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High-performance liquid chromatographic analysis of methocarbamol enantiomers in biological fluids

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ABSTRACT

Methocarbamol enantiomers in rat and human plasma were quantified using a stereospecific high-performance liquid chromatographic method. Racemic methocarbamol and internal standard, (*R*)-(−)-flecainide, were isolated from plasma by a single-step extraction with ethyl acetate. After derivatization with the enantiomerically pure reagent (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate, methocarbamol diastereomers and the (*R*)-flecainide derivative were separated on a normal-phase silica column with a mobile phase consisting of hexane–isopropanol (95:5, v/v) at a flow-rate of 1.6 ml/min. Ultraviolet detection was carried out at a wavelength of 280 nm. The resolution factor between the diastereomers was 2.1 (α = 1.24). An excellent linearity was observed between the methocarbamol diastereomers/internal standard derivative peak-area ratios and plasma concentrations, and the intra- and inter-day coefficients of variation were always < 9.8%. The lowest quantifiable concentration was 0.5 μg/ml for each enantiomer (coefficients of variation of 9.8 and 8.8% for (*S*)- and (*R*)-methocarbamol, respectively), while the limit of detection (signal-to-noise ratio 3:1) was approximately 10 ng/ml. The assay was used to study the pharmacokinetics of methocarbamol enantiomers in a rat following intravenous administration of a 120 mg/kg dose of racemic methocarbamol and to evaluate plasma and urine concentrations in a human volunteer after oral administration of a 1000-mg dose of the racemate. The method is suitable for stereoselective pharmacokinetic studies in humans as well as in animal models.

INTRODUCTION

(*R*,*S*)-Methocarbamol, (*R*,*S*)-3-(2-methoxyphenoxy)-1,2-propanediol-1-carbamate, is a well known skeletal muscle relaxant which has been commercially available for over three decades. It is formulated as a single entity and in combination with such analgesics as acetylsalicylic acid, acetaminophen and codeine for the relief of discomfort associated with acute, painful musculoskeletal conditions [1].

Human and animal studies show that about 98% of a dose is excreted in the urine in 72 h, with less than 1% as unchanged drug and the remainder as unconjugated and conjugated metabolites [2–5]. The two major metabolites isolated in humans and rats are inactive and have been identified as 3-(2-hydroxyphenoxy)-1,2-propanediol-1-carbamate and 3-(4-hydroxy-2-methoxyphenoxy)-1,2-propanediol-1-carbamate [2,3].

The literature contains only limited information on the pharmacokinetics of methocarbamol. The plasma half-life in humans ranges from 1 to 2 h [6,7] and is reported to be between 0.5 and 1 h in animal models (rat, dog and horse) [3,5,8]. There do not appear to be any major differences among species studied in the metabolism and disposition of methocarbamol [2]. Dose-dependent kinetics have been suggested in the rat [8] and the horse [5].

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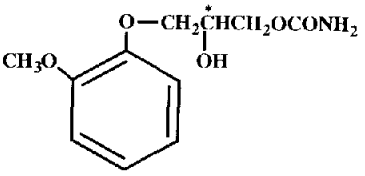

Fig. 1. Structure of (*R,S*)-methocarbamol. The asterisk denotes the location of the chiral center.

(*R,S*)-Methocarbamol is a chiral drug (Fig. 1) which is administered as a racemate. We are not aware of any published literature on the pharmacodynamic and pharmacokinetic properties of the enantiomers. In recent years it has been established that non-stereoselective data generated for racemic drugs can be misleading [9]. In light of these observations it is worthwhile to investigate methocarbamol enantiomers in order to obtain a complete and accurate understanding of the biological fate of this drug.

The specific purpose of this study was to develop a stereospecific assay [10] which could be used to determine the pharmacokinetics of methocarbamol enantiomers in humans and in animal models.

EXPERIMENTAL

Chemicals and reagents

(*R*)-(−)-Flecainide (internal standard) was a gift from Riker Labs. (St. Paul, MN, USA), while (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate (NEIC) and (*R*)-(−)-NEIC were purchased from Aldrich (Milwaukee, WI, USA). Racemic methocarbamol and (*R*)-methocarbamol were gifts from Whitehall-Robins (Mississauga, Canada) and Lee Labs. (Petersburg, VA, USA), respectively. Ethyl acetate was obtained from BDH (Edmonton, Canada) and was glass-distilled prior to use. Analytical-grade hexane and isopropanol were also obtained from BDH. HPLC-grade water was purchased from Canlab (Edmonton, Canada). β-Glucuronidase/arylsulfatase (EC 3.2.1.31/EC 3.1.6.1) from *Helix pomatia* was purchased from Boehringer Mannheim (Laval, Canada).

