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Note

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

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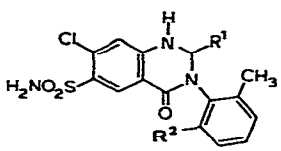
Metolazone (Fig. 1, Ia) is a recently introduced diuretic drug. Although a fluorimetric [1] and a high-performance liquid chromatographic (HPLC) [2] assay have been reported for the drug in urine, no method is currently available for metolazone in plasma, with the result that any information about its absorption and pharmacokinetics have been based on measurements of total radioactivity [3].



I (a) $R^1 = CH_3$; $R^2 = H$
(b) $R^1 = (CH_2)_3\,CH_3$; $R^2 = H$
(c) $R^1 = (CH_2)_2\,CH_3$; $R^2 = H$
(d) $R^1 = CH(CH_3)_2$; $R^2 = H$
(e) $R^1 = R^2 = CH_3$

Fig. 1. Structures of metolazone and its analogues.

In conjunction with studies of the pharmacokinetics and absorption of the drug in man a HPLC method utilising a fluorescence detector has been developed to quantify the drug in plasma and urine. The method and some initial results of its application are described in this report.

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EXPERIMENTAL

Apparatus

Chromatography was performed with a component system comprising an Applied Chromatography (Luton, Great Britain) Model 750/03 HPLC pump, Deci-linear gradient programmer, solvent composition optimising unit and a column (10 cm × 5 mm I.D.) fitted with a syringe injector (Shandon Southern Products, Runcorn, Great Britain) and slurry packed with C_8-alkyl silylated silica (5 μm, SAS-Hypersil, Shandon Southern Products) according to the method of Knox et al. [4]. The column was linked to a Perkin-Elmer (Beaconsfield, Great Britain) 3000 fluorescence spectrophotometer fitted with a 100-μl flow cell. The output from the fluorimeter was linked to a 10-mV potentiometric recorder (Servoscribe 1S, Smiths Industries, Cricklewood, Great Britain) and an integrator (Supergrator, Columbia Scientific Industries, Austin, TX, U.S.A.).

Materials

Metolazone (Ia) was obtained from G.D. Searle (High Wycombe, Great Britain) and its four analogues (Fig. 1) were kindly donated by Pennwalt (Rochester, NY, U.S.A.). All other reagents, and solvents which were redistilled before use, were of Analar quality, purchased from Hopkin and Williams (Chadwell Heath, Great Britain). Control plasma and urine samples were obtained from healthy human volunteers.

Selection of fluorimetric conditions and internal standard

The fluorescence spectra of metolazone and its analogues recorded in methanol—water (50:50), exhibited excitation and emission maxima at 230 and 420 nm respectively. The separation of metolazone from plasma components was optimised using flow programming and the metolazone analogues were chromatographed in the selected optimised solvent mixture methanol—water (35:65). This showed the 2-isopropyl analogue (Id) to be a suitable internal standard for a quantitative assay.

Plasma assay procedure

A 0.05-ml aliquot of a solution (1.25 μg/ml) of the internal standard (Id) in methanol—water (50:50) was added to plasma (2 ml) in an acid-washed amber glass tube. The plasma was then extracted with hexane (2 ml) on a partitioning extractor for 5 min, centrifuged and the hexane layer was discarded. Saturated potassium dihydrogen phosphate solution (2 ml) was added, mixed and the acidified plasma was extracted with chloroform (4 ml), centrifuged, the chloroform layer was transferred to an acid-washed amber glass Quick-Fit tube (MF 23/5) and evaporated to dryness under a nitrogen stream.

The residue was dissolved in 50 μl methanol—water (40:60) and 10-μl aliquots were analysed on a column of SAS-Hypersil (5-μm; 10 cm × 5 mm I.D.) with methanol—water (35:65) mobile phase at a flow-rate of about 2.3 ml/min, pressure 80—100 bar, with the fluorimeter set at 230 nm (excitation) and 420 nm (emission). The peak height ratio of metolazone and the internal standard, and the metolazone concentration in unknown samples were determined by the integrator. The calibration curve and the assessment

of the accuracy and precision of the assay method were obtained by the analysis of plasma samples containing added metolazone.

