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Novel high-performance liquid chromatographic method using solid-phase on-line elution for determination of metolazone in plasma and whole blood

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Abstract

A novel solid-phase on-line elution HPLC method employing fluorescence detection to measure metolazone in plasma and whole blood has been developed. The method is sensitive and selective for metolazone and linear over a dynamic range of 1–50 ng/ml with a sample requirement of 250 μl. The limit of quantitation for the method is 1 ng/ml and combined intra- and inter-day accuracy and precision had an error and coefficient of variation of 2.9 and 5.5%, respectively.

1. Introduction

Metolazone is a distal tubular diuretic employed in the management of hypertension and diuretic-resistant conditions [1,2]. The diuretic activity of metolazone relates to both the rate and extent of its urinary delivery [3] which, in turn, is linked by its plasma and indirectly by its whole blood concentrations [4]. Due to the limited and somewhat erratic nature of metolazone absorption accurate quantitation of low level plasma and/or whole blood quantities of metolazone is necessary [4].

Prior HPLC methods for determination of metolazone in plasma have utilized either ultraviolet [5] or fluorescence detection [6] but showed limitations either because of poor sensitivity [5] or because of excessive sample volume requirements (2 ml of plasma) [6]. A recently developed urine method [7] circumvented many of these problems and provided the background needed for development of a method for plasma metolazone with similar sensitivity and selectivity. Accordingly, we have developed a sensitive HPLC method for measuring plasma and whole blood concentrations of metolazone. This method utilizes on-line elution and fluorescence detection and was developed in conjunction with a pharmacokinetic and pharmacodynamic investigation of metolazone in chronic renal failure.

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2. Experimental

2.1. Chemicals and reagents

Pure metolazone and a metolazone isomer (Lot No. 812-256FB-00) (Fig. 1) employed as an internal standard were kindly donated by Fisons Pharmaceuticals (Rochester, NY, USA). Acetonitrile (Burdick and Jackson, Muskegon, MI, USA), monobasic and dibasic potassium phosphate (Fisher Scientific, Pittsburgh, PA, USA), methanol (Burdick and Jackson), and hexane (Fisher Scientific) were of HPLC grade. Phosphoric acid (85%) (Fisher Scientific) was of analytical grade. The C_2 ethyl sorbent cartridges were purchased from Analytichem International (Harbor City, CA, USA). Prepurified nitrogen was obtained from Airco Medical Products (Chester, VA, USA).

A stock solution of metolazone (1 mg/ml) was prepared in methanol–deionized water (70:30; v/v) and stored at 4°C in amber polypropylene bottles. A stock solution of metolazone isomer (1 mg/ml) was prepared in methanol–deionized water (90:10, v/v) and sonicated prior to storage at 4°C in amber polypropylene bottles. A working solution of metolazone was prepared by serially diluting 25 μl of stock metolazone solution with deionized water to achieve a final working concentration range for metolazone of 1–50 ng/ml. A working solution of metolazone isomer was prepared by serially diluting 50 μl of stock metolazone isomer solution with deionized water to achieve a final working concentration range for metolazone isomer of 1 μg/ml. Working standard and internal standard solutions were prepared monthly and stored in amber polypropylene bottles at 4°C to minimize photodegradation.

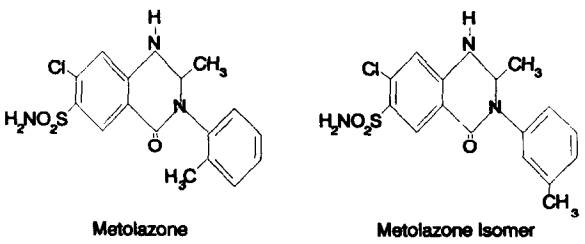

Fig. 1. Structure of metolazone and metolazone isomer.

2.2. HPLC instrumentation and operating conditions

An LKB Model 2150 pump and LKB Model 2152 liquid chromatographic controller (Gaithersburg, MD, USA) equipped with a Shimadzu RF-535 fluorescence detector (Tokyo, Japan) coupled to an advanced automated sample processor (AASP) (Varian, Walnut Creek, CA, USA), and Nelson 2600 chromatography data integration package (PE Nelson, Norwalk, CT, USA) were utilized. A Spherisorb ODS C_{18} column, 10 cm × 4.6 mm I.D. with 3-μm packing and a cartridge insert with 0.5-μm frits was used (Alltech, Deerfield, IL, USA). A C_{18} guard column, 3 cm, with 40–50 μm pellicular packing dry-packed with 2-μm frits was employed. The guard column was routinely changed after 100 injections to retard analytical column degradation.

