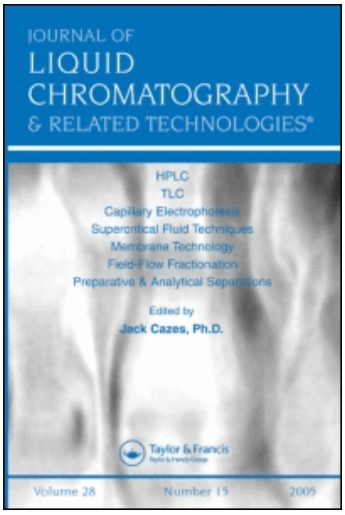

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DEVELOPMENT AND VALIDATION OF A CHIRAL HPLC METHOD FOR THE QUANTITATION OF METHOCARBAMOL ENANTIOMERS IN HUMAN PLASMA

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ABSTRACT

An isocratic chiral HPLC method was developed and validated for the quantitation of methocarbamol enantiomers in human plasma. Methocarbamol and an internal standard were extracted with ethyl ether. Chiral separation was achieved on coupled Spherisorb CN and Chiralcel OD columns with a mobile phase of ethanol - hexane (30:70, v/v). The detection was by UV at 272 nm. Linearity was established at 0.5 - 50 μg/ml ($r > 0.998$). Interday precision and accuracy of the calibration standards were demonstrated by 0.8 to 9.4% coefficients of variance (C.V.) and -5.2 to +3.8% relative error (R.E.). Quality controls showed interday precision and accuracy of 4.4 to 7.2% C.V. and +0.4 to +5.5% R.E. Recovery of methocarbamol enantiomers was 77 - 84%. No interconversion of the methocarbamol enantiomers was observed during process of storage, extraction nor chromatograph. Reproducibility and stability of the analytical columns were demonstrated.

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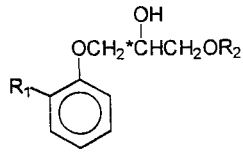

R_1	R_2	
$-OCH_3$	$-OCNH_2$	Methocarbamol
$-OCH_3$	-H	Guaifenesin
$-CH_3$	-H	Mephenesin (I.S.)

Figure 1. Chemical structures of methocarbamol, guaifenesin and mephenesin (I.S.). The asterisk denotes the location of the chiral center.

INTRODUCTION

Methocarbamol (MET) is a carbamate derivative used for the relief of discomfort. Guaifenesin (GUA), also biologically active, is the major degradation compound of MET [1]. The chemical structures of MET and GUA are shown in Figure 1. MET is administered as the racemate. The mechanism of the pharmacological action of MET in vivo has not been established [2]. Information is lacking for pharmacokinetics and pharmacodynamics of each of the MET enantiomers.

Previous methods using UV spectrophotometry after derivatization [3] and HPLC [4] for the quantitation of racemic MET lacked the sensitivity and selectivity. A more sensitive HPLC method for the routine quantitation of racemic MET in human plasma was developed and validated in our laboratory [5]. With this method, the lower limit of quantitation is 1 μg/ml with a signal to noise ratio of 20.

Alessi-Severini and co-workers have published a chiral HPLC method to determine MET enantiomers in biological fluids [6]. This method consists of a lengthy derivatization procedure (12 hours) and a long run time (50 minutes). Degradation of MET occurred during derivatization. Their results showed

differences in the pharmacokinetics of the enantiomers in the rat; the ratio of the two enantiomers (S/R) was 2.40. The ratio was 1.19 in one human plasma sample and 1.14 in one human urine sample.

A simple chiral HPLC method was developed and validated to facilitate the pharmacokinetic investigations of MET enantiomers. Separation of MET enantiomers from matrix interferences was achieved on coupled Spherisorb CN and Chiralcel OD columns with a mobile phase consisting of ethanol and hexane.

Very recently, Demian has reported using a Chiralcel OD column to separate enantiomers of several compounds including methocarbamol for the purpose of optical purity determination [7].

