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Assay of moguisteine metabolites in human plasma and urine: conventional and chiral high-performance liquid chromatographic methods

D. Castoldi *, A. Oggioni, M.I. Renoldi, E. Ratti, S. Di Giovine, A. Bernareggi

Boehringer Mannheim Italia S.p.A., Viale della Libertà Km. 0.750, 20052 Monza, Italy

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Abstract

Moguisteine is a novel peripheral non-narcotic antitussive agent. Pharmacokinetic studies in animal and in man showed that no unchanged drug is present in plasma, urine and faeces after oral administration. The main active metabolite, M1, is the free carboxylic acid of moguisteine, which maintains a stereogenic centre and consists of *R*(+)-M1 and *S*(−)-M1 enantiomers. M1 is partly metabolized to M2, its sulfoxidation derivative. A conventional HPLC method is described for the simultaneous determination of M1 and M2 in human plasma and urine after administration of therapeutic moguisteine doses. Plasma samples, previously acidified with phosphoric acid, are extracted with dichloromethane; urine samples are analyzed after appropriate dilution with methanol. Chromatography is performed using a Lichrosorb RP2 column and a linear gradient. M1 enantiomers can be determined in plasma extracts and urine samples by a chiral HPLC method using a β-cyclodextrin column. The analytical characteristics of both HPLC procedures proved to be adequate to analyze samples of subjects treated with therapeutic doses of moguisteine during clinical pharmacokinetic studies.

1. Introduction

Moguisteine, (*R*,*S*)-3-[2-(ethoxycarbonyl)-acetyl]-2-(2-methoxyphenoxymethyl)-thiazolidine (Fig. 1) is a novel peripheral non-narcotic antitussive agent [1]. The drug has been developed as a racemate because preclinical studies highlighted similar pharmacological and toxicological profiles for both moguisteine enantiomers (data on file, Boehringer Mannheim, Milan, Italia).

Moguisteine undergoes rapid and extensive metabolism following oral and intravenous administration of the racemate in animals and in man, and the unchanged drug cannot be detected in plasma and urine [2,3]. The main active metabolite corresponds to the free carboxylic acid, M1, which maintains the chiral centre and consists of a mixture of two enantiomers, *R*(+)-M1 and *S*(−)-M1 (Fig. 1). A secondary metabolite, M2, is formed by sulfoxidation of M1; this reaction generates a second stereogenic centre and M2 consists of a mixture of four optical isomers (Fig. 1). Conventional (non-chiral) chromatography may separate sulfoxide isomers in two peaks, coded M2/I and M2/II, each of them consisting of two enantiomers [2–4]. Conjugates

* Corresponding author.

