second experiment, plasma samples were spiked with a known amount of I (50, 200, 400 ng/ml) and the I.S. (100 ng/ml) and then extracted as described. After evaporation of the organic phase, 100 μl of the 1 μg/ml E.S. water solution were added. The solutions were evaporated to dryness, dissolved in the HPLC mobile phase and injected.

Derivatization

The reaction medium was acetonitrile–0.05% sodium bicarbonate solution (80:20, v/v); the pH

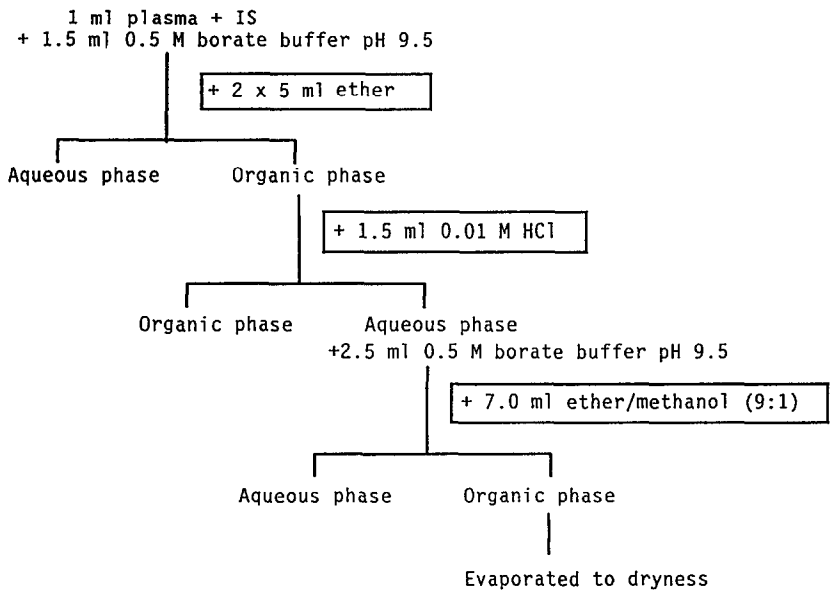

Fig. 2. Extraction scheme.

was adjusted to 7.5, at room temperature, by dropwise addition of 0.1 *M* HCl. The residue obtained after extraction and evaporation (free or total I, Fig. 2) was dissolved in the reaction medium (100 μl) and a 0.4% MBIC acetonitrile solution (10 μl) was added. The samples were allowed to stand at room temperature (25 ± 2°C) for 16 h and, finally, 0.001 *M* HCl (100 μl) was added. Aliquots of 50 μl were injected into the chromatographic system. The derivatization yield was optimized by adjusting temperature, time and reagent ratios of the reaction medium.

Calibration and calculation

Calibration curves were obtained using drug-free human plasma spiked with known amounts of I in a concentration range expected to include the unknown samples. Six concentrations were used for the calibration curve: 50, 100, 200, 400, 700 and 1000 ng/ml I, corresponding to 13.5, 27, 54, 108, 189 and 270 ng/ml +A and −A, and to 11.5, 23, 46, 92, 161 and 230 ng/ml +B and −B.

Peak-height ratios of each derivative to the I.S., obtained from plasma standards, were plotted against the concentration of each stereoisomer to generate the linear least-squares regression line. The responses related to each diastereoisomer obtained on three different days were used to calculate a global linear least-squares regression line. The analysis of variance (*F*-test, $\alpha = 0.05$) and lack of fit were used to confirm the significance of the regression and the adequacy of the linear model [12].

Precision and accuracy

The precision of the method was evaluated by calculating the intra-day and the inter-day coefficients of variation (C.V.), using plasma spiked with three different concentrations (100, 400 and 700 ng/ml) of I, corresponding to 27, 108 and 189 ng/ml +A and −A, and 23, 92 and 161 ng/ml +B and −B.

