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Short Communication

Development and validation of a high-performance liquid chromatographic method for the determination of methocarbamol in human plasma

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Abstract

An isocratic HPLC method was developed and validated for the quantitation of methocarbamol in human plasma. Methocarbamol and internal standard in 200 μl of human plasma were extracted with ethyl acetate, evaporated to dryness and reconstituted in water. Separation was achieved on a reversed-phase C_{18} column with a mobile phase of methanol–0.1 *M* potassium phosphate monobasic–water (35:10:55, v/v/v). The detection was by ultraviolet at 272 nm. Linearity was established at 1–100 μg/ml ($r > 0.999$). The limit of quantitation was designed as 1 μg/ml to suit pharmacokinetic studies. Inter-day precision and accuracy of the calibration standards were 1.0 to 3.6% coefficients of variance (C.V.) and −2.0 to +1.6% relative error (R.E.). Quality controls of 3, 20 and 70 μg/ml showed inter-day precision and accuracy of 2.5 to 3.6% C.V. and −0.9 to −0.4% R.E. Recovery of methocarbamol was 91.4–100.3% in five different lots of plasma. The method was shown to be applicable on different brands of C_{18} columns.

1. Introduction

Methocarbamol is a carbamate derivative used for the relief of discomfort associated with acute, painful skeletal muscular conditions. Guaifenesin, the major degradation compound of methocarbamol, is also biologically active [1]. The chemical structures of methocarbamol and guaifenesin are shown in Fig. 1. Methocarbamol is administered as the racemate though it has a chiral center. Like other muscle relaxants, the mechanism of action of methocarbamol *in vivo* has not been established, but it may be due to general nervous system depression [2]. Clinical studies have demonstrated the efficacy of methocarbamol compared to placebo and aspirin [3–6].

In order to determine and evaluate the relationship of dose, plasma methocarbamol concentration and therapeutic effect over time, a sensitive method capable of measuring 1 μg/ml of methocarbamol in plasma is desirable. Previous methods using UV spectrophotometry after derivatization [7] and HPLC [8] lacked sensitivity and selectivity. The HPLC method was used for a case study of fatal methocarbamol intoxication where the limit of quantitation (LOQ) was reported as 10 μg/ml and the methocarbamol

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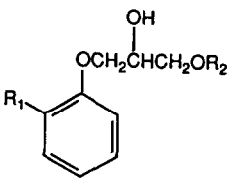

R_1	R_2	
$-OCH_3$	$-OCNH_2$	Methocarbamol
$-OCH_3$	-H	Guaifenesin
$-CH_3$	-H	Mephenesin (I.S.)

Fig. 1. Chemical structures of methocarbamol, guaifenesin and mephenesin (I.S.).

peak was not well-resolved from matrix interferences [8]. Alessi-Severini and co-workers have published a chiral HPLC method to quantify methocarbamol enantiomers in biological fluids [9]. Their method is useful to study methocarbamol enantiomers although a lengthy derivatization procedure (12 h) and a long run time (50 min) were used. Their results showed some differences in the kinetics of the enantiomers in the rat, the signal-to-noise ratio was 2.40. However, their results also showed that the signal-to-noise ratio was 1.19 in a human plasma sample and 1.14 in a human urine sample. Therefore, these results suggest that the enantiomers behave similarly in humans and the pharmacokinetic results of racemate paralleled that of the enantiomers. A simple isocratic HPLC method was developed in our laboratory and validated according to the pharmaceutical industry guidelines [10]. After extraction, methocarbamol and internal standard were separated from each other and from the matrix compounds on a C_{18} column. The method presented in our paper for the racemic drug is a much simpler and faster method to provide useful pharmacokinetic data than the stereoselective method of Alessi-Severini and co-workers.

2. Experimental

2.1. Materials and reagents

Methocarbamol and guaifenesin were from the United States Pharmacopeia and the internal standard (I.S.) mephenesin was from Aldrich (Milwaukee, WI, USA). The chemical structure of mephenesin is also shown in Fig. 1. All organic solvents were of HPLC-grade and were from Fisher (Fair Lawn, NJ, USA). Potassium phosphate monobasic, of analytical grade, was from Mallinckrodt (Paris, KT, USA). Deionized water was purified by a NANOpure system from Barnstead (Dubuque, IA, USA). Control human sodium fluoride plasmas were drawn in our laboratory from healthy volunteers.

2.2. Standards and quality controls

Two primary stock solutions of methocarbamol were prepared from separate weighings for standards and quality control samples (QCs). Water solutions of methocarbamol primary stock and substocks were prepared and stored at 4°C in polypropylene tubes. Working standards were prepared fresh daily by spiking 50 μl 4-fold concentrated solutions into 200 μl of blank control plasma by using Gilson pipettors from Rainin (Woburn, MA, USA). The pipettors were calibrated daily. The final concentrations of methocarbamol in human plasma standards were 1, 2, 5, 10, 25, 50, 75 and 100 μg/ml. Three levels of QCs, 3, 20 and 70 μg/ml were prepared, aliquoted and stored frozen at −70°C with the clinical samples to be analyzed.