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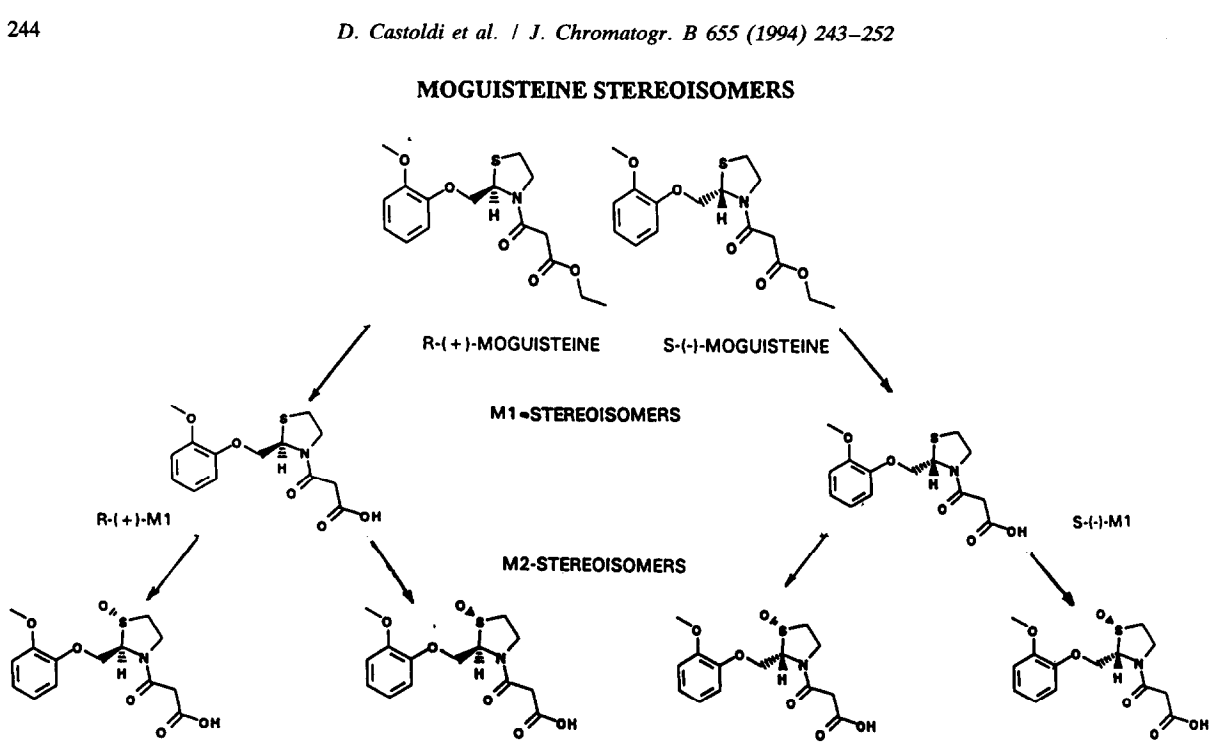

Fig. 1. Metabolic pathway of moguisteine.

of the carboxylic metabolites have never been detected.

This paper describes a conventional HPLC method for determining M1, M2/I and M2/II concentrations, and a chiral HPLC method to quantify *R*(+)-M1 and *S*(−)-M1 concentrations. Both methods are applied to human plasma and urine samples. These methods are currently used to study the pharmacokinetics of moguisteine metabolites in man.

2. Experimental

2.1. Chemicals

M1, M2/I, M2/II, *R*(+)-M1 and *S*(−)-M1 (enantiomeric purity >99%) were from Boehringer Mannheim Italia (Milan, Italy). Methanol and 2-propanol HPLC grade, sodium dihydrogen orthophosphate and analytical grade phosphoric acid were obtained from Merck (Darmstadt, Germany)

2.2. Non-chiral method

Sample handling

Plasma samples (1 ml) were acidified with 1 *M* phosphoric acid (0.2 ml) to pH 3–4 and extracted with dichloromethane (8 ml). After the extraction, the organic phase (7.5 ml) was dried under a nitrogen stream at room temperature. The residue was redissolved in 0.1 ml water–methanol (1:1, v/v). Aliquots of 20 μl were employed for chromatographic analysis.

Urine samples (0.2 ml) were analyzed directly (without extraction) after dilution with methanol (0.8 ml) and filtration through a Millex-GV 0.22-μm filter (Millipore). Aliquots of 10 μl were employed for chromatographic analysis.

Equipment and chromatographic settings

The HPLC system consisted of a Model 510 pump, a 6000A pump, a Wisp Model 710 B automatic injector, a 490 UV detector set at 230 nm (Waters, Milford, MA, USA). A Waters 840 (release 5.0) data and chromatography control station and a Perkin-Elmer (Cupertino, CA,

USA) chromatographic data management system (P.E. Nelson Access Chrom, release 1.7) running on a Microwax 3100 computer (Digital, Maynard, MA, USA) were also employed. The column was a Lichrosorb RP2, 7 μm, 250 × 4.6 mm I.D. from Service T.L. (Milan, Italy). Chromatographic separations were performed using a linear gradient with the following solutions: Solution A: 0.05 *M* phosphate buffer pH 3.4–methanol–isopropanol (85.5:9.5:5, v/v/v); Solution B: 0.05 *M* phosphate buffer pH 3.4–methanol–isopropanol (66.5:28.5:5, v/v/v). The phosphate buffer was prepared by mixing 0.05 *M* NaH_2PO_4 and 0.05 *M* H_3PO_4 (95:5, v/v). The flow-rate was 1 ml/min and the analysis was performed at room temperature.

