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Short communication

Determination of methocarbamol concentration in human plasma by high performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

A simple and reproducible high performance liquid chromatography–tandem mass spectrometric method was developed for methocarbamol analysis in human plasma. Methocarbamol and the internal standard (IS) were extracted by a protein precipitation method. Under isocratic separation condition the chromatographic run time was 3.0 min. The calibration curve was linear over a range of 150–12,000 ng/mL with good intraday assay and interday assay precision (CV% < 10.9%). The method was proven to be sensitive and selective for the analysis of methocarbamol in human plasma for bioequivalence study.

1. Introduction

Methocarbamol, the carbamate of guaifenesin, was developed for the treatment of skeletal muscle conditions of pain and discomfort [1]. It is formulated as a single entity (trade name: Robaxin®) or in combination with other active ingredients such as acetaminophen (trade name: Robaxacet®), ibuprofen (trade name: Robax Platinum®), and aspirin (trade name: Robaxisal®). The mechanism it works is unknown, but it is thought to depress the nervous system [2,3].

Methocarbamol is easily absorbed from the intestine and widely distributed in all body tissues, especially the liver and kidney [4]. Its metabolism is similar in rats, dogs, and humans, which is ring hydroxylation and o-demethylation at phase I and conjugation at phase II [4,5]. The sulfoconjugate is the main metabolite recovered in the urine. The structure of methocarbamol is shown in Fig. 1a(I) [6].

For pharmacokinetic or bioequivalence studies, a fast, selective and sensitive method for determination of methocarbamol concentration in plasma is highly desirable. Methods of methocarbamol analysis published in literature are mainly HPLC with liquid–liquid extraction. Obach et al. used HPLC with UV detection to determine plasma methocarbamol concentrations to study pharmacokinetics and bioavailability of methocarbamol in rats [7]. Ethyl acetate was used to extract methocarbamol from plasma. A volume of 0.5 mL plasma was required and a large volume of reconstituted extract residue, 150 μL, was injected into the column. The flow rate was very high at 3 mL/min. The study suggested a dose-dependent pharmacokinetic behavior of methocarbamol. Alessi-Severini et al. reported a stereospecific HPLC method with UV detection after derivatization to quantify methocarbamol enantiomers in biological fluids [8]. The sample preparation procedure was complicated and the derivatization step took 12 h. The run time was also long; around 50 min. A simple HPLC-UV method was developed by Weng et al. [9] for the analysis of methocarbamol in human plasma. A volume of 200 μL plasma was used and liquid–liquid extraction with ethyl acetate was performed. However, the selectivity of UV was still low and the run time needed was 13 min. Koupai-Abyazani et al. applied HPLC with UV detection to determine the methocarbamol in equine serum and confirmed with LC–MS. The extraction method needed two steps; enzyme hydrolysis followed by liquid–liquid extraction [10].

We have recently developed a liquid chromatography–tandem mass spectrometric method for the analysis of methocarbamol in human plasma. The mass spectrometry (MS) detection method has a much higher selectivity than UV, and can separate analytes from co-eluents based on their mass-to-charge ratios. The MS detection method also has higher sensitivity than UV, and thus less plasma is needed. Due to this advantage of sensitivity, a simple protein precipitation method can be applied which results in reducing the sample processing time compared to liquid–liquid extraction. This method requires only 50 μL of plasma and a run of 3.0 min for each sample. The method has been demonstrated to be easy to operate and robust for the determination of methocarbamol in human plasma for bioequivalence study.

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2. Experimental

2.1. Chemicals

Methocarbamol (99.8%) and tolbutamide (99.6%) were bought from Sigma–Aldrich (Oakville, ON, Canada). Ammonium formate (certified) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Methanol and acetonitrile were both HPLC grade from EMD chemicals Inc. (Gibbstown, NJ, USA). Deionized water was produced in-house using a Barnstead ultrapure water purification system (Dubuque, IA, USA).

