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Determination of methazolamide concentrations in human biological fluids using high performance liquid chromatography[1]

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Abstract

Methazolamide is a carbonic anhydrase inhibitor used to treat glaucoma. In vivo, methazolamide readily distributes into red blood cells. Therefore, both blood and plasma concentration data are needed in order to characterize the pharmacokinetics of methazolamide. In the present study, an analytical method using high performance liquid chromatography was validated for determination of methazolamide concentrations in several biological fluids. Through slight modification of a previously reported method for acetazolamide, another carbonic anhydrase inhibitor, methazolamide was readily quantitated in whole blood, plasma and urine. Sample preparation involved liquid–liquid extraction with ethyl acetate followed by a washing step using phosphate buffer (pH 8.0). After back extraction into glycine buffer (pH 10.0), samples were then washed with ether and injected onto the chromatograph. Chromatography was performed using a C-18, 5 μm reverse-phase column with UV detection at a wavelength of 285 nm. Mobile phase consisted of 0.05 M sodium acetate (pH 4.0) and acetonitrile (20%). The assay was validated over two standard concentration ranges from 1 to 100 μg ml^{-1}, concentrations reflective of those expected in vivo. Calibration curves were linear for all biological fluids and coefficients of variation for interday and intraday reproducibility studies were less than 8% (range 3.1–7.9%). The method was used to measure methazolamide concentrations in blood, plasma and urine following oral administration to five human subjects.

Keywords: Methazolamide; HPLC; Blood; Plasma; Urine; Carbonic anhydrase inhibitor

1. Introduction

Methazolamide (MTZ) is a carbonic anhydrase inhibitor (CAI) used clinically in the management of glaucoma [1]. Being a CAI, the disposition of MTZ is influenced by its substantial uptake into and sequestration by red blood cells. Consequently, when conducting pharmacokinetic studies with this compound, knowledge of drug

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Table 1
Data from calibration curves[a] of MTZ HPLC assay for various biological fluids

Fluid	Concentration range (μg ml^{-1})	Correlation[b] (r^2)	Slope[b]	Intercept[b]
Whole blood	1.0–10	0.9968 ± 0.0032	0.4816 ± 0.0380	0.147 ± 0.162[c]
	10–100	0.9976 ± 0.0024	0.0498 ± 0.0043	0.038 ± 0.065[c]
Plasma	1.0–10	0.9940 ± 0.0060	0.4862 ± 0.0266	0.040 ± 0.055[c]
Urine	10–100	0.9934 ± 0.0066	0.0334 ± 0.0022	−0.015 ± 0.026[c]

[a]Peak height ratio [MTZ:ACZ] vs. concentration.
[b]Data presented as mean ± S.D. of 5 calibration curves.
[c]Not significantly different from zero ($P > 0.1$).

concentrations in plasma, blood and urine are required for complete characterization of MTZ disposition. Although a GC/MS assay for MTZ is published in the literature [2], there is no reported method for quantitation of MTZ by high performance liquid chromatography (HPLC).

The purpose of this study was to validate an HPLC assay for MTZ in several biological fluids. MTZ was quantitated in human blood, plasma and urine through modification of a previously reported method for acetazolamide (ACZ) [3]. This analytical method was applied to a study of MTZ disposition in human subjects following oral administration.

2. Materials and methods

2.1. Chemicals

MTZ and ACZ (internal standard) were purchased from Sigma Chemical Company (St. Louis, MO). All solvents used for extraction and chromatography were obtained from J. and H. Berge Company (Plainfield, NJ). All solvents were HPLC grade.