To estimate the intra-day C.V., replicated spiked samples ($n = 5$) were analysed; concentrations were calculated using the appropriate daily standard curve. The inter-day C.V. was calculated by analysing duplicate spiked plasma samples, using the appropriate curve obtained daily on three consecutive days.

The accuracy was evaluated by calculating the relative error on the total number of samples assayed ($n = 12$) for each concentration.

RESULTS AND DISCUSSION

The chiral derivatization of I with MBIC, carried out according to the literature [13] in the sodium bicarbonate–acetonitrile (50:50, v/v) reaction medium, resulted in the simultaneous formation of the mono-MBIC derivatives of +B, −B, −A and +A, and of di-MBIC derivatives (Fig. 3).

It was noted that, by lowering the pH of the reaction medium, the peak height of di-MBIC derivatives was reduced, leading to selective formation of the urea derivatives when the pH was less than 7.5 (Fig. 4). Using a phosphate buffer instead of sodium bicarbonate at equimolar concentration, the results were the same. Furthermore, to obtain quantitative derivatization, different ratios of the sodium bicarbonate–acetonitrile mixture were assayed, and a ratio of 80:20 was found to be the most appropriate for compound I.

Under these conditions, the reaction time-course of I with MBIC was studied. The kinetics of derivatization and the detector sensitivity were the same for all four stereoisomers. To obtain more accurate results, a single stereoisomer (+A) was repetitively derivatized and the reaction yield was monitored by measuring the underivatized substrate and the corresponding urea derivative in the same chromatogram. A period of 16 h was established as the optimal reaction time (Fig. 5). The yield and reaction derivatization time did not change, even at a temperature of 40°C.

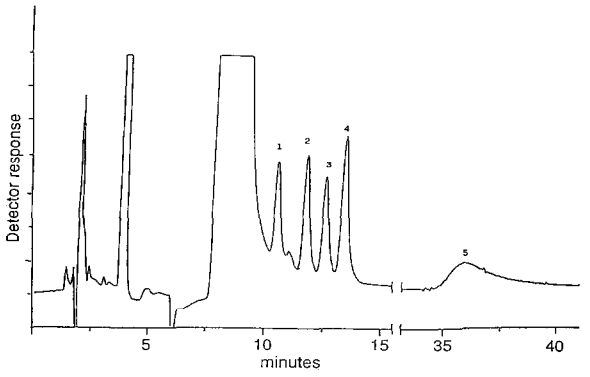

Fig. 3. Typical chromatogram obtained after MBIC derivatization of I conducted according to the literature [13], leading to the formation of urea derivatives (+B, −B, −A, +A) and urea-carbamate derivatives (di-MBIC derivatives, not chromatographically resolved under these conditions). Peaks: 1 = +B; 2 = −B; 3 = −A; 4 = +A; 5 = di-MBIC.

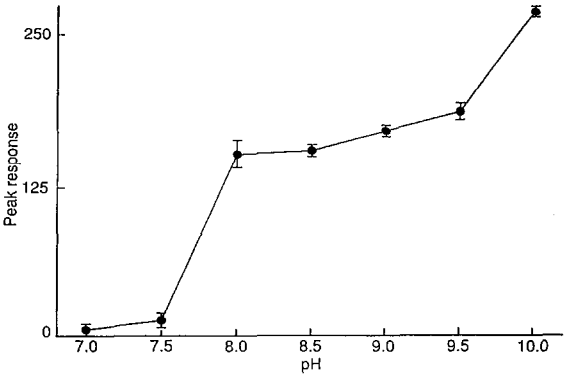

Fig. 4. Formation of di-MBIC derivatives of I (peak response. mean ± S.D., n = 3) as a function of pH.

Typical chromatograms obtained from plasma extracts after optimized derivatization are shown in Fig. 6. The order of elution of the diastereoisomers of I was established by separate derivatization of each optically pure stereoisomer [14].

Separation of vicinal peaks corresponding to the urea derivative of each stereoisomer was evaluated by the selectivity (α) and the resolution (R_s): the results are shown in Table I.