The mobile phase, pumped at a flow-rate of 1 ml/min, was monobasic potassium phosphate–acetonitrile (70:30, v/v) acidified to a pH of 3.0 with concentrated phosphoric acid. The excitation wavelength was 235 nm and the emission wavelength was 410 nm. Both excitation and emission wavelengths were optimized manually by use of a pure solution of the analyte. Injections were made via the AASP that uses on-line elution from the solid-phase C_2 ethyl sorbent cartridge into the HPLC system. All experiments were carried out at ambient temperature. With this mobile phase, the retention time for metolazone and metolazone internal standard were 5.83 and 7.00 min, respectively.

The Varian AASP conditions listed in Table 1 led to the highest recovery for the analytes of interest while still eliminating endogenous interfering substances from plasma. These conditions were determined during optimization of the valve reset and prepurge settings. The C_2 sorbent proved optimal as it is the most selective sorbent for metolazone and the internal standard. Other sorbents evaluated included C_{18}, C_8, phenyl and carboxymethyl. Hexane is utilized as

Table 1
Varian AASP conditions

Parameter	Value
Run time	2 min
Cycle time	8 min (external standard) 10 min (internal standard)
Valve reset	1 min
Wait time	6 s
Prepurge	5 × 25 μl with deionized water
Postpurge	10 × 25 μl with deionized water
AASP pressure	4.4 bar (nitrogen)
Prepstation pressure	1.3 bar (nitrogen)

a final wash step for the C_2 cartridge to eliminate cartridge plasticizers during injection.

2.3. Sample preparation and extraction

Whole blood samples were collected in heparinized tubes and split aliquotted. The whole blood aliquot as well as the plasma sample obtained by centrifugation (5 min, 580 *g*) were both stored in the dark in polypropylene tubes at −30°C. Plasma and whole blood samples were allowed to thaw by ambient temperature exposure alone and were shielded from outside light to prevent photodegradation of the metolazone. Plasma samples were subsequently mixed by inversion and vortex-mixed for 30 s and then centrifuged at 580 *g* for 10 min. Samples for whole blood analysis were vortex-mixed for 30 s to assure homogeneity of the sample. A 500-μl volume of whole blood is then pipetted into a 12 × 75 mm plastic culture tube whereupon 1500 μl of deionized water is added and the mixture is vortex-mixed for 15 s. Transfer to a microcentrifuge plastic bullet tube is then effected and the mixture is centrifuged for 5 min at 13 000 *g*.

Extraction was performed with the AASP prepstation, which uses nitrogen to create a positive-pressure purge of liquids through the C_2 ethyl sorbent cartridge, which is activated upon sequential addition of 1.5 ml of acetonitrile–methanol (50:50, v/v) and 1.5 ml of extraction buffer (25 ml K_2HPO_4). The extraction buffer was maintained at pH 7.0 using concentrated H_3PO_4. A 250-μl sample of plasma or prepared whole blood was mixed with 25 μl of internal standard and 250 μl of extraction buffer and applied to the previously conditioned C_2 ethyl sorbent cartridge. The retained analyte was sequentially washed with 1 ml of extraction buffer–methanol (90:10, v/v) and 1 ml of hexane and purged dry with nitrogen for 1 min in order to deactivate the C_2 ethyl sorbent. The nitrogen purge removes residual liquid from the cartridge which, in turn, prevents undesirable chemical reactions prior to injection. Cassettes, containing a maximum of 10 samples, were then placed on the Varian AASP for on-line elution into the HPLC system.

2.4. Quantitation

A standard curve was prepared for each assay by spiking blank plasma with aliquots from an aqueous working metolazone solution (250 ng/ml) to concentrations of 1, 5, 10, 25 and 50 ng/ml. Blank drug-free plasma samples and standards were each carried through the extraction procedure. Calibration curves were obtained with normal linear regression of known drug concentration *versus* peak area. Quality control samples of known concentrations (7.5, 15 and 37.5 ng/ml) were prepared identically to the standards but from a different stock solution. Subject sample concentrations and concentrations of quality control samples were calculated by using the regressed equation of a straight line $y = mx + b$ where y is the peak area, m is the slope, b is the y-intercept, and x is concentration in (ng/ml). The limit of quantitation for the method was defined as the lowest standard assayed at which a ± 20% accuracy was obtainable. The limit of detection for metolazone was calculated as 3 standard deviations from a forced baseline integration of the metolazone time window in biologic blanks ($n = 5$).

