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Note

Quantitation of metolazone in urine by high-performance liquid chromatography with fluorescence detection

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Metolazone is a distal tubular diuretic, commonly employed in diuretic-resistant conditions [1,2], whose rate and extent of absorption has proven to be somewhat unpredictable [3]. Diuretic responsiveness relates to both the time-course of urinary delivery as well as to the amount of drug reaching the urine [4]. Thus, accurate quantitation of low-level amounts of urinary metolazone is necessary to correctly characterize either its onset or duration of action.

Various methods [5–8] have been reported for the analysis of metolazone in urine including fluorescence spectrophotometry [5], high-performance liquid chromatography (HPLC) with fluorescence spectrophotometry [6] and reversed-phase liquid chromatography with UV detection [7]. Whereas early methods were characterized by a lack of sensitivity [5], subsequent methodologies have proven more sensitive with as little as 5 ng/ml metolazone detectable in urine [6]. Though sensitive plasma methods exist they have not been adapted for drug analysis in urine [7].

Despite reasonable sensitivity of available methodologies all existing methods require liquid–liquid extraction thereby increasing cost and the demand for technical time. Accordingly, we have developed a sensitive HPLC method utilizing fluorescence detection, capable of assaying small quantities of metolazone in unextracted urine, in conjunction with a pharmacokinetic and pharmacodynamic study of metolazone use in congestive heart failure.

EXPERIMENTAL

Chemicals and reagents

Pure metolazone was kindly donated by Fisons Pharmaceuticals (Rochester, NY, U.S.A.). Acetonitrile (Burdick and Jackson, Muskegon, MI, U.S.A.), monobasic potassium phosphate (Fisher Scientific, Pittsburgh, PA, U.S.A.) and metha-

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nol (Fisher Scientific) were HPLC grade. Phosphoric acid (85%) (Fisher Scientific) was of analytical-reagent grade.

A stock solution of metolazone (100 μg/ml) was prepared in 70% methanol and stored at 4°C. A working solution of metolazone was prepared by diluting 100 μl of metolazone with 900 μl of methanol achieving a final concentration of 10 μg/ml. Separate working solutions were prepared in blank urine for both standards and controls to ensure dilution accuracy. All frozen standards and controls were stored frozen in 1-ml aliquots in 75 mm × 12 mm polypropylene culture tubes at −20°C in the dark to avoid photodegradation and appeared to be stable for at least four months.

HPLC instrumentation and operating conditions

An LKB Model 2150 pump and LKB Model 2152 liquid chromatography controller (Gaithersburg, MD, U.S.A.) equipped with a Shimadzu RF-535 fluorescence detector (Tokyo, Japan), WISP Model 712 autosampler (Milford, MA, U.S.A.) and Nelson 2600 chromatography data integration package (Nelson Analytical, Paramus, NJ, U.S.A.) were utilized. A Nucleosil C_{18} column, 15 cm × 4.6 mm I.D., with 5-μm packing and a cartridge insert with 2-μm frits was used (Alltech, Deerfield, IL, U.S.A.). A C_{18} guard column, 3 cm, with 40–50 μm pellicular packing dry-packed with 2-μm frits was employed. The mobile phase, pumped at a flow-rate of 1 ml/min, was monobasic potassium phosphate–acetonitrile (65:35, v/v) acidified to a pH of 3.0 with concentrated phosphoric acid. The excitation wavelength was 240 nm and the emission wavelength was 450 nm. Both excitation and emission wavelengths were optimized manually by use of a pure solution of the analyte. All experiments were carried out at ambient temperature.

Sample preparation

Urine samples were stored in the dark at −20°C in polyethylene scintillation vials. Samples were allowed to thaw by ambient temperature exposure alone and were shielded from outside light to prevent photodegradation of the metolazone. Samples were subsequently mixed by inversion and vortexed for 15 s. Unfiltered urine (100 μl) was then mixed with 300 μl of deionized water, and a 40-μl aliquot was placed onto the column. The guard column was routinely repacked after 100 injections due to the non-filtration of urine and to protect the analytical column.

Volunteer study

Two male congestive heart failure patients [72 kg (A); 94 kg (B)] were given a single 5-mg oral dose of metolazone (Zaroxylyn tablets, 5 mg, Pennwalt Pharmaceuticals, Rochester, NY, U.S.A.) with 120 ml of water following an overnight fast. Urine samples were collected at periodic intervals over the ensuing 48 h and stored frozen until analysis.

RESULTS AND DISCUSSION

Chromatography

Chromatograms from a blank normal subject urine, a blank and treated urine from a congestive heart failure patient and human urine spiked with known concentrations of metolazone are shown in Fig. 1. All peaks were well resolved and were not interfered with by endogenous substances. Under the conditions described, the retention time of metolazone was 7.1 min and the limit of detection for this method was 4.2 ng/ml. Variation of the pH of the mobile phase was not found to have significant effects on the resolution of the peaks or retention volumes.