EXPERIMENTAL

Materials and Reagents

MET and GUA were from the United States Pharmacopeia and the internal standard (I.S.) mephenesin was from Aldrich (Milwaukee, WI, U.S.A.). The chemical structure of mephenesin is also shown in Figure 1. Dehydrated alcohol (ethanol) was purchased from Quantum (Newark, NJ, U.S.A.). All other organic solvents were of HPLC grade and were from Fisher (Fair Lawn, NJ, U.S.A.). Deionized water was purified by a NANOpure™ system from Barnstead (Dubuque, IA, U.S.A.). Control human sodium fluoride plasmas were drawn in our laboratory from healthy volunteers.

Standards and Quality Controls

Two primary MET stock solutions prepared from separate weighings were used to prepare standards and quality control samples (QCs). Water solutions of MET primary stock and substocks were prepared and stored at 4°C in polypropylene tubes. Working standards were prepared fresh daily by spiking 100 µl 4-fold concentrated solutions into 400 µl of blank control plasma using Gilson pipettors from Rainin (Woburn, MA, U.S.A.). The pipettors were calibrated daily.

The concentrations of calibration standards of each enantiomer were 0.5, 1, 2.5, 5, 12.5, 25, 37.5 and 50 μg/ml. Three levels of QCs, 1.5, 10, and 35 μg/ml for each enantiomer were prepared, aliquoted, and stored frozen at - 20°C with the clinical samples to be analyzed.

Instrumentation

The HPLC system consisted of a Waters 501 HPLC pump (Milford, MA, U.S.A.), a Perkin-Elmer ISS-100 autosampler (Norwalk, CT, U.S.A.), a Waters 484 UV detector set at 272 nm and a VG™ Multichrom data system for VAX™/VMS (Manchester, England). The HPLC autosampler tray was cooled at 4°C by a Brinkman RM6 cooling system (Westbury, NY, U.S.A.).

A Spherisorb CN column, 5 μm, 25 cm x 0.46 cm i.d. (Phase Separation Inc., Norwark, CT, U.S.A.) and a Chiralcel OD column, 5 μm, 25 cm x 0.46 cm i.d. purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.) were coupled in series. The columns were used at room temperature. The mobile phase was ethanol - hexane (30:70, v/v). The flow rate was 2.0 ml/min and the run time was 15 min.

Data Treatment

Chromatograms were measured using a VG™ Multichrom data system. The raw data output was acquired on a VG™ Chromserver and then transferred to the VAX™/VMS. A weighted 1/y linear regression was used to determine slopes, intercepts and correlation coefficients, where y = the ratio of the compound peak height to the I.S. peak height. The resulting parameters (y-intercept and slope) were used to calculate concentrations from the equation:
concentration = [y - (y-intercept)]/slope.

Extraction Procedure

To 400 μl plasma sample, 100 μl of I.S. water solution (200 μg/ml) was added. After mixing, 5 ml of ethyl ether was added to extract the compounds of interest by shaking for 15 min. The ethyl ether layer was decanted to another tube after

freezing the aqueous layer in a dry ice - acetone bath. The ethyl ether extract was evaporated to dryness under a stream of nitrogen and the residue reconstituted in 200 µl of ethanol - hexane (30:70, v/v). Fifty µl was injected onto the chiral HPLC system.

RESULTS AND DISCUSSION

HPLC separation

Figure 2 shows the chromatograms of unextracted enantiomers of MET, GUA, and I.S. Excellent chiral separations of MET and GUA enantiomers were achieved. The I.S. enantiomers were partially separated. Figure 3 shows chromatograms of extracted blank control plasma, the standard at limit of quantitation (L.O.Q) and a QC (35 µg/ml) for the chiral assay. The reproducibility of the HPLC method was demonstrated on Figure 2(B) showing chromatograms of the same samples obtained on another lot of the analytical columns.