2.2. High performance liquid chromatography and mass spectrometry

Chromatographic separation was obtained using an Agilent series 1100 system (Santa Clara, CA, USA) with a Phenomenex (Torrance, CA, USA) synergi Polar-RP column (75 mm × 2.0 mm, 4 μm) which was maintained at 28 °C with an Agilent series 1100 column heater. The mobile phase was prepared by mixing 650 mL of methanol and 350 mL of 10 mM ammonium formate. The flow rate for the mobile phase was set at 0.220 mL/min. Detection was performed by an AB/Sciex (Concord, ON, Canada) API2000 triple quadrupole mass spectrometer with a turbo-ion spray interface in the positive ion mode. The MS/MS transitions (m/z) were: methocarbamol, 242.0 → 118.2; tolbutamide (IS), 271.2 → 155.0, which are shown in Fig. 1a(II) and b(II). The ion source was heated to 480 °C and the spray voltage was set at 2000 V. The nebulizer gas, auxiliary gas, collision-activated dissociation (CAD) gas, and curtain gas were applied at 65, 75, 5 and 30 psi, respectively. The collision energy was set at 15 V for methocarbamol and 27 V for tolbutamide. The software Analyst 1.4.1 (Sciex, Concord, ON, Canada) was used to control the LC–MS/MS system and acquire the data.

2.3. Preparation of stock solution and standards

Stock solutions of methocarbamol (1.00 mg/mL) and IS (500 μg/mL) were prepared in methanol and stored at −20 °C. Working solutions of methocarbamol (100 and 10.0 μg/mL) were prepared daily by dilution of stock solution with methanol/deionized water, 1/1 (V/V). The IS working solution containing 50.0 μg/mL tolbutamide was diluted from the IS stock solution with 90/10 (V/V) deionized water/methanol. This solution was also prepared daily.

Before preparing the standard calibration curve, the plasma with additive K_3 EDTA (Biochemed Services, Winchester, VA) used for spiking was tested to make sure it was free of interferences with drug and IS. Appropriate amounts of methocarbamol working solutions were then spiked into the plasma to achieve the calibration curve points equivalent to 150, 300, 1000, 2000, 5000, 10,000 and 12,000 ng/mL of methocarbamol. Quality control (QC) samples were prepared in a similar way to calibration standards at a low level (450 ng/mL), mid-level (3000 ng/mL) and high level (8000 ng/mL). QC samples were extracted in triplicate and included with each calibration curve. QC samples were also prepared with three other different plasma sources to examine the matrix effects for methocarbamol and the IS. The samples were stored at −20 °C after the preparation.

2.4. Extraction procedure

A 50 μL aliquot of human plasma was put into a 2.0 mL microcentrifuge tube by an autopipette. 50 μL of the IS working solution containing 50 μg/mL tolbutamide and 900 μL of acetonitrile were added to the aliquot and were mixed for approximately 30 s on a VX-2500 multi-tube vortexer (VWR Scientific, Bridgeport, NJ). After waiting 5 min, the mixture was mixed again for around