2.5. Volunteer study

A male end-stage renal disease patient being maintained with chronic hemodialysis was administered daily doses of furosemide in conjunction with single daily oral doses of metolazone

(Zaroxylyn tablets, 2.5 mg, Fisons, Rochester, NY, USA) ranging from 2.5 to 5.0 mg. Metolazone administration was started on the day immediately preceding the first sampling day and was continued throughout the study. The dose was increased to 5.0 mg/day on the day immediately preceding the fourth and last sampling day. Plasma and whole blood samples were obtained throughout the study with all samples being obtained prior to that days dose of metolazone. Samples were stored frozen pending analysis.

3. Results and discussion

3.1. Chromatography

Chromatograms from normal subject blank plasma, a normal subject plasma sample spiked with 5 ng/ml of metolazone with and without internal standard, and a plasma and whole blood sample from the metolazone-treated chronic renal failure patient are represented in Fig. 2. All peaks were well resolved and there was no interference from any endogenous substances. Under the conditions described, the retention time for metolazone was 5.83 min and for metolazone internal standard 7.40 min. The limit of detection for metolazone was 0.02 ng/ml and the limit of quantitation was 1 ng/ml ($n = 5$).

Absolute recovery of metolazone during the extraction procedure was determined by comparing area counts for direct injection *versus* extracted 10 and 25 ng/ml standards ($n = 3$/each level). The mean recoveries were 90 and 93% for the 10 and 25 ng/ml levels, respectively. The average plasma recovery was 92%. The average recovery for whole blood, employing the same 10 and 25 mg/ml standards, was 91%. The mean absolute recovery for the internal standard was 94% ($n = 6$).

Method selectivity was indicated by the absence of interferences from the biological drug-free blank. Co-medications likely to be administered with metolazone, including bumetanide (not extractable at extraction buffer pH), furosemide (not extractable at extraction buffer pH), hydrochlorothiazide (no peak), ibuprofen (no peak), and indomethacin (not extractable at extraction buffer pH), were also assayed. These drugs at concentrations of 10 μg/ml did not

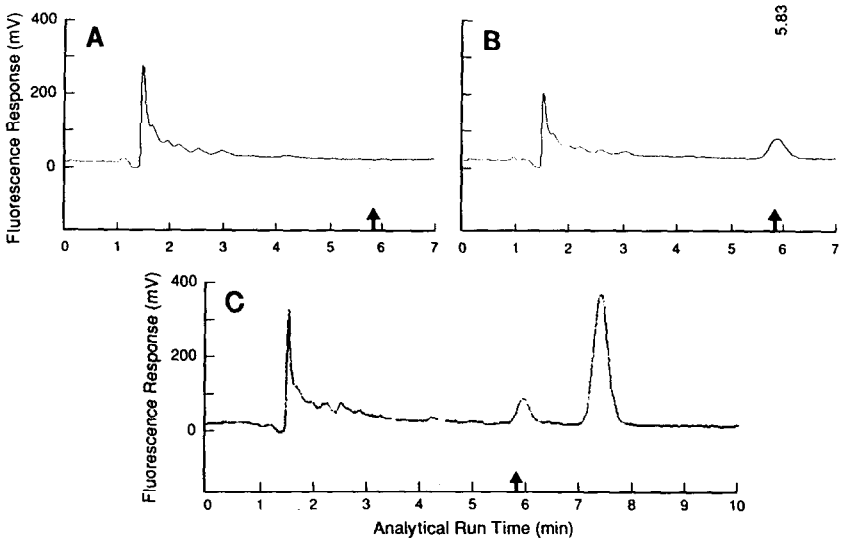

Fig. 2. Chromatograms of (A) blank normal subject plasma, (B) human plasma spiked with 5 ng/ml metolazone, (C) human plasma spiked with 5 ng/ml metolazone and 100 mg/ml of metolazone isomer. Peaks at 5.8 and 7.4 min are metolazone and metolazone isomer, respectively.

create interfering peaks or extend analytical run times.