The stereo-configuration of each enantiomer peak was not identified because the reference substances of each MET enantiomer are not available. MET enantiomer peaks were designated as MET-E1 and MET-E2 based on their elution order on the chromatogram. The enantiomers of the IS were partially separated from each other and the first peak (I.S.-E1) was used as the internal standard peak. Using several different mobile phases we were unable to separate MET-E1 from a matrix interference peak by using Chiralcel OD column alone. Figure 4 shows a chromatogram obtained on a Chiralcel OD column alone with a mobile phase ethanol - hexane (30:70, v/v). Coupling a CN column in front of the Chiralcel OD enable MET-E1 complete separation from the matrix peak as shown in Figure 3. Six control lots of control plasma were screened and none of them interferred the quantitation of the MET enantiomers as shown in Figure 5.

The GUA enantiomers had retention times of 5.2 min (GUA-E1) and 6.7 min (GUA-E2) on the chiral HPLC system. GUA-E2 coeluted with MET-E1. However, this should not cause any quantitation problem because GUA was less than 0.4% of the MET as determined by the previously developed achiral method [5]. The same proportion of GUA was also presented in an unextracted,

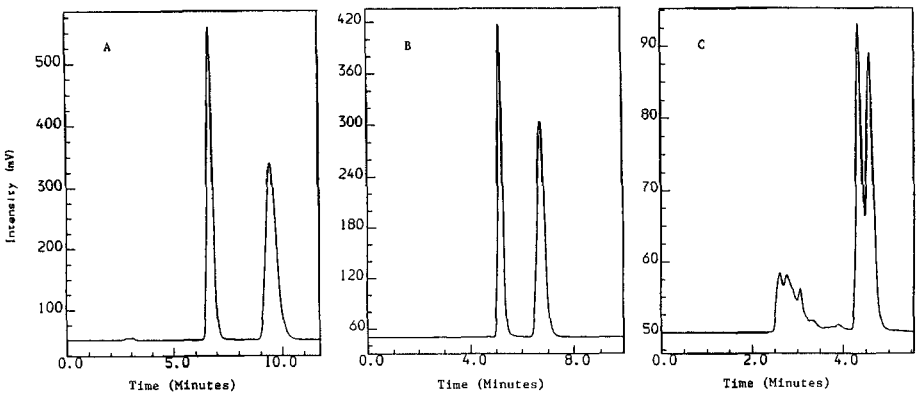

Figure 2. Chromatograms of chiral separation of unextracted methocarbamol (A), guaifenesin (B), and I.S. (C).

Columns: Spherisorb CN, 5μm, 25 x 0.46 cm i.d. and Chiralcel OD, 5 μm, 25 x 0.46 cm i.d. in series. Mobile phase: ethanol - hexane (30:70, v/v). Flow rate: 2.0 ml/min. Detection: UV at 272 nm.

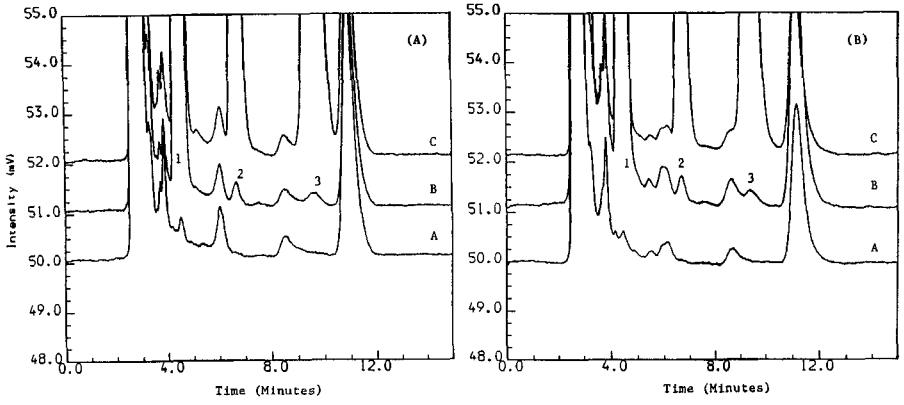

Figure 3. Chromatograms of chiral separation of methocarbamol enantiomers in human plasma on two lots (A and B) of Spherisorb CN and Chiralcel OD column.