The samples were injected after an adequate equilibration time (30 min, 100% solution A). The composition of the eluent changed from 100% A to 16% A and 84% B according to a linear gradient in 50 min. Subsequently, the column was purged at least for 40 min with 100% solution B.

Stock solution

A stock solution was prepared by dissolving M1, M2/I and M2/II in a mixture of water–methanol (1:1, v/v) in order to obtain concentrations of 100 mg/l for each metabolite; the stock solution was prepared weekly and stored at 4°C.

Calibration curves in plasma and urine

For each metabolite, calibration curves were obtained by adding adequate volumes of the stock solution to drug-free human plasma or urine in order to cover concentrations ranging from 0.05 to 5 mg/l in plasma and from 5 to 750 mg/l in urine.

Plasma and urine samples spiked at 5 concentration levels were analyzed as described above and peak areas were plotted as a function of sample concentrations. Three replicates per concentration level were analyzed. Moreover, calibration samples were run on each day of analysis. Quantitation was performed by external standard method.

2.3. Chiral method

Sample handling, equipment and chromatographic settings

Plasma and urine samples were prepared as described above for the non-chiral method. The chromatographic separation was performed at room temperature using the equipment previously described, on a β-cyclodextrin column, Cyclobond 10 × 0.46 cm I.D. (Astec, Whippany, NJ, USA). A precolumn, 5 × 0.46 cm I.D., with the same characteristics of the analytical column was employed; moreover a RP18 Guard-Pak precolumn (Waters, part number 80040) was inserted between the pump and the sampler. The mobile phase consisted of 0.05 *M* sodium phosphate buffer pH 3.4–methanol (93:7, v/v) at a flow-rate of 1.2 ml/min. The absorbance was measured at 230 nm.

Stock solution

A stock solution of *R*(+)-M1 and *S*(−)-M1 was prepared by dissolving the enantiomers in methanol in order to obtain concentrations of 1 mg/ml for both compounds; the stock solution was prepared weekly and stored at 4°C.

Calibration curves in plasma and urine

The calibration samples were obtained by adding known volumes of the stock solution to blank plasma or urine to obtain final concentrations ranging from 0.2 to 3.0 mg/l for both enantiomers in plasma and from 25 to 300 mg/l in urine. Three samples per each of the 5 concentration levels were analyzed. Quantitation was performed by external standard method.

2.4. Method validation

Linearity

The linearity of the analytical methods was tested by plotting the analytical response (peak area) as a function of the analyte concentration in spiked blank plasma and urine samples.

Recovery

The recovery of the methods in plasma and urine was calculated by replicate analysis of

blank samples spiked with known amounts of analytes, as the percent ratio of the concentration found to the concentration added. Peak areas obtained from extracted plasma samples or diluted urine samples containing known concentrations of analytes, were compared to peak areas obtained from direct injection of the corresponding amount of each analyte in standard solution.

Accuracy

The accuracy of the methods in plasma and urine was evaluated by replicate analysis of blank samples spiked with known amounts of analytes, at two concentration levels. The accuracy was calculated as the percent ratio of the concentration measured to the concentration added in plasma and urine samples. Quantitation was performed by using the calibration curve set up in biological matrices.

Precision

The precision of the methods was evaluated in plasma and urine by replicate analysis of blank samples spiked with known amounts of analytes, at two concentration levels for two consecutive days. It was calculated as the coefficient of variation (C.V.%) of independent replicate determinations at each of the two concentration levels.

3. Results

3.1. Non-chiral method

Specificity

Chromatograms corresponding to standard solutions, blank plasma and blank urine samples, plasma and urine samples spiked with M1, M2/I, M2/II and samples collected from subjects treated orally with 200 mg moguisteine, are shown in Fig. 2. Metabolites were eluted within 40 min from the start of the chromatogram. Unchanged moguisteine, injected as standard solution or added to blank urine and plasma samples (after inhibition of esterase activity), eluted approximately 20 min later (peak not shown in the HPLC tracings).