The structures of mono-MBIC derivatives were checked by submitting the sample to HPLC–MS analysis. The spectra of the four chromatographic peaks show the same qualitative fragmentation pattern (Fig. 7). Characteristic signals are the protonated molecular ion at m/z 505, the fragment ion at m/z 487 due to the loss of water, and the base peak at m/z 234.

Although the spectra were consistent with the structure of mono-MBIC derivatives, they did not indicate if the reaction occurred at the amino or the phenolic group. Therefore, the derivative of a single enantiomer (−B) was isolated and investigated by ^{1}H NMR. The spectrum of the compound (Fig. 8) shows a downfield shift (1.15 ppm) of the proton H-2 with respect to the free base of −B, indicating that the isocyanate gives rise to a urea derivative rather than a carbamate derivative.

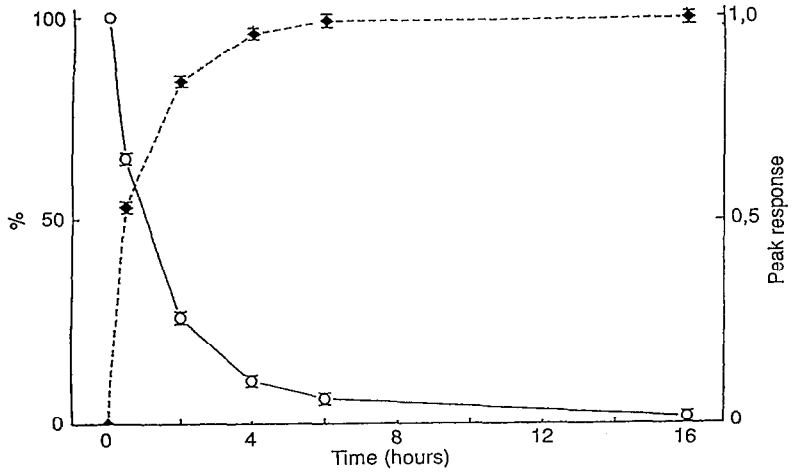

Fig. 5. Time-course of the reaction of +A (250 ng) with MBIC at pH 7.5: (○) percentage (mean ± S.D., n = 3) of underivatized +A remaining in the samples; (◆) corresponding peak response of urea derivative formed.

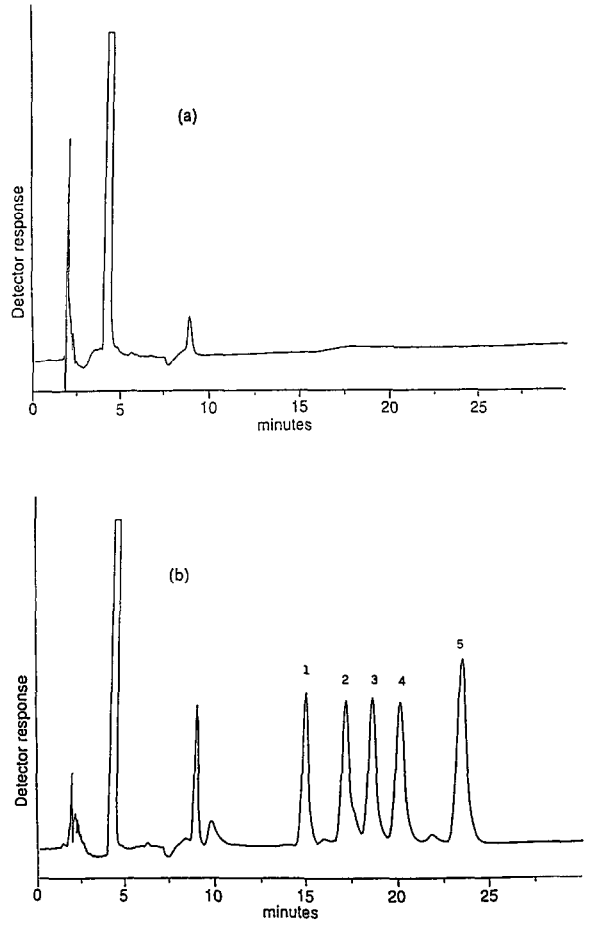

Fig. 6. Chromatograms of plasma extracts after optimized MBIC derivatization reaction. (a) Blank sample; (b) sample spiked with 400 ng/ml I as racemates. Peaks: 1 = +B (t_R = 14.8); 2 = −B (t_R = 16.9); 3 = −A (t_R = 18.2); 4 = +A (t_R = 19.9); 5 = I.S. (t_R = 23.3).