A stock solution of (*R,S*)-methocarbamol was obtained by dissolving 10 mg in 100 ml of water. Working solutions of methocarbamol in plasma and urine were then prepared to give final concentrations of 0.5, 1, 2, 5, 10 and 30 μg/ml. A 10 μg/ml aqueous working solution of the internal standard was prepared by dissolving 1 mg of (*R*)-(−)-flecainide acetate in 100 ml. The 0.2% solution of (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate in ethyl acetate was made by diluting 20 μl of reagent in 10 ml of solvent.

Instrumentation

The high-performance liquid chromatographic (HPLC) system (Waters Scientific, Mississauga, Canada) consisted of a 590 pump, a 481 variable-wavelength UV detector set at 280 nm and a 3390A integrator–recorder (Hewlett-Packard, Mississauga, Canada). Electron-impact mass spectra were recorded on a double-focusing magnetic sector instrument (Kratos MS-50, Manchester, UK). Optical rotations of the derivatives and of (*R*)-methocarbamol were measured in a Perkin-Elmer 241 polarimeter (Rexdale, Canada). UV spectra were recorded on a PU 8700 scanning UV- visible spectrophotometer (Philips, UK). NMR spectra were recorded on a high-resolution Bruker AM 300 Fourier transform spectrometer (Bruker Spectrospin, Milton, Canada) with tetramethylsilane as internal standard. Samples were mixed with an IKA Vibrax UXR mechanical shaker (Terochem, Edmonton, Canada), centrifuged with a Clay-Adams centrifuge (Parsippany, NJ, USA), and heated in a Reactitherm block (Pierce, Rockford, IL, USA).

Chromatographic conditions

A Partisil 5 silica analytical column (250 mm × 4.6 mm I.D.) (Whatman, Clifton, NJ, USA) was used for the chromatographic separation of the diastereoisomers. The mobile phase was a mixture of hexane and isopropanol (95:5, v/v) and the flow-rate was 1.6 ml/min. A silica Prep-Nova-PAK HR column (6 μm, 100 mm × 25 mm I.D. PrepPak cartridge, Waters Scientific) was used for the semi-preparative separation of methocarbamol diastereomers. The reversed-phase analytical column, μBondapak C_{18} (5 μm, 300 mm × 3.9 mm I.D.), was purchased from

Waters Scientific. The fused-silica Chirasil-Val III gas chromatographic capillary column (50 m) was obtained from Terochem (Edmonton, Canada), while the DB-5 (50 m) and DB-1 (50 m) columns were purchased from J & W Scientific (Rancho Cordova, CA, USA).

Sample collection

One rat was sacrificed (cardiac puncture) 1.5 h after intravenous administration of a 30 mg/kg dose of (*R,S*)-methocarbamol. Another rat underwent surgical incannulation of the jugular vein. A 120 mg/kg dose of (*R,S*)-methocarbamol was administered intravenously and blood samples were collected from the catheter at 0, 15, 30, 60, 90, 120 and 180 min. In one human subject a blood sample was taken by venipuncture 3 h after a 1-g oral dose. The 6-h urine output was collected and a 1:100 diluted sample was analysed directly according to the above procedure. A similar aliquot was incubated with β-glucuronidase/sulfatase at 37°C for 18 h and subsequently analysed. After centrifugation, plasma samples were stored at −20° until ready for analysis.

Sample analysis

Following addition of 0.3 ml of internal standard solution, plasma and diluted urine (1:100, v/v) samples were basified with 1 ml of 1 *M* NaOH, and 5 ml of ethyl acetate were added. After mixing on the mechanical shaker (20 min), the samples were centrifuged at 1000 *g* (5 min). The organic phase was separated and evaporated to dryness under a stream of nitrogen. The residues were reconstituted in 1 ml of distilled ethyl acetate, and 80 μl of the NEIC reagent solution were added to each tube. The mixtures were vortex-mixed for 5 s and heated at 85°C for 12 h. After derivatization the samples were evaporated and reconstituted in 0.2 ml of distilled ethyl acetate. Aliquots of 0.03–0.15 ml were injected into the HPLC apparatus.