The RP2 column was chosen after evaluation of RP8, RP18, cyano- and diol-stationary phases. Unlike the others, the RP2 column gave both symmetric peaks and baseline separations of the moguisteine metabolites, characterized by slightly different polarity. Peaks interfering with the assay have never been detected in the analysis of plasma or urine samples collected from untreated subjects.

Limit of detection

The minimum detectable concentration for M1, M2/I and M2/II was 0.02 mg/l in plasma and 2 mg/l in urine for a signal-to-noise ratio of 3.

Limit of quantitation

The minimum measurable plasma concentrations of M1, M2/I and M2/II were 0.05 mg/l (M1) and 0.025 mg/l (M2/I and M2/II). In urine, the minimum measurable concentration was 5 mg/l for M1 and 3.75 mg/l for M2/I and M2/II. The signal-to-noise ratio was 5 for both plasma and urine. These values cover the concentrations of interest in clinical pharmacokinetic studies.

Linearity and calibration curves

The detector response for each metabolite of moguisteine was linear in the range from 10 to 1500 ng injected. In plasma, the calibration curve for M1 was linear in the range from 0.05 mg/l to 5 mg/l and from 0.025 mg/l to 250 mg/l for M2/I and M2/II; in urine, calibration curves were linear from 5 mg/l to 750 mg/l (M1) and from 3.77 mg/l to 750 mg (M2/I and M2/II).

The correlation coefficients of the calibration curves in plasma were 0.9999 for M1, 0.9999 for M2/I and 0.9991 for M2/II. In urine, the correlation coefficients were 0.9989 for M1, 0.9992 and 0.9996 for M2/I and M2/II, respectively.

The intercept values were not significantly different from zero. Moreover, assuming the curvilinear model $y = a + bx + cx^2$, the values for the coefficient of the quadratic term (c) were found to be included in the confidence interval around the zero value (95% confidence limit). This indicates that deviation from linearity may be neglected.