Urine assay procedure

A 0.05-ml aliquot of a solution (11 μg/ml) of the internal standard (Id), in methanol—water (50:50) was added to the urine (0.5 ml) in an acid-washed amber glass tube. Saturated potassium dihydrogen phosphate solution (0.5 ml) was added, mixed, and the acidified urine was extracted with chloroform (1 ml). The chloroform extract was separated by centrifugation and evaporated to dryness. The residue was dissolved in 0.5 ml methanol—water (40:60) and 0.01-ml aliquots were analysed as described above for the plasma extracts.

RESULTS AND DISCUSSION

The chromatographic conditions provided an efficient resolution of metolazone and the internal standard from the endogenous plasma and urine components as shown in Figs. 2 and 3, although a small peak with a retention time close to that of metolazone was observed. The slurry packed SAS-Hypersil reversed-phase columns (10 cm × 5 mm I.D.) gave 4200—5500 theoretical plates for metolazone and the internal standard which had retention times of about 4 and 8 min respectively. Column performance deteriorated gradually during the analysis of plasma extracts, but the high efficiency could be regained by replacing the ballotini beads and re-packing the first 2—3 mm of the column with a concentrated slurry of the stationary phase. However, after the analysis of some 150—200 plasma samples column performance could not be improved by this technique and a new column was prepared.

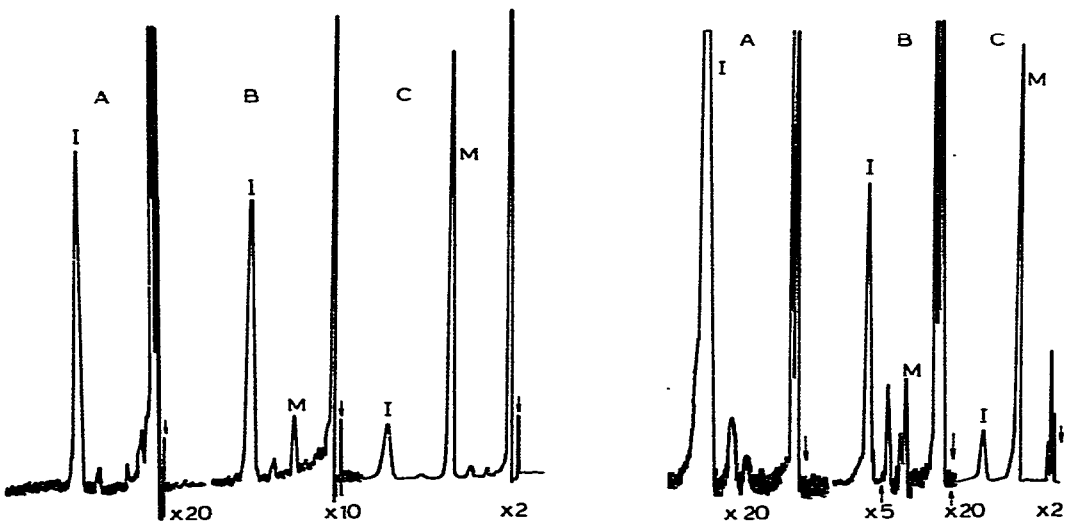

Fig. 2. Chromatograms of extracts from plasma samples containing (A) 0, (B) 3 and (C) 124 ng/ml of metolazone. M = Metolazone; I = internal standard.

Fig. 3. Chromatograms of extracts of urine samples containing (A) 0, (B) 50, and (C) 5000 ng/ml of metolazone. M = Metolazone; I = internal standard.

There was a linear correlation between the metolazone concentration in plasma and the metolazone/internal standard peak height ratio over the concentration range 0—200 ng/ml. The analysis of quality control plasma samples showed a satisfactory level of accuracy and precision (Table I). Plasma levels of 4.7 ng/ml could be accurately measured with a between-assay coefficient of variation of 8.1%. The limit of detection was about 1 ng/ml with a 2-ml plasma sample.