Accuracy and precision

The accuracy and precision of the method were evaluated by analysing four replicates of spiked plasma samples at each of three concentrations against a calibration curve. The accuracy was evaluated as error percent, [(mean of measured − mean of added) / mean of added] × 100, while the precision was given by the inter-day and intra-day coefficients of variation (C.V.). The inter-assay reproducibility was also evaluated by computing the mean ± S.D. of the slope for different calibration curves at four different days.

RESULTS AND DISCUSSION

Methocarbamol is a relatively simple molecule which appeared to be readily amenable to gas chromatographic analysis. However, reactions with trifluoroacetic or pentafluoropropionic anhydrides, followed by chromatography on a Chirasil-Val III column, failed to resolve the enantiomers.

Efforts were then directed to the preparation of diastereomers by reaction with enantiopure derivatizing reagents including (−)-menthyl chloroformate, 1-[(4-nitrophenyl)sulfonyl]-L-prolyl chloride, (−)-α-chlorophenylacetyl chloride and (*R,R*)-O,O-diacetyltartaric acid anhydride. These reagents did not react with (*R,S*)-methocarbamol, either at room temperature or after heating. However, following reaction with (*S*)-(+)-NEIC, the peak representing underivatized methocarbamol disappeared from the gas chromatographic trace (DB-5 column). Unfortunately, chromatographic peaks corresponding to the diastereomers were not observed on this trace presumably as a consequence of thermal instability (numerous unidentified peaks were evident in the chromatograms). Gas chromatography was nevertheless useful during method development since the derivatization reactions could be optimized by monitoring for the disappearance of underivatized methocarbamol.

The reaction with NEIC does not occur to any significant extent under ambient conditions and while higher temperatures (up to 110°C) could reduce the reaction time to about 4–6 h, the increased proportion of interfering by-products (as evident by HPLC) was unacceptable. The reaction was considered complete when the peak of (*R,S*)-methocarbamol disappeared from the gas

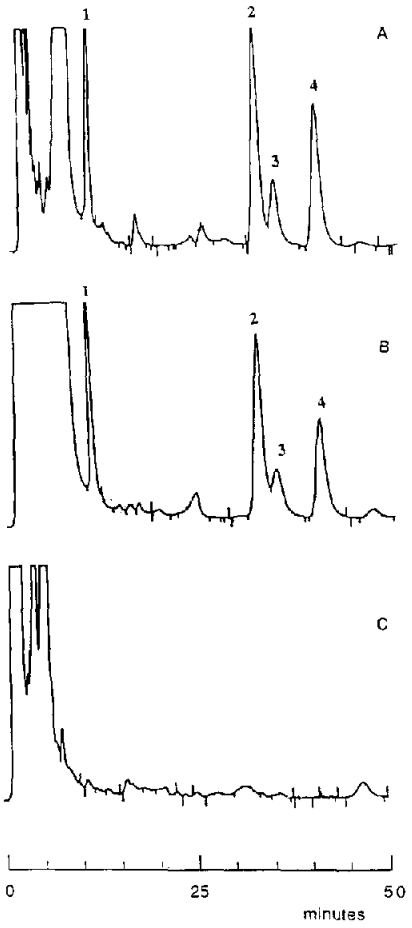

Fig. 2. Chromatograms of (A) blank rat plasma spiked with 5 μg of racemic methocarbamol and internal standard, (B) plasma sample taken from a rat 1.5 h after intravenous administration of a 30 mg/kg dose of racemic methocarbamol and (C) blank rat plasma. Peak 1 is the derivatized (*R*)-flecainide; peaks 2 and 4 are the diastereomers of (*S*)- and (*R*)-methocarbamol, respectively; peak 3 is unidentified.

chromatogram; this was accomplished after heating for 12 h at 85°C. Efforts to facilitate the reaction by means of microwave irradiation were unsuccessful.

Methocarbamol enantiomers were not resolved under reversed-phase HPLC conditions but were separated by normal phase with satisfactory results ($\alpha = 1.24$, $R_s = 2.10$). This allowed sufficient discrimination of an unidentified interfering peak from the first eluting diastereomer (Fig. 2). The diastereomers of methocarbamol eluted at 33 and 41 min (Fig. 2) while the internal standard, (*R*)-(−)-flecainide, derivative eluted at 12 min. Guaifenesin, a possible product of methocarbamol decomposition, does not interfere with the assay as it was found to elute, after derivatization, at approximately 20 min under our experimental conditions.