2.3. Instrumentation

The HPLC system consisted of a Beckman 110B solvent delivery module (Berkeley, CA, USA), a Perkin-Elmer ISS-100 autosampler (Norwalk, CT, USA), a Waters 484 UV detector (Milford, MA, USA) set at 272 nm and a VG Multichrom data system for VAX/VMS (Manchester, England). The tray of the autosampler was cooled at 4°C by a Brinkman RM6 cooling

system (Westbury, NY, USA). The flow-rate was 0.8 ml/min, and the run time was set at 13 min. The analytical column, a Microsorb C_{18} column of 3 μm, 10 × 0.46 cm I.D., was from Rainin. For some experiments, a Hypersil C_{18} column of 3 μm, 10 × 0.46 cm I.D. from Alltech (Deerfield, IL, USA) and a Partisil C_{18} column of 5 μm, 10 cm × 0.46 cm I.D. from Whatman (St. Louis, MO, USA) were also used. The columns were maintained at 40°C by a Fiatron CH-30 column heater (Oconomowoc, WI, USA). The mobile phase was methanol–0.1 *M* potassium phosphate monobasic–water (35:10:55, v/v/v).

2.4. 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 is 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:

$$\text{concentration} = [y - (y\text{-intercept})]/\text{slope}.$$

2.5. Extraction procedure

To 200 μl plasma sample, 100 μl of an aqueous I.S. solution (200 μg/ml) were added. After mixing, 5 ml of ethyl acetate were added to extract methocarbamol and I.S. by shaking for 15 min. A 3-ml volume of the organic extract was evaporated to dryness under a stream of nitrogen and reconstituted in 500 μl of water. A 50-μl aliquot was injected onto the HPLC.

3. Results and discussion

3.1. HPLC separation

Since no methods were available for determining low concentrations of methocarbamol in human plasma, an HPLC method suitable for pharmacokinetic studies was developed. Fig. 2 shows chromatograms of extracted blank control plasma, standard at 1 μg/ml and QC at 70 μg/ml. Methocarbamol and I.S. were well-resolved from matrix peaks, possible concomitant drugs, and from each other. The minor peak in front of the methocarbamol peak was confirmed to be guaifenesin by injecting a guaifenesin reference solution. The guaifenesin was less than 0.4% of the methocarbamol peak area and therefore did not interfere with the quantitation of methocarbamol. The same proportion of guaifenesin was also presented in an unextracted, freshly-prepared methocarbamol reference solution, indicating that guaifenesin was not formed upon storage, extraction or injection procedure. The analytical column maintained integrity after more than five hundred injections indicated by the fact that very consistent capacity factors of methocarbamol and I.S. (3.2 and 7.7) and calculated plate numbers for methocarbamol (N = 11 000) were achieved throughout the entire study. Selectivity against ibuprofen and

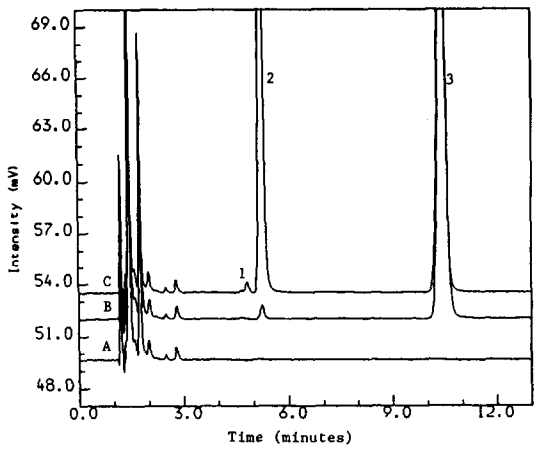

Fig. 2. Chromatograms of (A) blank control plasma, (B) standard at 1.0 μg/ml, and (C) QC at 70 μg/ml. Column: Microsorb C_{18}, 3 μm, 10 × 0.46 cm I.D., maintained at 40°C. Mobile phase: methanol–0.1 *M* potassium phosphate monobasic–water (35:10:55, v/v/v). Flow-rate: 0.8 ml/min. Detection: UV at 272 nm. Peak identification: 1 = guaifenesin, 2 = methocarbamol, 3 = mephenesin (I.S.).

acetaminophen was investigated and no interferences were observed.

3.2. Extraction

We developed a simple procedure to extract methocarbamol and I.S. from plasma. Though clean chromatograms were also obtained with the extracts from chloroform, methylene chloride and ethyl ether, ethyl acetate gave the best recovery. Table 1 summarizes recoveries of methocarbamol and I.S. from human plasma drawn from five different volunteers. Consistent recoveries of methocarbamol over the whole standard concentration range and I.S. were obtained from all five lots of plasma tested. None of the tested plasmas showed interferences with methocarbamol and I.S.