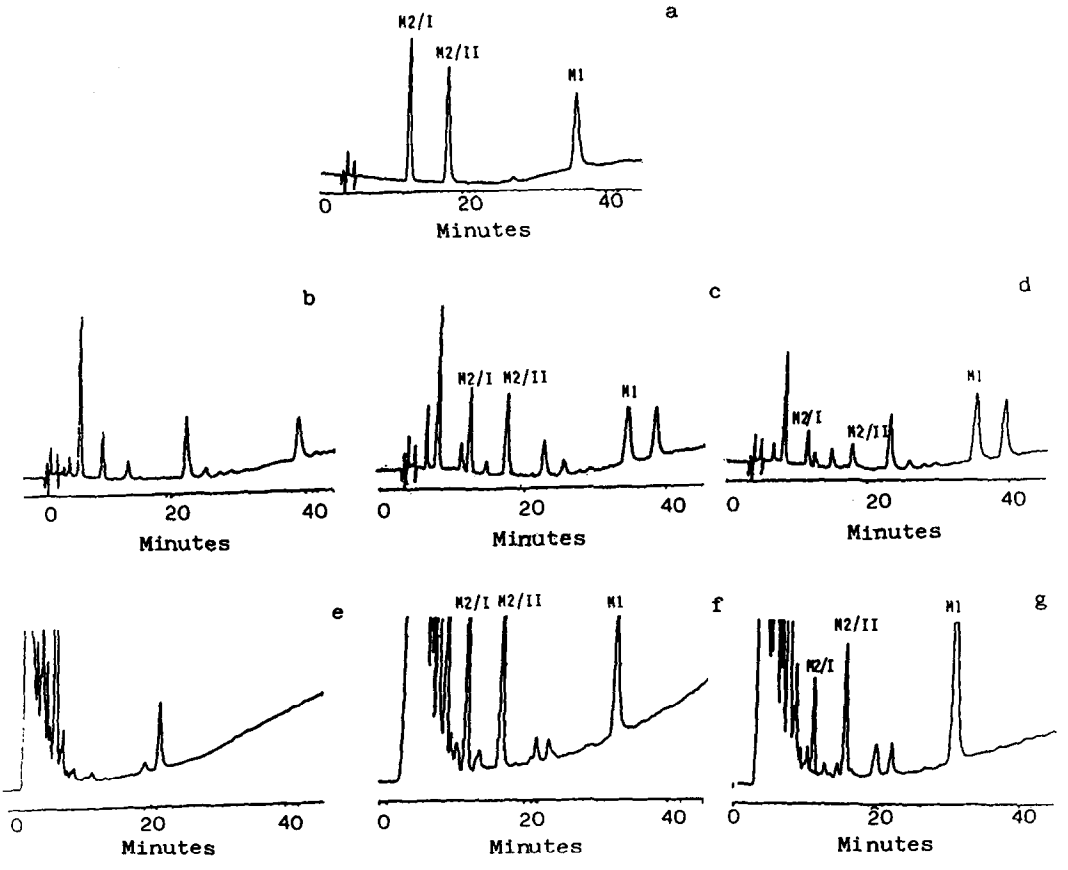

Fig. 2. Representative chromatograms obtained with the non-chiral method. (a) Standard solution of M1, M2/I and M2/II (200 ng injected); (b) blank plasma sample; (c) plasma sample enriched with M1, M2/I and M2/II (1 mg/l); (d) plasma sample obtained from a subject treated with 200 mg moguisteine; (e) blank urine sample; (f) urine sample enriched with M1, M2/I and M2/II (150 mg/l); (g) urine sample obtained from a subject treated with 200 mg moguisteine.

Recovery

Plasma recovery of all moguisteine metabolites after sample extraction with dichloromethane was rather incomplete (Table 1). For this reason, quantitation of sample concentrations was performed by interpolation on calibration curves set up in the biological matrices. On the other hand, recovery proved to be complete in urine, where sample handling consisted simply in dilution with methanol (Table 2).

Accuracy and precision

Accuracy and precision (C.V.%) of the non-chiral method were satisfactory and in keeping with international standards for analytical method validation [5].

In plasma, method accuracy and precision for M1 determination were found to be 102.1% at 0.25 mg/l and 94.8% at 3 mg/l; for M2/I and M2/II determination, accuracy and precision were respectively 94.0% and 115.4% at 0.125 mg/l, 95.4% and 93.8% (at 1.5 mg/l (Table 3).

In urine, the accuracy was found to be 98.3% at 25 mg/l and 98.6% at 500 mg/l for M1, 96.9% and 99.8% at 18.75 mg/l, 101.1% and 102.8% at 375 mg/l respectively for M2/I and M2/II (Table 4).

The described analytical procedure is currently employed for clinical pharmacokinetic studies.

Table 1
Recovery of the non-chiral analytical method for the determination of M1, M2/I and M2/II in human plasma ($n = 3$)

Concentration added (mg/l)	M1		M2/I		M2/II	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
0.025			84.12	18.96	44.29	11.95
0.05	88.25	13.84				
0.125			65.25	16.17	74.08	20.70
0.25	89.64	4.84				
0.50			61.30	4.54	75.70	6.31
1.00	84.00	3.89				
1.50			63.10	3.25	73.15	2.26
2.50			64.93	3.07	74.69	6.32
3.00	83.65	0.99				
5.00	85.97	3.21				

Table 2
Recovery of the non-chiral analytical method for the determination of M1, M2/I and M2/II in human urine ($n = 3$)

Concentration added (mg/l)	M1		M2/I		M2/I	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
3.75			104.06	23.78	84.90	12.50
5.00	117.65	27.03				
18.75			103.08	14.82	102.94	11.19
25.00	100.28	11.42				
187.5			100.09	7.22	105.20	8.00
250.0	100.92	9.67				
375.0			99.71	8.79	103.52	9.98
500.0	100.27	9.74				
750.0			99.01	8.28	103.35	10.01
1000.0	101.05	9.73				

Table 3
Accuracy and precision of the non-chiral analytical method for the determination of M1 M2/I and M2/II in human plasma ($n = 10$)

Compound	Concentration added (mg/l)	Concentration found (mg/l)	Accuracy (%)	Precision (C.V.%)
M1	0.250	0.255	102.1	6.2
	3.000	2.844	94.8	3.8
M2/I	0.125	0.118	94.0	12.1
	1.500	1.431	95.4	6.5
M2/II	0.125	0.144	115.4	12.0
	1.500	1.408	93.8	6.0