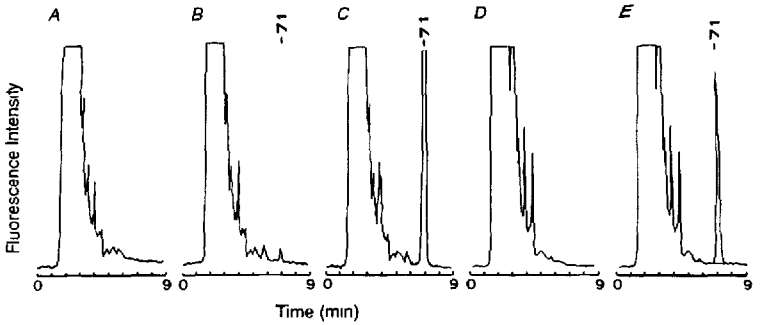

Fig. 1. Chromatograms of (A) blank normal subject urine, (B) human urine spiked with 25 ng/ml metolazone, (C) human urine spiked with 1000 ng/ml metolazone, (D) congestive heart failure blank urine and (E) patient dosed with metolazone Peak at 7.1 min = metolazone

Though other diuretic compounds were not administered in the course of this patient study, diuretics likely to be co-administered with metolazone were evaluated as to their retention times in this system. Accordingly bumetanide (retention time, 23.4 min), furosemide (retention time, 8 min) and chlorothiazide (no peak) and hydrochlorothiazide (no peak) were found not to interfere with metolazone analysis. In addition, other commonly administered medications such as ibuprofen and acetaminophen had no peak while salicylic acid had a retention time of 4.9 min

Linearity, precision and sensitivity

A standard curve was constructed for metolazone by plotting calculated peak areas *versus* standard concentrations. For the analysis in urine, the standard curve was linear over the range studied (25–1000 ng/ml) with correlation coefficients of 0.999904 ± 0.00010 (S.D.). Intra-day precision was evaluated by replicate analysis ($n = 4$) of a pooled urine sample containing metolazone at various concentrations (Tables I and II). Inter-day precision was similarly evaluated over one month ($n = 10$).

TABLE I

INTRA-DAY VARIABILITY OF METOLAZONE IN URINE (n = 4)

Spiked concentration (ng/ml)	Calculated concentration (ng/ml)	Coefficient of variation (%)
40	41.8	1 9
250	252.7	1 5
750	762 6	1 7

TABLE II

INTER-DAY VARIABILITY OF METOLAZONE IN URINE (n = 10)

Spiked concentration (ng/ml)	Calculated concentration (ng/ml)	Coefficient of variation (%)
40	39 8	5.5
250	249 4	2.5
750	749 2	2 6

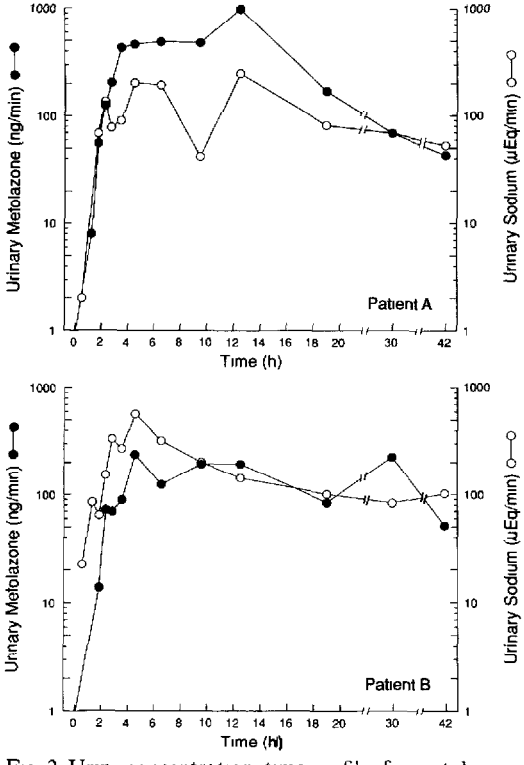

Fig 2 Urine concentration–time profiles for metolazone. Patient A and patient B were each administered 5 mg of metolazone.

Pharmacokinetic study

Fig. 2 shows the urine metolazone (ng/min) and sodium excretion (μequiv./min) rate–time profiles for two patients following a single oral 5-mg dose of metolazone. Metolazone was first detected in the urine between 1 and 1.5 h after its administration and reached its peak excretion rate between 11 and 14 h after its administration. Sodium excretion paralleled that of metolazone with excretion increasing starting at 1–1.5 h and peaking between 11 and 14 h (patient A) and between 6 and 8 h (patient B) following administration of metolazone. Though metolazone was detectable in urine throughout the 48 h of the study, sodium excretion was noticeably curtailed at the later stages of the study suggesting an attenuation of the diuretic response.

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