The di-MBIC derivative was also isolated, and its structure was confirmed by ^{1}H NMR analysis [14].

The combined use of a highly sensitive coulometric detector and good extraction techniques allowed quantitation of each stereoisomer at low levels. Another important factor was found to be the mobile phase pH, because it was noted that the degree of oxidation (and detector response) of urea derivatives increased when the pH was changed from low to high values (data not reported).

Linear calibration curves were calculated in the 11.5–230 ng/ml concentration range for +B and −B, and 13.5–270 for −A and +A. Table II

TABLE I

SELECTIVITY (α) AND RESOLUTION (R_s) OF UREA DERIVATIVES OF I

$\alpha = k'_2/k'_1$, where k'_2 and k'_1 are the capacity factors of peaks. $R_s = 2(t_{R2} - t_{R1})/(W_1 + W_2)$ where t_{R2} and t_{R1} are the retention times and W_1 and W_2 are the widths, at half-height, of the peaks of the diastereoisomers.

	α	R_s
+B/−B	1.17	6.10
−B/−A	1.10	3.69
−A/+A	1.09	3.42

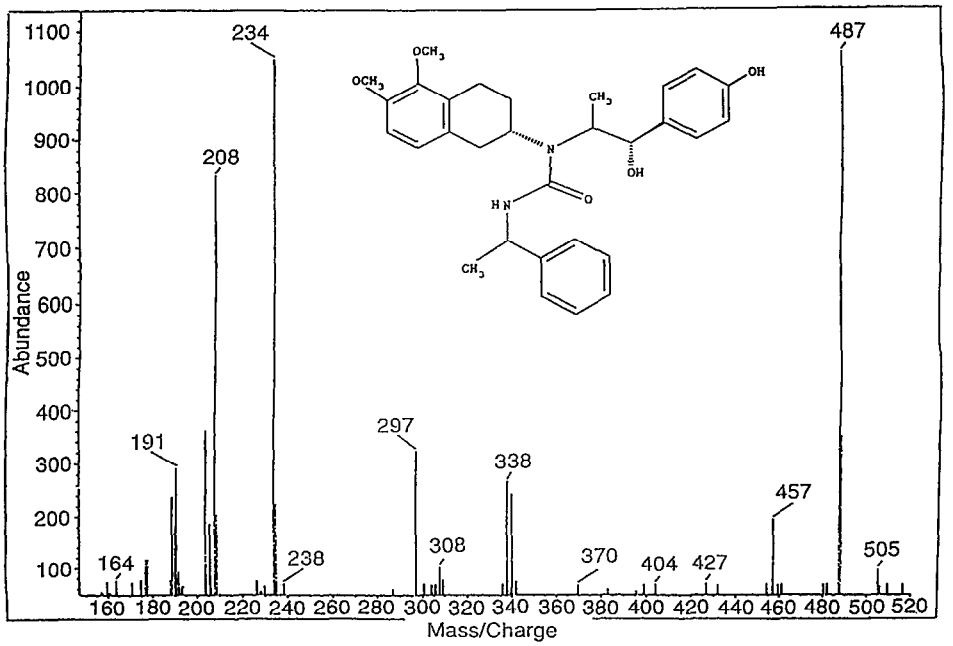

Fig. 7. Positive ion spectrum obtained from the chromatographic peak assigned as the mono-MBIC derivative of −B.

shows the mean (±S.D.) slope, intercept and correlation coefficient for each stereoisomer obtained from three different curves. The analysis of variance with the F-test ($\alpha = 0.05$) and lack of fit were used to confirm the significance of the regression and the adequacy of the linear model.