Table 4
Accuracy and precision of the non-chiral analytical method for the determination of M1, M2/I and M2/II in human urine ($n = 10$)

Compound	Concentration added (mg/l)	Concentration found (mg/l)	Accuracy (%)	Precision (C.V.%)
M1	25.00	24.60	98.3	3.0
	500.00	492.90	98.6	3.0
M2/I	18.75	18.20	96.9	3.3
	375.00	379.00	101.1	2.4
M2/II	18.75	18.70	99.8	4.9
	375.00	385.70	102.8	3.6

Fig. 3 shows the time course of mean plasma levels of M1, M2/I and M2/II in healthy volunteers after a single oral administration of 200 mg moguisteine.

3.2. Chiral separation

Specificity

Representative chromatograms of standard solutions of $R(+)$-M1 and $S(-)$-M1, blank plasma and urine, plasma and urine samples fortified with $R(+)$-M1 and $S(-)$-M1 and plasma and urine samples collected from a subject treated with moguisteine, are shown in Fig. 4.

No or negligible interferences due to plasma or urine components and to the other moguisteine metabolites, were observed in the HPLC tracings.

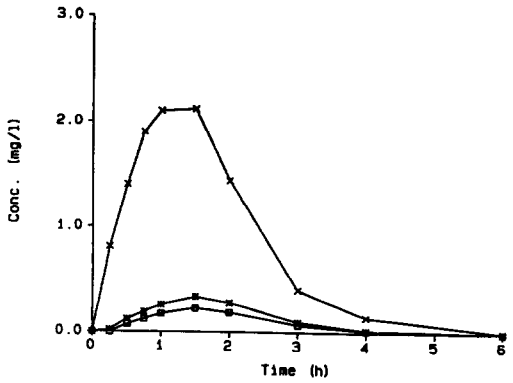

Fig. 3. Mean plasma concentration–time profiles for M1 (×), M2/I (□) and M2/II (*) after a single oral administration of 200 mg moguisteine (tablets) to 12 healthy subjects.

Limit of detection

The minimum detectable concentrations of $R(+)$-M1 and $S(-)$-M1 in plasma and urine samples were 0.1 mg/l and 10 mg/l, respectively at a signal-to-noise ratio of 3.

Limit of quantitation

The minimum measurable concentrations of $R(+)$-M1 and $S(-)$-M1 in plasma and urine samples were 0.2 mg/l and 25 mg/l, respectively, at a signal-to-noise ratio of 5.

3.2.4 Linearity and calibration curves

The linearity of the detector response was proved after injection of amounts of $R(+)$-M1 and $S(-)$-M1 ranging from 20 to 600 ng. The detector response was linear with correlation coefficients (r) of 0.9975 and 0.9999 for $R(+)$-M1 and $S(-)$-M1, respectively.

The calibration curves of the enantiomers were linear over the concentration range from 0.2 to 3 mg/l in plasma and from 25 to 300 mg/l in urine.

The correlation coefficients (r) of the calibration curves in plasma were 0.9900 and 0.9945 for $R(+)$-M1 and $S(-)$-M1 respectively; in urine, 0.9950 for $R(+)$-M1 and 0.9962 for $S(-)$-M1.

Recovery

The recovery of $R(+)$-M1 from human plasma over the concentration range from 0.2 to 3 mg/l varied, on the average, from 73.9% to 123.0% and that of $S(-)$-M1 from 53.2% to 103.8% (Table 5).