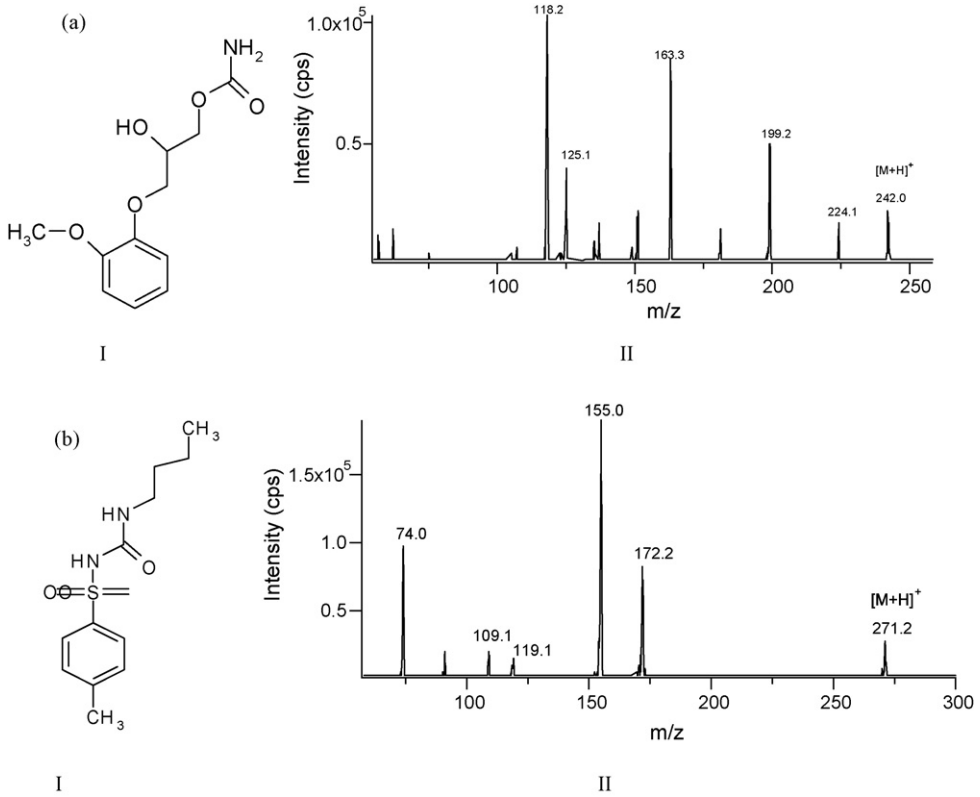

Fig. 1. Methocarbamol and tolbutamide structures and their product ion mass spectra of $[M+H]^+$. (a) I: Structure of methocarbamol. II: Product ion mass spectra of $[M+H]^+$ of methocarbamol. (b) I: Structure of tolbutamide. II: Product ion mass spectra of $[M+H]^+$ of tolbutamide.

Table 1
The intraday assay precision and accuracy ($n = 6$).

Nominal concentration (ng/mL)	Mean of calculated concentration (ng/mL)	Accuracy (%)	Precision (CV%)
150	137	91.3	6.6
450	451	100.2	1.6
3000	3010	100.3	1.5
8000	7776	97.2	1.0

30 s, followed by centrifuging for 5 min at 9100 × *g*. 50 μL of the supernatant was transferred to a culture tube containing 1.0 mL of reconstitution solution (methanol/10 mM ammonium formate, 50/50, V/V) and was mixed well. Only 5 μL was injected into the LC–MS/MS system.

2.5. Method validation

The developed method has been validated for specificity, linearity, precision and accuracy, matrix effects, recovery, and stability according to US Food and Drug Administration (FDA) bioanalytical method validation guidance [11].

3. Results and discussion

3.1. Specificity

Blank plasma samples from ten different sources were extracted with and without the IS to evaluate the specificity of the method. It was found that the method was selective for methocarbamol and the IS. No endogenous interference was observed at the retention time of methocarbamol and the IS. The chromatogram of an extracted blank plasma sample is shown in Fig. 2a. Fig. 2b shows the chromatogram of an extracted plasma sample (8000 ng/mL).

3.2. Linearity and sensitivity

Standard calibration curves of seven points that correspond to methocarbamol concentrations ranging from 150 to 12,000 ng/mL were extracted and analyzed. A blank plasma sample (containing no drug and no IS) and a zero sample (with only IS) were also extracted. The extracted blank sample was placed to follow the highest concentration sample. These two samples were analyzed in order to confirm the absence of interferences and the blank sample was also used for assessing the level of any carryover. The calibration curve was constructed based on the peak area ratio of methocarbamol to the IS versus methocarbamol concentration. The weighted least squares regression analysis with a weighting factor of $1/x^2$ was carried out for calibration curves.

The result shows a good linear relationship over the range of 150–12,000 ng/mL for methocarbamol. The correlation coefficients were higher than 0.9982, with an average of 0.9989. A typical standard calibration curve is described by the weighted least squares regression as: $y = 0.000090x + 0.002006$ ($R^2 = 0.9982$), where y is the peak area ratio of methocarbamol to the IS, and x corresponds to the concentration of methocarbamol added to plasma ranging from 150 to 12,000 ng/mL.

The limit of quantification (LOQ) was set at 150 ng/mL based on the expected plasma concentration level of methocarbamol in the subject samples at five times the elimination half-life for the bioequivalence study. The limit of detection (LOD) was around 0.04 ng/mL with a signal-to-noise (S/N) ratio of 3. Fig. 2c shows the chromatogram of an extracted LOQ sample.

The blank plasma sample and zero sample indicate that there were no interferences for methocarbamol and the IS, and the blank plasma sample also shows no carryover for methocarbamol and the IS.