Separate stock solutions of 1 mg ml^{-1} MTZ and ACZ were prepared weekly by dissolution in 0.005 M sodium hydroxide. Solutions of 0.1 M glycine (pH 10.0) and 0.1 M phosphate buffer

Table 2
Summary of data detailing reproducibility of HPLC assay for various biological fluids

Biological fluid	Interday reproducibility[a]		Intraday reproducibility[b]	
	Conc. (μg ml$-^1$)[c]	cv%[d]	Conc. (μg ml$-^1$)[c]	cv%[d]
Whole blood	70	4.70	70	3.10
	30	3.62	30	6.83
	7.0	5.44	7.0	3.75
	3.0	6.30	3.0	4.15
Plasma	7.0	4.13	7.0	4.04
	3.0	5.73	3.0	6.23
Urine	7.0	4.56	70	5.20
	3.0	5.93	30	7.91

[a]Established over 5 days.
[b]Ten check samples processed at each concentration.
[c]Concentration of check sample processed.
[d]Coefficient of variation based upon average of concentrations measured by HPLC.

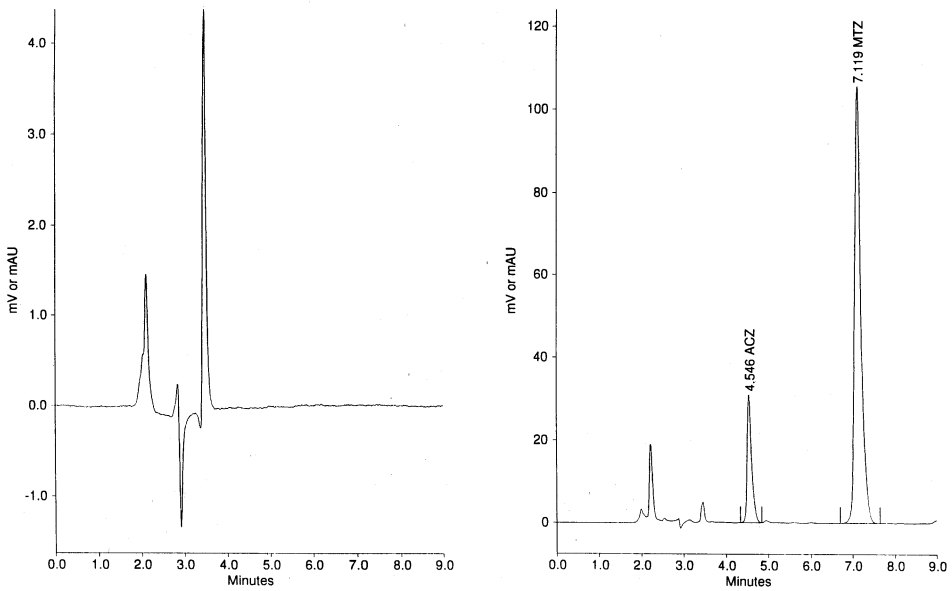

Fig. 1. Sample chromatograms of an extract of blank blood (left panel) and blood containing MTZ and ACZ.

(pH 8.0) were prepared every other week. All solutions were stored at room temperature.

2.2. Extraction procedure

Whole blood and plasma were acquired from the New York Blood Center (New York, NY) and urine was obtained from drug-free volunteers. 500 μl of sample (blood, plasma, or urine) was added to a 16×125 mm borosilicate glass tube. 20 μl of internal standard was added. 2.5 ml of 50% ammonium sulfamate was then added and the tube was sealed and vortexed for 30 s. At this point, tubes containing whole blood were placed in boiling water for 30 s and then quickly placed in cold water. 5 ml of ethyl acetate added, and the tube again was sealed and vortexed. The mixture was then centrifuged at $3000 \times g$ for 10 min. The organic layer was transferred to an identical tube containing 5 ml phosphate buffer (pH 8.0). The tube was sealed, vortexed and centrifuged as previously stated. The organic layer was then transferred to a 13×100 mm tube containing 500 μl glycine buffer (pH 10.0) and the resultant mixture vortexed (30 s) and centrifuged for 5 min. The organic layer was then aspirated and discarded. 500 μl of ether was added to the remaining glycine layer and the tube was then sealed and vortexed for 1 min. The ether phase was then removed and discarded. In order to remove any residual ether from the sample, the tube was allowed to vent for ≈ 30 min. 20 μl of sample was then injected onto the chromatograph.