The limits of quantitation, defined as the amount of each stereoisomer per ml of plasma giving a signal-to-noise ratio of 10 [15], were 0.59 ng/ml for +B, 1.50 ng/ml for −B, 1.07 ng/ml for −A, and 1.15 ng/ml for +A.

The intra- and inter-assay precision (C.V. %) and the accuracy of the method at different concentrations of each stereoisomer are summarized

TABLE II

LINEAR REGRESSION PARAMETERS FOR THE STANDARD CURVES AND ANALYSIS OF VARIANCE OF THE GLOBAL CALIBRATION LINES

MS = mean squares; df = degrees of freedom. F_1 = MS regression (df = 1) / MS residual (df = 16); F_2 = MS lack of fit (df = 4) / MS pure error (df = 12). $F_1 > F_{tab}(1,16,0.95) = 4.49$: regression significant; $F_2 < F_{tab}(4,12,0.95) = 3.26$: lack of fit not significant.

	+B	−B	−A	+A
Slope ($\times 10^{-3}$) (mean ± S.D., $n = 3$)	4.87 ± 0.07	5.03 ± 0.06	4.48 ± 0.04	5.03 ± 0.06
Intercept ($\times 10^{-2}$) (mean ± S.D.; $n = 3$)	−1.22 ± 0.18	−1.60 ± 0.39	−1.83 ± 0.51	−2.46 ± 0.14
r^2 (mean ± S.D.; $n = 3$)	0.9992 ± 0.0010	0.9995 ± 0.0004	0.9988 ± 0.0009	0.9977 ± 0.0014
F_1	24 797.96	12 739.12	12 676.28	6025.03
F_{tab}	4.49	4.49	4.49	4.49
F_2	2.87	0.89	2.79	1.96
F_{tab}	3.26	3.26	3.26	3.26

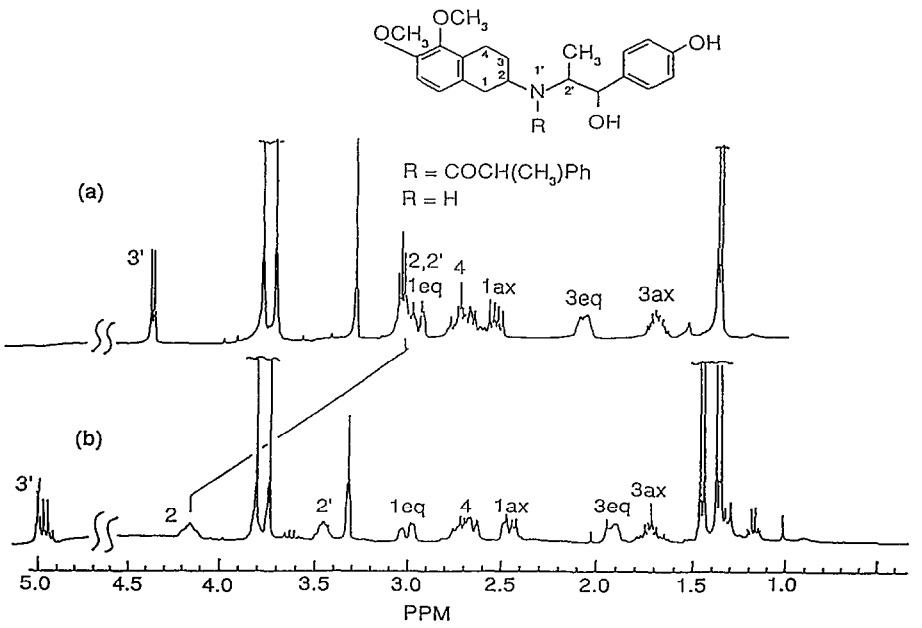

Fig. 8. Diagnostic downfield shift (1.15 ppm) of the H-2 proton in the mono-MBIC derivative of − B (b) with respect to the free base (a).