3.3. Precision and accuracy

The intraday assay precision and accuracy were determined by analyzing six replicates of the QC samples at three concentration levels (450, 3000 and 8000 ng/mL) and LOQ. The precision (CV%) at the LOQ was 6.6% and the accuracy was 91.3%. For QC levels, the precision was between 1.0% and 1.6%, and the accuracy ranged from 97.2% to 99.7%. The results are summarized in Table 1.

The interday assay precision and accuracy were determined by six repeated analyses of the QC samples and LOQ on six different days. For two of the six days, six replicates of QC samples at each concentration level and LOQ were analyzed. For other days, three replicates of QC samples at each concentration level and LOQ were analyzed. The sample concentrations were determined by standard calibration curve prepared and analyzed on the same day.

The interday assay precision (CV%) of the QC samples was 1.4–2.6% and accuracy was between 97.3% and 102.0%. For LOQ, the precision was 10.9% and the accuracy was 98.0%. The results are shown in Table 2.

3.4. Matrix effects

For bioanalysis using LC–MS/MS, there may be co-eluted species that affect the ionization efficiency of analytes at the interference. They may suppress or enhance the MS signal and impact the reproducibility and accuracy of the method. In order to develop a robust method for bioequivalence studies, matrix effects need to be minimized and consistent for different sources of plasma.

During the method development, the matrix effects on the drug and IS were evaluated. A reference solution containing 21.0 ng/mL methocarbamol and 126 ng/mL IS was prepared in reconstitution solution, and 1.0 mL of this solution was mixed with 50 μL of extraction supernatant of a blank plasma sample to mimic the matrix of extracted samples. The final concentrations of this mixed solution were 20.0 ng/mL methocarbamol and 120 ng/mL IS. Another pure reference solution (20.0 ng/mL methocarbamol and 120 ng/mL IS) was prepared only in reconstituted solution. The MS responses of the mixed solution were compared with those of the pure reference solution to evaluate the matrix effects. It was found that the co-eluents from the plasma extraction suppressed

Table 2
The interday assay precision and accuracy ($n = 24$).

Nominal concentration (ng/mL)	Mean of calculated concentration (ng/mL)	Accuracy %	Precision (CV%)
150	147	98.0	10.9
450	459	102.0	2.6
3000	2997	99.9	2.3
8000	7787	97.3	1.4