The stability of metolazone and the internal standard on the C_2 sorbent was demonstrated by extracting standard curves in plasma followed by timed injections over an 8-hr period. Regression analysis of the standard curves over time showed no significant differences in the slope, *y*-intercept, and correlation coefficient. Control samples undergoing repetitive freeze–thaw cycles over ten months remained stable.

3.2. Linearity, precision, and sensitivity

A standard curve was constructed for metolazone by plotting calculated peak areas *versus* standard concentrations. For the analysis in plasma or whole blood, the method demonstrated good linearity over a dynamic range of 1 to 50 ng/ml determined by normal linear regression with mean correlation coefficients of 0.9985 and 0.9998 using internal and external standard calculations, respectively ($n = 5$). External standardization was utilized for all post method validation data manipulations as it demonstrated superior linearity over internal standardization and reduced the analytical run time.

The accuracy of the method was evaluated by calculating the percent difference between the concentrations of metolazone in the control samples, as determined with the HPLC method, and the known concentrations of metolazone in spiked blank plasma samples. The values listed in Table 2 reflect combined intra-day and inter-day statistics. The HPLC method is accurate, as indicated by the observation that all the percentage standard errors were < 2.9 (15 observations per control level). The precision of the method for combined intra- and inter-day analyses was determined by calculating the percentage coefficient of variation at each concentration of metolazone added to blank plasma samples (Table 2). Excellent precision for the method is indicated by coefficient of variations of 5.5% or less at all concentrations of metolazone tested (15 observations/control level).

Table 2
Combined intra- and inter-day accuracy and precision of metolazone in plasma

Spiked concentration (ng/ml)	Calculation concentration (ng/ml)	Mean error (%)[a]	Coefficient of variation (%)
7.5	7.3	(−)2.9	4.2
15.0	15.2	1.0	5.5
37.5	36.8	(−)1.9	1.2

[a] (−) = Negative mean error.

3.3. Pharmacokinetic study

Table 3 and Fig. 3 both show the plasma and whole blood metolazone concentrations in one subject. Three determinations at a daily dose of 2.5 mg and one determination at a dose of 5.0 mg/day are presented. With the 2.5-mg dose of metolazone, accumulation occurred as represented by an increase in whole blood and plasma

Table 3
Whole blood, plasma and hematocrit-corrected plasma metolazone concentrations following metolazone administration in a dialysis patient

Dose (mg)	Whole blood concentration (ng/ml)	Plasma concentration (ng/ml)	Hematocrit-corrected plasma concentration[a] (ng/ml)
2.5	31.1	29.7	20.5
2.5	63.2	67.2	46.4
2.5	52.6	62.3	43.0
5.0	126.2	161.2	111.2

[a] Patient's hematocrit during the study was 31 vol%. Hematocrit-corrected plasma concentration = (plasma concentration of metolazone) × (1 − hematocrit).

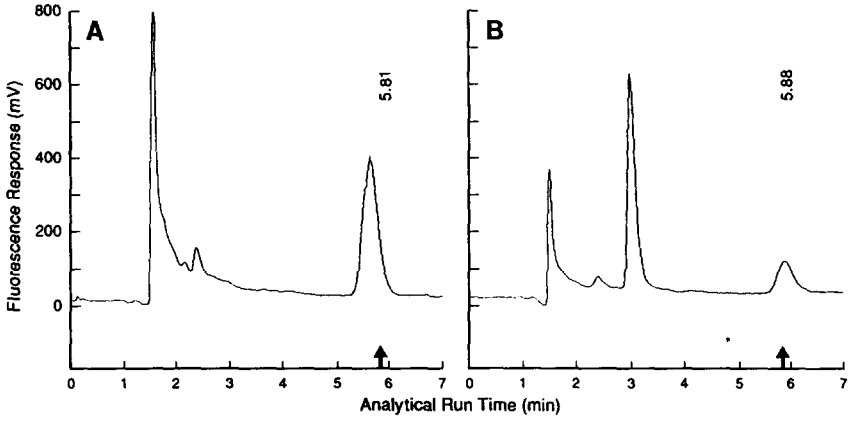

Fig. 3. (A) Chromatogram of trough plasma 24 h after administration of 2.5 mg of metolazone to a patient. (B) Chromatogram of whole blood 24 h after administration of 2.5 mg of metolazone to a patient. The metolazone concentration found was 8 ng/ml (1:4 dilution).

concentrations between the first and second sample days. A further concentration increase occurred with an increment in the dose from 2.5 to 5.0 mg/day.

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