For the purpose of structure elucidation, the diastereomers were isolated from the appropriate HPLC eluent fractions. Samples submitted for mass spectral analyses were obtained by combining several fractions eluting from the analytical column, while optical rotation measurements and NMR analyses were determined following isolation of the respective diastereomers on the preparative HPLC system.

The direct inlet electron-impact mass spectra of the corresponding diastereomer fractions were superimposable. While they lacked the molecular ion (*m/z* 438), they contained fragments of diagnostic value which indicated that derivatization takes place on the alcohol OH. Plausible fragments are suggested in Fig. 3. NMR spectra also confirmed the structures of the two derivatives.

The UV spectra of the diastereomers were superimposable and showed maxima at 206 and 280 nm. The latter was used as the HPLC analytical wavelength.

The optical rotations of the diastereomers were determined by polarimetry (sodium D line, 589 nm). The first-eluting derivative was levorotatory while the second-eluting derivative was dextrorotatory. A sample of pure (*R*)-methocarbamol in methanol examined under the same conditions gave a positive optical rotation.

The (+)-diastereomer that elutes at 41 min under our chromatographic conditions corresponds to (*R*)-(+)-methocarbamol as confirmed by derivatizing and injecting a sample of pure (*R*)-methocarbamol. By exclusion the (−)-derivative was considered derived from (*S*)-(−)-methocarbamol. Reaction of the racemate and the pure (*R*)-(+)-methocarbamol with (*R*)-(−)-NEIC was equally successful and showed the expected transposition of retention times of the derivatives.

The extraction of methocarbamol isomers and

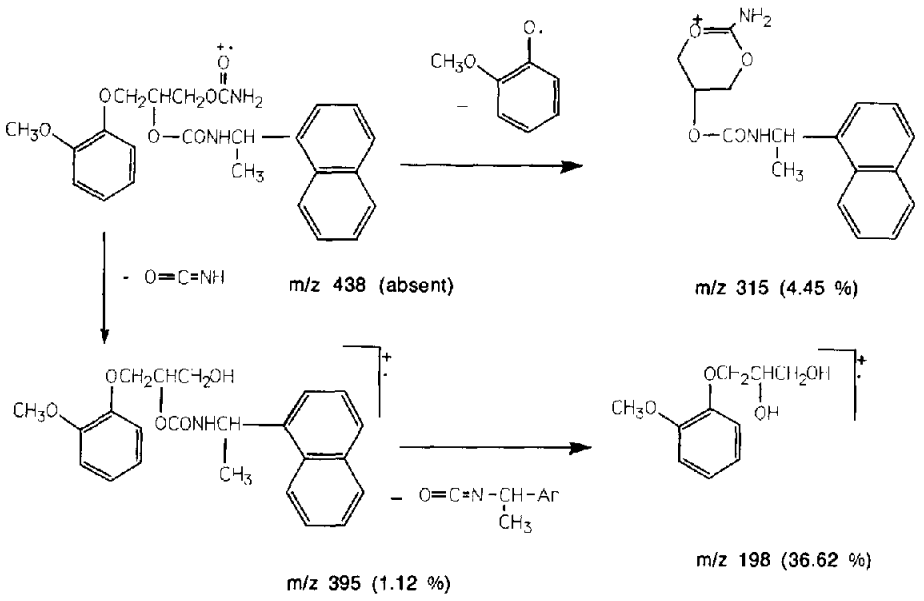

Fig. 3. Electron-impact mass spectral structures and proposed fragmentation mechanisms of the most diagnostic fragments of methocarbamol derivatives.

(*R*)-(−)-flecainide was performed under alkaline conditions in order to eliminate possible interferences from phenolic metabolites and endogenous plasma constituents, and to facilitate a complete extraction of the basic internal standard. Ethyl acetate was preferred to diethyl ether, hexane and chloroform as extraction solvent because it gave a better recovery of methocarbamol. Samples of 1 and 10 μg/ml were analysed in duplicate and compared to samples derivatized directly without extraction. The amount of methocarbamol recovered was 0.93 and 0.94 μg/ml (mean 93.5%) at the low concentration and 9.0 and 8.7 μg/ml (mean 89.5%) at the high concentration.