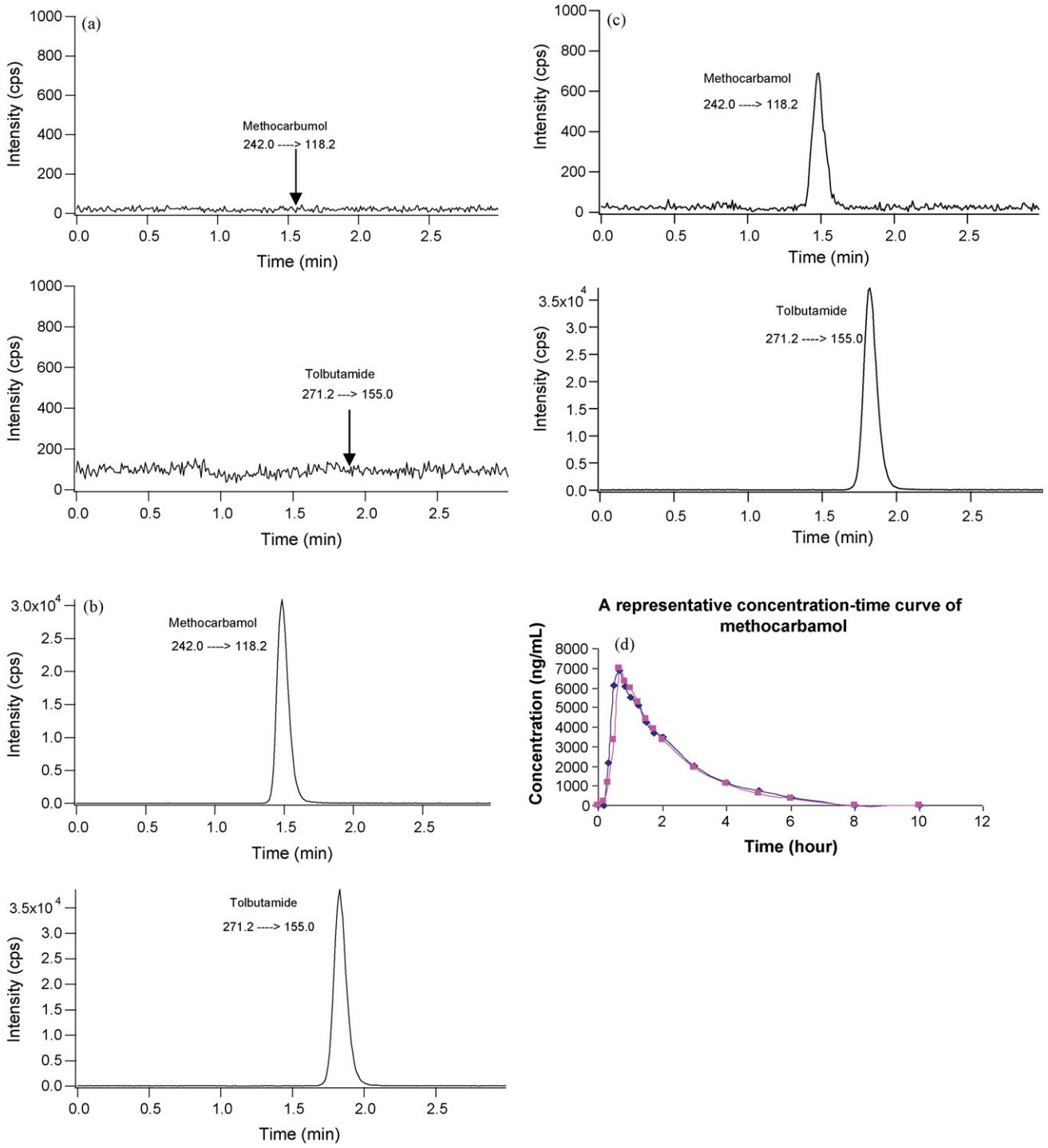

Fig. 2. Chromatograms of extracted samples and a representative concentration–time curve of methocarbamol. (a) Chromatogram of an extracted blank plasma sample. (b) Chromatogram of an extracted plasma sample (8000 ng/mL methocarbamol, 50.0 μg/mL tolbutamide). (c) Chromatogram of an LOQ. (d) A representative concentration–time curve of methocarbamol.

the MS signals here. With a mobile phase consisting of 80/20 (V/V), methanol/10 mM ammonium formate, the matrix effect was around −40% for the drug and around −35% for the IS. Different percentages of methanol in the mobile phase were examined. When the percentage of methanol decreased, the matrix effects reduced while the MS signals also decreased. Considering the two factors, the best mobile phase was determined to be 65/35 (V/V), methanol/10 mM ammonium formate. For the final method, the matrix effects were determined to be approximately −15% for the drug and IS.

To confirm that the level of matrix effects in this method was consistent with various plasma sources, three different concentration levels of QC samples from three other donors were tested. Results indicated that the MS responses of methocarbamol and IS were consistent with the three different plasma sources, which means that plasma samples from various sources have similar matrix effects for the methocarbamol and IS using this method. Reproducible and accurate results were achieved. The precision (CV%) of the QC samples was 0.8–2.3%, and the accuracy was between 97.3% and 99.7%. The results are shown in Table 3.

Table 3
Precision and accuracy for plasma samples from three different sources ($n = 6$).

	Nominal concentration (ng/mL)	Mean of calculated concentration (ng/mL)	Accuracy %	Precision (CV%)
Matrix 1	450	441	98.0	2.3
	3000	2990	99.7	1.0
	8000	7782	97.3	1.3
Matrix 2	450	448	99.6	2.0
	3000	2962	98.7	1.7
	8000	7783	98.0	0.8
Matrix 3	450	449	99.3	1.6
	3000	2934	97.8	1.3
	8000	8045	99.1	1.2

3.5. Recovery

In order to obtain the true extraction recovery, the matrix effect factor needs to be considered. Reference QC solutions were prepared in reconstitution solution and mixed with the extraction supernatant of blank plasma samples in order to get a similar matrix as the extracted samples. The final concentrations of the reference QC solutions were the same as the QC samples. The recovery of methocarbamol and the IS was then determined by comparing the peak areas of the extracted QC samples at three different concentrations (450, 3000, 8000 ng/mL) with those of reference QC solutions.