A: blank control plasma, B: standard at 0.5 μg/ml for each enantiomer and C: QC at 35 μg/ml for each enantiomer. Columns: Spherisorb CN, 5μm, 25 x 0.46 cm i.d. (Lot # A and B) and Chiralcel OD, 5 μm, 25 x 0.46 cm i.d. (Lot # A and B) in series. Mobile phase: ethanol - hexane (30:70, v/v). Flow rate: 2.0 ml/min. Detection: UV at 272 nm. Peak identification: 1 = mephenesin (I.S.) enantiomers, 2 = methocarbamol enantiomer 1 (MET-E1), 3 = methocarbamol enantiomer 2 (MET-E2).

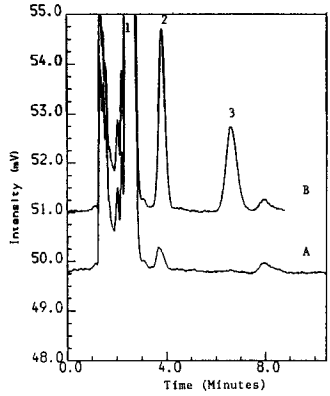

Figure 4. Chromatograms of chiral separation of methocarbamol enantiomers in human plasma on a Chiralcel OD column alone.

A: blank control plasma, B: standard at 2.5 μg/ml for each enantiomer. See Figure 3 for peak identification.

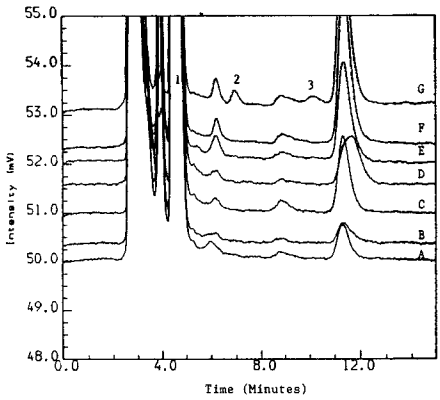

Figure 5. Chromatograms of screening six lots of blank control human plasma for the chiral separation of methocarbamol enantiomer.

A-F: six lots of control human plasma spiked with the I.S., G: standard at 0.5 μg/ml for each enantiomer. See Figure 3 for peak identification.

freshly-prepared MET reference solution, indicating that GUA was not formed upon storage, extraction or injection procedures [5].

Figure 6 shows that after 280 injections, a slight decrease in the resolution of I.S. enantiomers was observed for the Chiralcel OD column. The I.S. enantiomer resolution changed from 0.82 to 0.76 and the resolution of MET-E1 and MET-E2 changed from 4.2 to 4.1. The calculated plate number per column, calculated from MET-E1, decreased from 3070 to 2710 after 280 injections. This slight loss of performance did not affect the accurate determination of MET enantiomers.

We compared the results of using racemic I.S. to those of using only one enantiomer, I.S.-E1, on two validation curves. The C.V. values and mean results were analyzed by an F-test and a Student's t-test ($P = 0.05$) [8]. In no instance were the difference by the F-test or the t-test significant. The single I.S. enantiomer was prepared by injecting 100 µl of racemic I.S. (20 mg/ml in mobile phase) onto the chiral HPLC system described here with a mobile phase ethanol - hexane (5:95, v/v). The fractions corresponding to I.S.-E1 (retention time = 17.5 min) and I.S.-E2 (retention time = 20.0 min) were collected and evaporated to dryness under nitrogen. The enantiometric purities for I.S.-E1 and I.S.-E2 were 99.9% and 99.5% as determined by the afore-mentioned HPLC. The I.S.-E1 residue was taken up by 10 ml of water and this solution was used as the working I.S. in these two comparative curve runs. I.S.-E1 was stable for at least 9 days at 4 °C. No I.S.-E2 was formed through the extraction and chromatography process. Since the pure I.S.-E1 was not commercially available, we considered it was easier to use the racemate as the I.S.