2.3. Chromatography

The HPLC system consisted of a Thermo Separation Products P1000 constant flow pump and a UV1000 ultraviolet detector (Thermo Separation Products, Riviera, FL). Output from the detector was processed using an IBM P75 personal computer (IBM, Research Triangle Park NC) with PC1000 integration software (Thermo Separation Products). Separation was achieved using an Altima 4.6×250 mm, 5 μm C-18 reverse phase column (Alltech Associates, Deerfield IL) and a

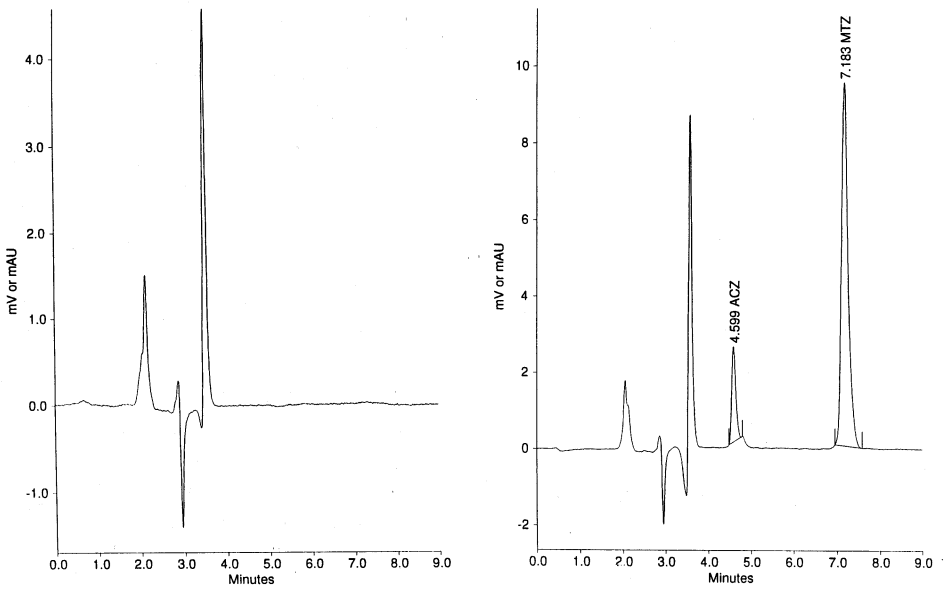

Fig. 2. Sample chromatograms of an extract of blank plasma (left panel) and plasma containing MTZ and ACZ.

mobile phase which consisted of acetonitrile (20%) and 0.1 M sodium acetate (pH 4.0). The mobile phase flow rate and detection wavelength were 1 ml min^{-1} and 285 nm, respectively.

2.4. Standard concentrations

Known quantities of MTZ were added to blank whole blood, plasma and urine. For whole blood analysis, two ranges of standard concentrations were utilized: 1–10 μg ml^{-1} (1, 2, 4, 6, 8, 10 μg ml^{-1}) and 10–100 μg ml^{-1} (10, 20, 40, 60, 80, 100 μg ml^{-1}). Only the lower concentration range was employed for the plasma assay, while the upper concentration range (10–100 μg ml^{-1}) was used for urine. The concentration of internal standard for these lower and upper ranges of standards was 5 and 50 μg ml^{-1}, respectively. Check samples representing two concentrations within each standard range were prepared prior to assay validation. For the lower concentration range, check samples of 3 and 7 μg ml^{-1} were utilized. Likewise, concentrations 30 and 70 μg ml^{-1} were used for the upper standard range.

2.5. Assay validation/data analysis

Interday assay reproducibility was assessed over a period of five days. Calibration curves were constructed by plotting peak height ratio (MTZ:ACZ) against MTZ concentration. The precision of the assay was confirmed through HPLC analysis of check samples. Intraday analysis was determined upon replicate analysis of 10 check samples. Absolute recovery of MTZ from whole blood, plasma and urine was calculated as the relative PHR between equal drug concentrations of sample and glycine buffer. In the latter sample, glycine buffer was spiked with drug and injected directly onto the chromatograph.