TABLE I

BETWEEN-ASSAY ACCURACY AND PRECISION OF PLASMA METOLAZONE ASSAY

Metolazone concentration (ng/ml)			C.V. (%)	Recovery (%)
Theory	Measured	*n*		
107	105	2	—	98.1
51.4	52.8	4	1.1	103
25.7	25.0	6	1.4	97.2
10.3	10.7	5	4.1	104
4.7	4.89	3	8.1	104
			Mean	101 ± 3.3% (S.D.)

There was a linear correlation between the metolazone concentration in urine and the metolazone internal standard peak height ratio over the concentration range 0—5000 ng/ml. Analysis of quality control urine samples showed a satisfactory level of accuracy and precision (Table II), and 50 ng/ml concentrations could be measured with a between-assay coefficient of variation of 7.8%. The limit of detection was about 5 ng/ml with a 0.5-ml urine sample.

Assay interference by metolazone metabolites was not specifically investigated. However, some 80% of the administered compound is excreted unchanged by man [3], and the known metabolites of metolazone are more polar than the drug (Pennwalt Corporation, unpublished data) and would be expected to elute in the solvent front region of the chromatogram. Thus assay interference by metabolites in human samples seems unlikely.

TABLE II

BETWEEN-ASSAY ACCURACY AND PRECISION OF URINARY METOLAZONE ASSAY

Metolazone concentration (ng/ml)			C.V. (%)	Recovery (%)
Theory	Measured	*n*		
5000	5070	4	2.4	101
2000	1979	3	0.4	99.0
50	51.8	5	7.8	104
			Mean	101 ± 2.5% (S.D.)

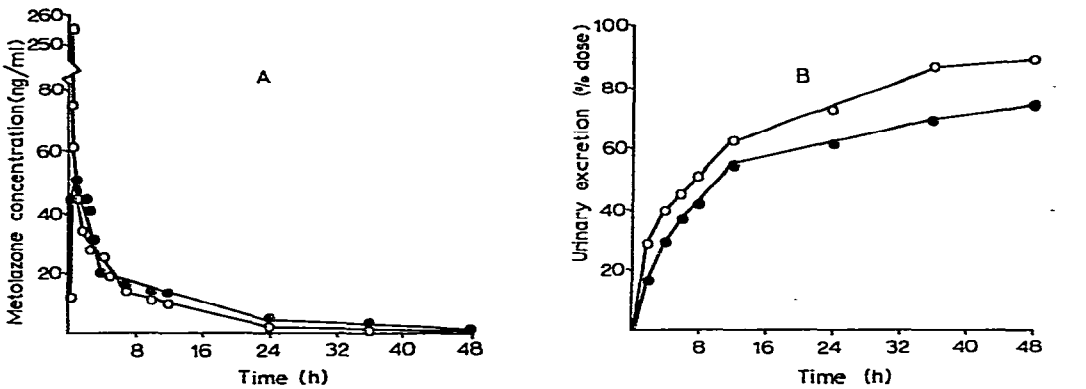

Fig. 4. Plasma levels (A) and urinary excretion (B) of metolazone in a healthy man following single 5-mg intravenous (○) and oral (●) doses of the drug.

The method allowed the measurement of plasma levels and urinary excretion of metolazone in man for up to 48 h after single intravenous or oral solution doses containing 5 mg of metolazone, as shown in Fig. 4.

Metolazone is structurally related to the thiazide diuretics, e.g. hydroflumethiazide, hydrochlorothiazide, and some of these might interfere in the assay during the analysis of samples from patients receiving metolazone in combination with other drugs. The specificity of the assay would therefore have to be determined relative to the co-administered drugs.

Until recently the most common analytical methods for the quantitation of the thiazide drugs have been fluorimetry [1, 5] and gas chromatography [6—8]. However, direct fluorimetry may lack specificity, and all these drugs must be derivatized prior to gas chromatographic analysis, which makes these assays more time consuming. The combination of fluorescence detection and HPLC described in this report therefore offers a new sensitive, specific and simpler quantitative method for this class of diuretic drugs in biological fluids. Applied to metolazone this technique has provided an assay some 50—100 times more sensitive than previously published assays [1, 2] and allowed its accurate and precise determination in plasma for the first time. It seems probable that this assay procedure will also be applicable to the determination of metolazone in whole blood.

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