Extraction

We developed a simple procedure to extract MET and the I.S. from plasma. The recoveries of MET from human plasma were close to 100% using ethyl acetate as an extraction solvent [5]. However, for the chiral assay, extraction using ethyl acetate lead to down field peaks which interfered with the subsquent chromatographic run. A less polar organic, ethyl ether, was therefore used for the extraction. The recoveries at 0.5, 25, and 50 µg/ml of each enantiomer were 79% ($n = 2$, C.V. = 8.1%), 84% ($n = 2$, C.V. = 1.7%) and 84% ($n = 2$, C.V. = 7.5%) for

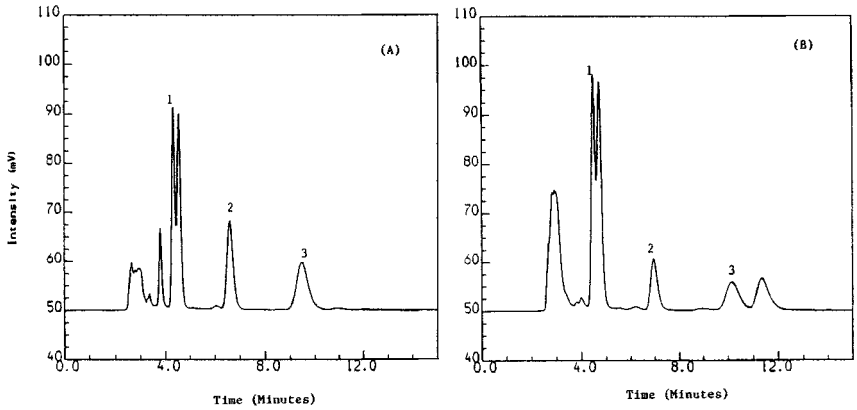

Figure 6. Chromatograms of injections # 15 (A) and # 280 (B) of chiral separation of methocarbamol enantiomers.

Peak identification: 1 = I.S. enantiomer 1 (I.S.-E1), 2 = methocarbamol enantiomer 1, 3 = methocarbamol enantiomer 2.

MET-E1, 77% (n = 2, C.V. = 3.7%), 84% (n = 2, C.V. = 0.8%) and 84% (n = 2, C.V. = 7.5%) for MET-E2. The I.S.-E1 recovery was 98% (n = 6, C.V. = 7.0%).

Validation performance

Five validation curves were run on five separate days. We observed consistent slopes and good correlation coefficients ($r > 0.9983$) through out these validation runs. Table 1 shows the interday linearity and precision data of each standard concentration. The L.O.Q. was 0.5 μg/ml (signal to noise ratio = 10 for MET-E1 and 7 for MET-E2). Tables 2 shows the interday and intraday accuracy and precision of QCs. The accuracy and precision data show that the chiral method is consistent and reliable with low error and imprecision for standards and QCs at the entire concentration range. The current standard curve range is suitable for pharmacokinetic studies.

Stability

In order to mimic the analysis of authentic clinical samples, stabilities of processing (freeze-thaw, benchtop), chromatography (on-system and re-injection)