In urine, the recovery over the concentration

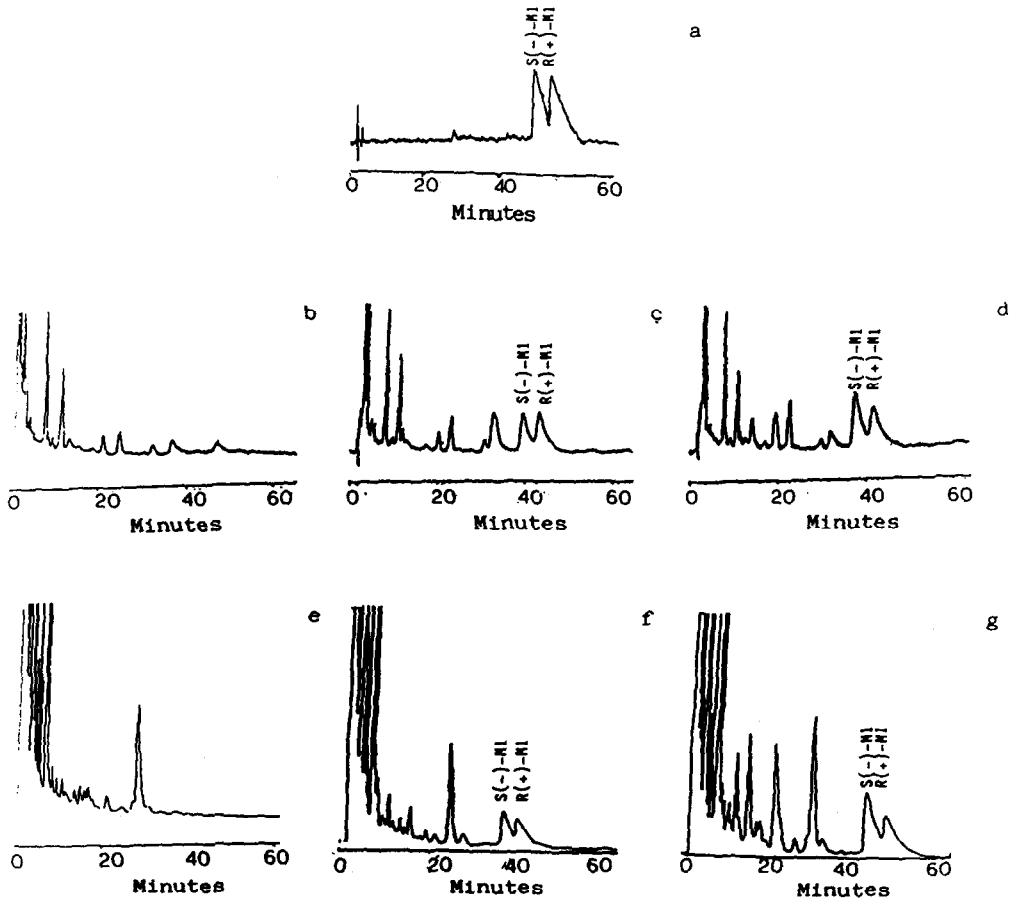

Fig. 4. Representative chromatograms obtained with the chiral method. (a) Standard solution of $R(+)$-M1 and $S(-)$-M1 (200 ng injected); (b) blank plasma sample; (c) plasma sample enriched with $R(+)$-M1 and $S(-)$-M1 (1 mg/l); (d) plasma sample obtained from a subject treated with 200 mg moguisteine; (e) blank urine sample; (f) urine sample enriched with $R(+)$-M1 and $S(-)$-M1 (100 mg/l); (g) urine sample obtained from a subject treated with 200 mg moguisteine.

Table 5
Recovery of the chiral analytical method for the determination of $R(+)$-M1 and $S(-)$-M1 in human plasma ($n = 3$)

Concentration added (mg/l)	$R(+)$-M1		$S(-)$-M1	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
0.2	73.9	6.9	53.2	18.7
0.4	123.0	4.1	78.7	10.2
1.0	110.7	10.4	93.6	13.6
2.0	78.3	17.1	90.8	8.8
3.0	75.1	24.0	103.8	17.6

Table 6
Recovery of the chiral analytical method for the determination of $R(+)$-M1 and $S(-)$-M1 in human urine ($n = 3$)

Concentration added (mg/l)	$R(+)$-M1		$S(-)$-M1	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
25.0	75.0	4.5	71.7	4.1
50.0	53.6	1.0	82.6	3.5
100.0	84.5	1.8	87.9	5.1
200.0	67.2	9.5	84.1	13.3
300.0	66.8	11.1	78.2	7.1

range from 25 to 300 mg/l varied from 53.6% to 84.5% for $R(+)$-M1 and from 71.7% to 87.9% for $S(-)$-M1 (Table 6).