The overall extraction recovery was 94.2% for methocarbamol and 98.1% for the IS, respectively. The precision (CV%) of the extraction yield at the three QC concentration levels was 4.9% for methocarbamol.

3.6. Stability

Freeze–thaw stability, processed sample stability, short-term stability at room temperature, and long-term stability at −20 °C were evaluated. To determine the stability, the QC samples of three concentration levels together with a standard calibration curve were prepared freshly, and then they were analyzed with the stability samples at the same time. The acceptance criterion is that precision and accuracy deviation values must be less than 15% for at least 67% stability samples at each concentration level.

For freeze–thaw stability evaluation, each cycle included thawing QC samples unassisted at room temperature until they were completely thawed and re-freezing them at–20 °C for at least 12 h. The freeze–thaw cycle was repeated two more times and then analyzed on the third cycle. The results revealed that methocarbamol in human plasma are stable for at least three freeze–thaw cycles.

Processed sample stability was evaluated by leaving the extracted QC samples in the autosampler at 4 °C for a certain time after the immediate injection. The samples were then re-injected. Responses of the QC samples for both injections were compared. The result showed that extracted methocarbamol and IS are stable in the autosampler at 4 °C for at least 100 h.

For short-term stability, QC samples were left on bench at room temperature for certain time and then analyzed. The result showed that methocarbamol is stable at room temperature for at least 3.7 h. Methocarbamol is stable at −20 °C for at least 86 days for long-term stability.

In order to verify whether the response of the MS was consistent from the first injection to the last injection for a same sample within a big batch, reinjection reproducibility was also evaluated. Within a same batch, QC samples were injected and then re-injected after certain injections. The responses were compared to see if they kept constant under the conditions defined by the method. It was found that responses of samples were consistent for reinjection after more than 100 injections.

3.7. Application to bioequivalence study

This method was applied to a single dose bioequivalence study in healthy volunteers under fasting conditions. The blood samples were collected over a period of 12 h following an oral dose of 500 mg tablets. The blood samples were immediately centrifuged and plasma was separated from blood cells and stored at −20 °C until the analysis was complete. An example of plasma concentration–time curve of methocarbamol from one volunteer is shown in Fig. 2d. The results show that the test and the reference products have a similar profile. Both t_{max} are around 0.7 h, and the C_{max} are approximately 7000 ng/mL.

4. Conclusion

A highly selective and sensitive HPLC–MS/MS method was developed and validated for the determination of methocarbamol concentration in human plasma. The method offers a simple, fast and efficient extraction procedure and analysis for methocarbamol. The results demonstrate that the method is robust, which delivers processed sample stability for over 100 h and reinjection reproducibility for over 100 injections. It is suitable for pharmacokinetic or bioequivalent studies with a large number of samples.

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References

[1] E.B. Carpenter, South. Med. J. 51 (1958) 627.
[2] J.K. Elenbaas, Am. J. Hosp. Pharm. 37 (1980) 1313.
[3] W.W. Muir, R.A. Sams, S. Ashcraft, Am. J. Vet. Res. 45 (1984) 2256.
[4] R.B. Bruce, L.B. Turnbull, J.H. Newman, J. Pharm. Sci. 60 (1971) 104.
[5] A.D. Campell, F.K. Coles, L.L. Eubank, E.G. Hut, J. Pharmacol. Exp. Ther. 131 (1961) 18.
[6] M.J. O'Neil (senior ed.), The Merck Index, Merck Research Laboratories, Whitehouse Station, NJ, 13th ed., 2001, p. 1070.
[7] R. Obach, J. Prunonosa, A. Menargues, M. Nomen, J. Valles, Biopharm. Drug Dispos. 9 (1988) 501.
[8] S. Alessi-Severini, R.T. Coutts, F. Jamali, F.M. Pasutto, J. Chromatogr. 582 (1992) 173.
[9] N.D. Weng, J.W. Lee, J.D. Hulse, J. Chromatogr. B 654 (1994) 287.
[10] M.R. Koupai-Abyazani, B. Esaw, B. Laviolette, J. Anal. Toxicol. 21 (1997) 301.
[11] Guidance for industry, bioanalytical method validation. US Department of Health and Human Services. Food and Drug Administration, Center for Drug Evaluation and Research (CDER), May, 2001.