TABLE III

PRECISION AND ACCURACY OF THE METHOD

Amount added (ng/ml)	C.V. (%)		Accuracy total[c] (%)
	Intra-day[a]	Inter-day[b]	
Stereoisomer (+B)			
23	1.31	5.20	+0.41
92	1.11	2.21	−2.04
161	1.44	3.29	+3.21
Stereoisomer (−B)			
23	1.98	6.42	+4.45
92	0.65	0.56	−1.50
161	1.20	3.39	+3.27
Stereoisomer (−A)			
27	1.19	5.18	−0.81
108	0.95	1.93	−1.83
189	1.52	2.85	+2.88
Stereoisomer (+A)			
27	0.72	7.24	+4.45
108	1.23	0.38	−0.13
189	1.06	3.20	+3.07

[a] Calculated as $(s/x) \cdot 100$, where x is the mean amount found ($n = 5$) and s is its standard deviation.

[b] Calculated according to the previous formula, where x is the between-days mean amount found ($n = 6$) and s is its standard deviation.

[c] Calculated as $[(x - \mu)/\mu] \cdot 100$ where x is the total mean amount found ($n = 12$) and μ is the amount added.

in Table III. The results showed a good reproducibility between experiments.

The plasma recovery is reported in Table IV. The data refer to racemates of I, because the derivatization of the amount extracted was demonstrated to be selective and almost quantitative. The data showed a good recovery for both I and the I.S. Moreover, the recovery ratio of the drug to the I.S. was reproducible at different concentrations.

Similar chromatograms were obtained from human and rat plasma. The method was applied to determine free and total stereoisomers of I in plasma after oral administration of 50 mg/kg of the drug to rats. Typical chromatograms obtained after 15 min are shown in Fig. 9.

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TABLE IV

RECOVERY OF I AND THE I.S.

I added (ng/ml)	I.S. added (ng/ml)	Recovery (mean ± S.D. n = 5) (%)		I/I.S. recovery ratio (mean ± S.D.)
		I	I.S.	
50	100	75.1 ± 4.8	81.4 ± 1.6	0.922 ± 0.047
200	100	75.2 ± 3.2	85.2 ± 2.9	0.882 ± 0.009
400	100	76.4 ± 4.2	87.4 ± 3.1	0.871 ± 0.025

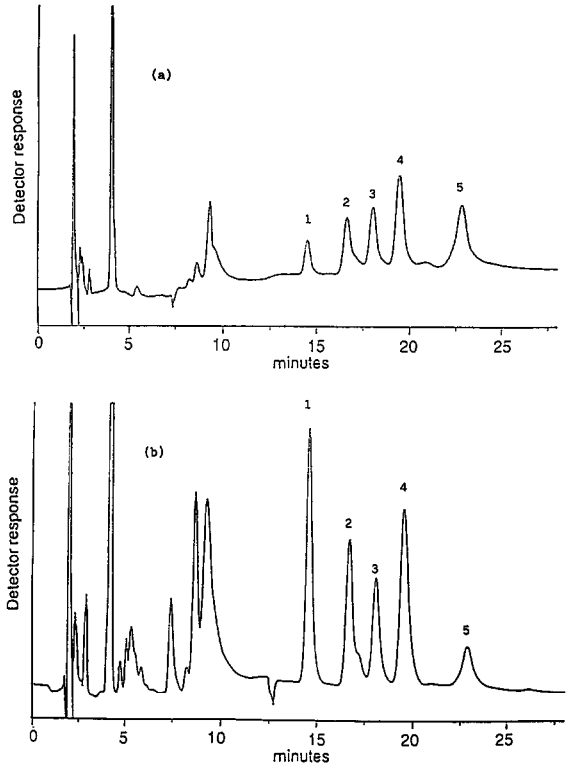

Fig. 9 Chromatograms obtained from rat plasma after oral administration of the drug (50 mg/kg) as racemates. Samples were submitted to the extraction procedure for the determination of free (a) and total (b) stereoisomers of I. Peaks: 1 = +B; 2 = −B; 3 = −A; 4 = +A; 5 = I.S.

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