TABLE 1
Interday Precision and Accuracy of Methocarbamol Enantiomer Standards

Standard curve number	Methocarbamol enantiomer (μg/ml) 0.50	1.00	2.50	5.00	12.5	25.0	37.5	50.0	r
				MET-E1					
A	0.49	1.03	2.61	4.95	11.9	25.0	36.9	51.3	0.9990
B	0.48	1.15	2.52	4.76	11.9	24.9	37.2	51.2	0.9992
C	0.50	0.96	2.42	5.19	13.2	25.3	37.0	49.5	0.9996
D	0.45	0.98	2.82	4.93	12.9	25.5	37.3	49.2	0.9995
E	0.45	1.07	2.53	5.01	12.4	25.3	37.3	49.3	0.9995
Mean	0.47	1.04	2.58	5.01	12.4	25.3	37.3	50.1	
C.V. (%)	4.9	7.3	5.8	4.0	4.9	1.2	0.8	2.1	
R.E. (%)	-5.2	+3.8	+3.2	+0.3	-0.8	+1.0	-0.7	+0.2	
				MET-E2					
A	0.48	1.07	2.64	4.96	11.8	24.7	36.7	51.8	0.9983
B	0.60	1.01	2.31	4.52	12.2	25.4	37.1	51.0	0.9993
C	0.51	0.92	2.41	5.20	13.4	25.7	37.1	48.9	0.9988
D	0.50	0.86	2.90	4.98	12.6	25.4	37.1	49.7	0.9997
E	0.49	0.98	2.49	5.19	12.3	25.7	38.0	48.8	0.9991
Mean	0.52	0.97	2.55	4.97	12.5	25.4	37.2	50.0	
C.V. (%)	9.4	8.4	9.0	5.5	4.8	1.6	1.3	2.6	
R.E. (%)	+3.2	-3.2	+2.0	-0.6	-0.3	+1.5	-0.8	+0.1	

TABLE 2
Precision and Accuracy of Methocarbamol Enantiomers Quality Controls

	MET-E1 (μg/ml) 1.50	10.0	35.0	MET-E2 (μg/ml) 1.50	10.0	35.0
Interday (n = 30)						
Mean	1.58	10.6	35.7	1.51	10.5	35.9
C.V. (%)	5.4	5.2	7.2	7.7	4.4	7.2
R.E. (%)	+5.2	+5.5	+2.0	+0.4	+5.4	+2.5
Intraday (n = 6)						
Mean	1.59	10.2	34.3	1.60	10.1	34.0
C.V. (%)	1.8	1.8	2.7	1.6	1.5	4.6
R.E. (%)	+5.2	+1.7	-2.1	+6.8	+1.0	-2.9

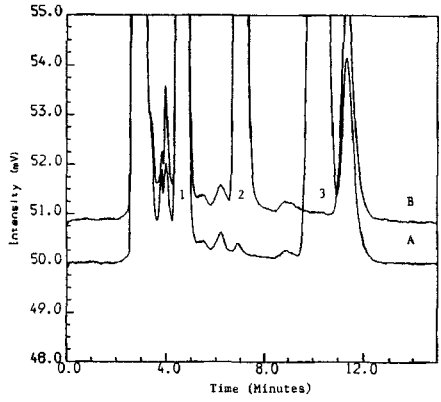

Figure 7. Chromatograms of methocarbamol enantiomer 1 (A) and enantiomer 2 (B). The samples have gone through a storage period of 9 days at -20°C, three cycles of freeze-thaw, 3 hours on bench prior to extraction and 32 hours on the autosampler before injection.

See Figure 3 for peak identification.

and sample storage were established [5]. No degradation of MET was observed after those stability tests [5]. Stability of single enantiomer was also examined. Small amounts of pure MET enantiomers were isolated by the chiral method described here. Figure. 7 shows the chromatograms of single MET enantiomers through nine days of storage in plasma at -20 °C, three cycles of freeze-thaw, two hours on bench-top at room temperature prior to extraction and 32 hour on an autosampler before injection. Interconversion of the enantiomers was insignificant. There were only 0.1% of MET-E2 in MET-E1 and 0.3% of MET-E1 in MET-E2 in these stability test chromatograms.

CONCLUSIONS

A simple chiral HPLC method was developed for the analysis of methocarbamol enantiomers in human plasma. This chiral HPLC method is suitable for pharmacokinetic studies of drug interaction as well as dose-efficacy of formulations with the single methocarbamol enantiomer. Stability of methocarbamol and its

enantiomers during storage, extraction and injection has been established. The analytical column demonstrated good robustness and no column batch to batch variability was observed.

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