2.6. Disposition of mtz in humans following oral administration

Following an overnight fast, five healthy human subjects received a 1.5 mg kg^{-1} oral dose of MTZ. Blood and plasma samples were collected at 0, 0.5, 1, 2, 3, 4, 8, 12, 24 and 36 h post-dose. Urine was collected in predetermined intervals

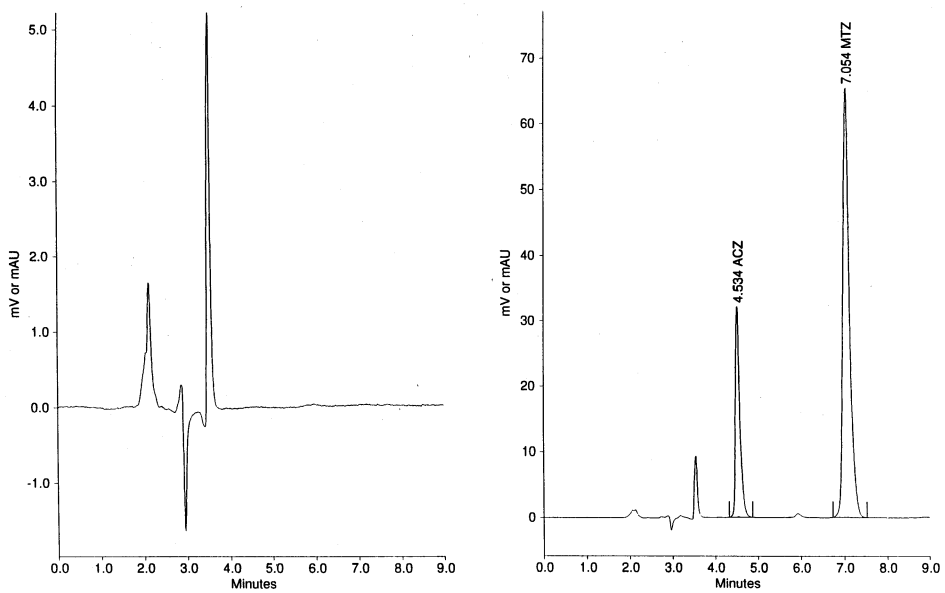

Fig. 3. Sample chromatograms of an extract of blank urine (left panel) and urine containing MTZ and ACZ.

over the duration of the study. MTZ was quantitated in all samples using the HPLC assay described above. MTZ red blood cell concentrations (C_{RBC}) were estimated from blood (C_B) and plasma (C_P) concentrations using the following formula [4]:

$$C_{RBC} = \frac{C_B - (1 - Hct)C_P}{Hct}$$

where Hct represents the hematocrit.

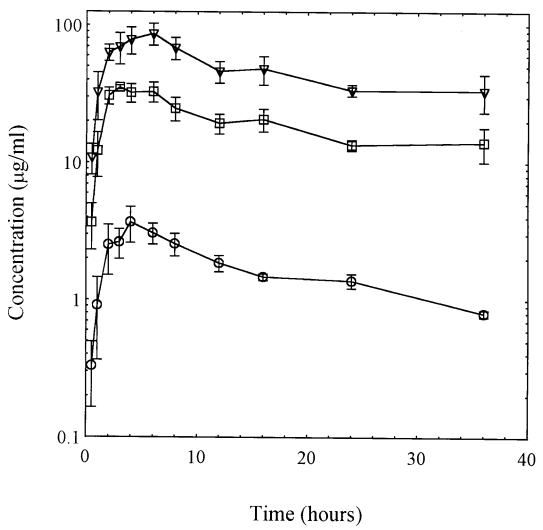

Fig. 4. Plot of mean (S.D.) MTZ concentrations versus time following oral administration to human subjects (○, plasma; □, blood; △, RBC).