Excellent linearity was observed between methocarbamol derivatives/internal standard derivative peak-area ratios and plasma concentrations (range evaluated: 1–30 μg/ml racemate). Typical calibration curves gave the following equations: $y = -0.05 + 0.86x$ for (*S*)-methocarbamol and $y = -0.10 + 0.99x$ for (*R*)-methocarbamol with $r = 0.998$ and 0.999, respectively. Intra-day precision and accuracy (Table I) were satisfactory as were the inter-day precision and

TABLE I

INTRA-DAY ACCURACY AND PRECISION ($n = 4$)

Concentration (μg/ml)			Error (%)		C.V. (%)	
Theoretical	Measured		(*S*)	(*R*)	(*S*)	(*R*)
	(*S*)	(*R*)				
5.00	5.50	5.50	10	10	0.91	0.91
1.00	0.96	0.89	−4	−11	2.08	1.12
0.50	0.52	0.51	4	2	3.80	3.90

TABLE II

INTER-DAY ACCURACY AND PRECISION (n = 4)

Concentration (μg/ml)			Error (%)		C.V. (%)	
Theoretical	Measured		(*S*)	(*R*)	(*S*)	(*R*)
	(*S*)	(*R*)				
5.00	5.10	5.10	2	2	3.9	5.9
0.50	0.51	0.52	2	4	9.8	8.8

accuracy (Table II). The slopes of the curves did not change significantly on four different days; the mean for (*S*)-methocarbamol was 0.83 ± 0.05 and 1.03 ± 0.03 for (*R*)-methocarbamol.

The lowest quantifiable concentration was set at 0.5 μg/ml for each enantiomer. If a 3:1 signal-to-noise ratio criterion is applied, a detection limit of approximately 10 ng/ml could be claimed.

In order to assess the applicability of this analytical method to pharmacokinetic studies, plasma levels of methocarbamol enantiomers were measured in one rat at 1.5 h after intravenous administration of a 30 mg/kg dose of racemic methocarbamol. The concentration of (*S*)-methocarbamol was 2.38 μg/ml whereas the concentration of (*R*)-methocarbamol was 0.99 μg/ml (*S*/*R* enantiomer ratio = 2.40). A plasma sample taken from a human volunteer 3 h after a 1-g oral dose contained 2.57 and 2.16 μg/ml (*S*)- and (*R*)-enantiomer, respectively (*S*/*R* = 1.19). A 6-h urine specimen contained 12.8 and 11.2 μg/ml (total output of 3.61 and 3.16 mg) (*S*/*R* = 1.14). An aliquot of the 6-h urine specimen was analysed following incubation with β-glucuronidase/sulfatase at 37°C for 18 h. In this case the total output was 62.6 mg for (*S*)-methocarbamol and 51.3 mg for (*R*)-methocarbamol (*S*/*R* = 1.22). The recovery of the (*R*,*S*)-methocarbamol initial dose excreted in 6 h as unchanged drug was therefore 0.68% while the total drug (unchanged plus conjugated) was 11.3%. All these single-point data are comparable with previously reported non-stereoselective data [2,3,6,7].

Plasma time-courses of methocarbamol enantiomers in one rat after intravenous administration of a 120 mg/kg dose of racemic methocarbamol are depicted in Fig. 4. Plasma concentrations of the two enantiomers differ significantly. The cause for this observed stereoselectivity remains to be determined.

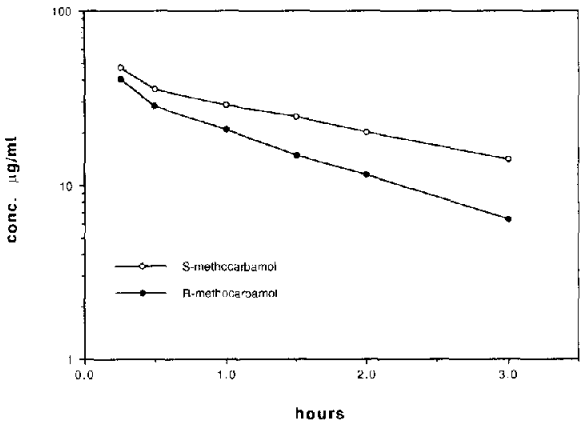

Fig. 4. Plasma concentration time profiles of methocarbamol enantiomers in one rat following a single intravenous dose of 120 mg/kg racemic methocarbamol.

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