3.3. Validation performance

Five validation curves were run on five separate days. We observed consistent slopes and excellent correlation coefficients ($r > 0.9997$) throughout these validation runs. Table 2 shows the inter-day accuracy and precision data of the standard concentrations. The standards show a linear range of 1–100 μg/ml, with an LOQ of 1 μg/ml. Table 3 shows the inter-day and intra-day accuracy and precision of QCs. The accuracy and precision data show that this method is consistent and reliable with a very low error and imprecision for standards and QCs over the entire concentration range. The current standard curves range is suitable for pharmacokinetic studies. At 1 μg/ml the signal of the methocarbamol peak is at least 20 times the background noise indicating that a lower concentration is attainable if necessary. Since the baseline noise is very low the LOQ can be further improved several folds by using a larger volume of plasma sample and by reconstituting the extract residue in less water.

Table 1
Recovery of methocarbamol and I.S. from five different lots of plasma

Plasma lot	Methocarbamol recovery (%)			I.S. recovery (%)
	1 μg/ml	25 μg/ml	100 μg/ml	
1	97.9 (3; 3.2)	97.3 (3; 2.6)	100.3 (3; 2.4)	98.7 (9; 1.6)
2	98.9 (3; 3.7)	95.6 (3; 4.7)	98.6 (3; 0.6)	97.3 (9; 1.8)
3	98.1 (3; 1.0)	95.0 (3; 1.8)	97.5 (3; 2.2)	96.7 (9; 0.9)
4	98.8 (2; 0.4)	91.4 (2; 0.4)	96.1 (3; 2.2)	93.1 (7; 1.5)
5	99.8 (3; 1.8)	95.2 (3; 3.5)	96.2 (3; 3.0)	95.0 (9; 0.9)

Number of determination (n) and C.V. (%) values are given in parentheses.

Table 2
Inter-day precision and accuracy of methocarbamol standards ($n = 5$)

Concentration added (μg/ml)	Concentration found (mean) (μg/ml)	C.V. (%)	R.E. (%)
1.00	0.98	1.6	−2.0
2.00	2.01	3.6	+0.6
5.00	5.00	1.1	0
10.0	10.2	1.1	+1.6
25.0	25.2	1.9	+0.8
50.0	49.1	2.1	−1.8
75.0	75.6	1.5	+0.8
100	100	1.0	+0.1

Table 3
Precision and accuracy of methocarbamol quality controls

Concentration added (μg/ml)	Concentration found (mean) (μg/ml)	C.V. (%)	R.E. (%)
Inter-day ($n = 30$)			
3.00	2.99	3.6	−0.4
20.0	19.7	2.5	−1.3
70.0	69.4	2.9	−0.9
Intra-day ($n = 6$)			
3.00	2.97	2.5	−1.1
20.0	19.9	1.3	−0.8
70.0	68.2	1.7	−2.6

3.4. Stability

Stabilities of processing (freeze-thaw, benchtop), chromatography (on-system and re-injection) and sample storage were established. QCs were subjected to various cycles of freezing and thawing and then analyzed. After two cycles of freeze-thaw, the values of QCs were 98.9–100.0% of those obtained after one cycle. After three cycles of freeze-thaw, the values of QCs were 97.9–99.9% of those obtained after one cycle. Benchtop stability after 3 h at 22°C was 98.5–101.8% of the values obtained with immediate processing. The on-system stability after 23.5 h was 100.5–102.7% compared to the original values. The stability of a sample re-injected after 45.5 h was 98.5–101.0% of the original value. Extracted samples were stored in a refrigerator at 4°C for 72 h prior to injection. The results were 101.4–102.0% of the normal samples. Sample storage stability was tested after QCs were stored at −70°C for 10.5 months. The values of the stored samples were 94.6–102.3% of the values at the original assay for methocarbamol. After storage the amount of guaifenesin in QCs was not increased. We also tested sample collection stability. The result of immediate plasma centrifugation at 20°C was 100.8% of that after centrifugation at 4°C. The result for blood samples standing at 22°C for one hour before centrifugation at 20°C was 101.8% of that obtained after immediate plasma centrifugation at 4°C. No degradation of methocarbamol was observed during the sample collection procedure. These extensive stability data for sample collection allow a wide range of conditions such as normally encountered in a large phase III study where samples may be collected at multiple sites under different conditions.

3.5. Column variability

In order to test the robustness of the method, three validation curves were separately run on three different brands of C_{18} columns. The results for samples injected on the three different C_{18} columns showed variations of 100.0–101.5% from the Microsorb column for the Hypersil column and 99.1–100.5% for the Partisil column. Good separation of methocarbamol and I.S. from potential interferences was achieved on all three columns. The C.V. values and the mean results were analyzed by an F-test and a Student's t-test ($P = 0.05$) [11]. In no instance were the F-test or the Student's t-test significant. The capacity factors of guaifenesin, methocarbamol and I.S. were 2.5, 2.8 and 6.1 on the Hypersil column and 2.8, 3.3 and 6.2 on the Partisil column.

4. Conclusions

A simple and robust HPLC method has been developed for the analysis of methocarbamol in human plasma. Only 200 μl of plasma and a simple extraction step were required. This method has been successfully used in our laboratory for the analysis of large numbers of human plasma samples from pharmacokinetic studies. Stability of methocarbamol during sample collection, storage, extraction and injection has been established. Guaifenesin, the major degradation product of methocarbamol, can also be monitored. The analytical column demonstrated excellent robustness. This method can be applied on different brands of C_{18} columns.

5. References

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