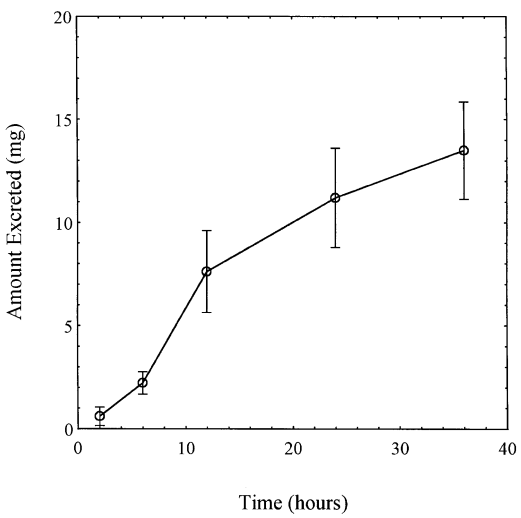

Fig. 5. Plot of mean (S.D.) amount of MTZ excreted into urine versus time following oral administration to human subjects.

3. Results

Table 1 contains linear regression results for calibration curves for all biological fluids studied. Mean correlation coefficients (r^2), slopes and intercepts are included in the table. Calibration curves were linear over each concentration range studied and intercepts were not significantly different from zero. Table 2 summarizes the assessment of both interday and intraday reproducibility of the method. Data presented in Table 2 are the coefficients of variability (CV%) for each check sample processed.

Absolute recovery of MTZ from whole blood, plasma and urine were 52, 46 and 34% respectively. Sample chromatograms for drug in blood, plasma and urine are displayed in Figs. 1–3. Included in these figures are chromatograms of blank 'drug-naive' sample and sample containing MTZ and internal standard (ACZ).

The results of the drug disposition study are presented in Figs. 4 and 5. Fig. 4 displays the concentration–time profile for MTZ in plasma, blood and red blood cells. The urinary excretion profile of MTZ (cumulative amount excreted versus time) is depicted in Fig. 5.

4. Discussion

There is limited information in the literature regarding MTZ. While Maren et al. have published an overall review of the drug's pharmacology and disposition [1], they provide no analytical method for MTZ. Previously, Bayne et al. have published a GC/MS assay for quantitation of MTZ in blood, plasma and urine [2]. However, there is no reported HPLC method for quantitating MTZ in biological fluids.

MTZ is a methylated derivative of a tautomer of ACZ. Unlike MTZ, an HPLC assay for ACZ has been previously reported [3]. In the present study, MTZ was readily quantitated in several biological fluids by HPLC through modification of that assay using ACZ served as the internal standard. Modifications were slight and included use of a different column, different mobile phase and higher detection wavelength. As indicated in Tables 1 and 2, the assay displayed linearity across all concentration ranges studied and both interday and intraday variability was less than 8%. Like ACZ, MTZ is a relatively polar compound (octanol:water partition coefficient approximately 3.5×10^{-2}) [5] and would therefore be expected to coextract with constituents present in the sample matrix, creating difficulties in drug detection and quantitation. As demonstrated in Figs. 1–3, however, blank samples of blood, plasma, and urine do not show any interfering peaks at 4.5 and 7.1 min, the retention times for internal standard and MTZ. Chapron and White attribute the successful separation of ACZ from any interfering endogenous substances to the phosphate buffer washing step in the extraction procedure [3]. Consequently, while the use of multiple extraction steps may have compromised the absolute recovery of drug from sample, the assay method allowed for separation of drug with good sensitivity.

The HPLC assay describe herein was successfully utilized to study the disposition of MTZ in humans following oral administration. Fig. 4 depicts mean concentration–time profiles for MTZ in plasma, blood and red blood cells from five subjects. Inspection of this graph reveals evidence of significant red blood cell uptake of drug. The urinary excretion profile of MTZ is included in Fig. 5. Preliminary data analysis suggests that the elimination half-life of MTZ from blood may be as long as 100 h (unpublished data), a phenomenon which can be attributed to its extensive sequestration by red blood cells.

In summary, an HPLC assay for MTZ in whole blood, plasma and urine was validated in the present study. The assay displayed excellent reproducibility and was linear over standard concentration ranges of 1–10 μg ml^{-1} and 10–100 μg ml^{-1}. The assay was used to quantitate drug concentrations in human biological fluids following oral dosing.

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