Accuracy and precision

In plasma accuracy and precision were 107.5% (C.V.% 8.9) and 90.6% (C.V.% 11.4) for $R(+)$-M1 and $S(-)$-M1, respectively at a concentration of 1.0 mg/l (Table 7).

In urine accuracy and precision were 117.2% (C.V.% 15.7) and 103.1% (C.V.% 29.5) for $R(+)$-M1 and $S(-)$-M1, respectively at a concentration of 100 mg/l (Table 8).

The described analytical procedure is currently employed for clinical pharmacokinetic studies.

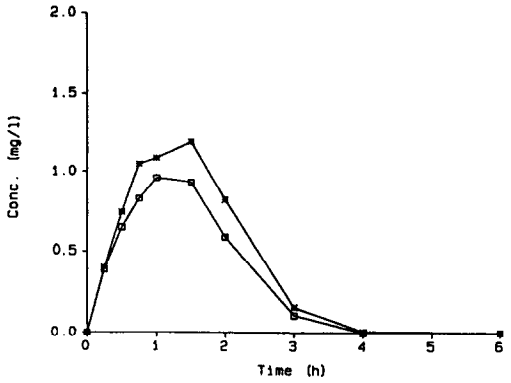

Fig. 5. Mean plasma concentration–time profiles for $R(+)$-M1 (*) and $S(-)$-M1 (×) after a single oral administration of 200 mg moguisteine (tablets) to 12 healthy subjects. The AUCs for the two enantiomers of M1 were not significantly different.

Fig. 5 shows the time course of the mean plasma concentrations of $R(+)$-M1 and $S(-)$-M1 in healthy volunteers treated orally with a single 200 mg moguisteine dose.

4. Discussion

The non-chiral method for measurement in human plasma and urine of concentrations of the

Table 7
Accuracy and precision of the chiral analytical method for the determination of $R(+)$-M1 and $S(-)$-M1 in human plasma ($n = 10$)

Compound	Concentration added (mg/l)	Concentration found (mg/l)	Accuracy (%)	Precision (C.V.%)
$R(+)$-M1	1.000	1.075	107.5	8.9
$S(-)$-M1	1.000	0.906	90.6	11.4

Table 8
Accuracy and precision of the chiral analytical method for the determination of $R(+)$-M1 and $S(-)$-M1 in human urine ($n = 3$)

Compound	Concentration added (mg/l)	Concentration found (mg/l)	Accuracy (%)	Precision (C.V.%)
$R(+)$-M1	100.0	117.2	117.2	15.7
$S(-)$-MI	100.0	103.1	103.1	29.5

moguisteine metabolites M1, M2/I and M2/II, is characterized by high specificity and good accuracy and precision. Extensive application of the method for the assay of plasma and urine samples of subjects treated with therapeutic doses of moguisteine (200 mg orally), confirmed the usefulness of the method in clinical pharmacokinetic studies. The long run-time of the gradient analysis is necessary for the elution of other peaks with retention times longer than 40 min (not shown in Fig. 2). Moreover the described chromatographic conditions allow the detection of the possible presence of unchanged moguisteine in plasma samples.

The chiral assay allows the satisfactory separation of the enantiomers of M1 in plasma and urine. It is worth mentioning that the performance of β-cyclodextrin columns varies from one column to another and the efficiency decreases rapidly. We selected columns characterized by a sufficient stability capable of performing 80 to 100 consecutive analyses. Chiral columns have usually less chromatographic efficiency than conventional columns. Probably the efficiency of the chiral column can be preserved by using a switching procedure. However, the efficiency of the chromatographic system may then decrease and become insufficient to analyze the M1 enantiomers.

The limits of detection and quantitation are higher than those of the non-chiral method used for the determination of M1, M2/I and M2/II in plasma and urine. However the accuracy and the precision of the method for the determination of M1 enantiomers in plasma and urine are considered acceptable for a chiral assay in biological fluids. This method proved to be adequate for determining plasma and urine concentrations of $R(+)$-M1 and $S(-)$-M1 in clinical pharmacokinetic studies after therapeutic doses of moguisteine.

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