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Note

Determination of the thiazide diuretic bemetizide in the plasma and urine of humans by high-performance liquid chromatography

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Thiazides are among the most commonly used diuretic and antihypertensive agents, but until recently few methods have been reported for their determination in biological fluids. Earlier methods of measurement, involving hydrolysis and colorimetric determination of their diazotized amino degradation products [1], lacked sensitivity and specificity, Fluorimetric thin-layer chromatography [2] and spectrofluorimetry [3] have also been employed. Gas chromatographic determinations [4—6] utilising electron-capture detection are somewhat lengthy, involving several extraction steps as well as derivatisation. More recently, high-performance liquid chromatography (HPLC) has been applied to the measurement of hydrochlorothiazide in serum after gel filtration [7] and in serum and urine after solvent extraction and back extraction into alkali [8].

This paper describes a method for the measurement of the thiazide diuretic bemetizide [3-(α-methylbenzyl)-6-chloro-7-sulphamoyl-3,4-dihydro-1,2,4-benzothiadiazin-1,1-dioxide; Fig. 1]* in plasma and urine by HPLC in a reversed-phase mode. An internal standardisation technique was employed using the structurally related thiazide, cyclopenthiazide as the internal standard. The method is simple, rapid and sensitive and has been applied to the measurement of drug in human plasma and urine.

*Present in Diucomb® produced by Sanol Schwarz-Monheim GmbH, Monheim, G.F.R.

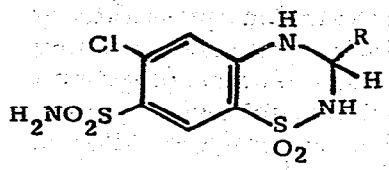

Fig. 1. Chemical structure of bemetizide (R = —$CH(CH_3)C_6H_5$) and cyclopenthiazide (R = —$CH_2C_5H_9$).

EXPERIMENTAL

Materials

Standard solutions of bemetizide in methanol were prepared at concentrations of 5 μg/ml and 20 μg/ml, and cyclopenthiazide (3-cyclopentylmethyl-6-chloro-7-sulphamoyl-3,4-dihydro-1,2,4-benzothiadiazin-1,1-dioxide) (the internal standard) was prepared at 20 μg/ml. Both thiazides were supplied by Sanol Schwarz-Monheim (Monheim, G.F.R.). Reagents were of analytical grade and inorganic reagents were prepared in freshly glass-distilled water.

Extraction

Plasma samples (2 ml) were pipetted into centrifuge tubes and spiked with internal standard (5 μl, 100 ng). Urine samples (1 ml) were spiked with internal standard (20 μl, 400 ng). Sodium hydrogen carbonate (200 mg) was then added, and the mixture was extracted by shaking it with diethyl ether (5 ml) for 1 min. After centrifugation, the ether layer was carefully transferred to a pointed centrifuge tube and evaporated to dryness under a stream of nitrogen at ambient temperature. The residue was washed to the bottom of the tube by the addition of further small volumes of diethyl ether, which were evaporated to dryness. The residue was dissolved in methanol (20 μl), and as much as possible injected into the chromatograph.

High-performance liquid chromatography

The chromatograph consisted of an M 6000 A pump (Waters Assoc., Stockport, Great Britain) fitted to a Cecil 212 variable-wavelength UV monitor (Cecil Instruments, Cambridge, Great Britain) operated at 271 nm. Injection was by syringe (50 μl) via a Waters Assoc. U6K universal injector. The column was 30 cm × 4 mm I.D., prepacked with μBondapak C_{18} (particle size 10 μm) (Waters Assoc.). Chromatography was performed in reversed-phase mode using a solvent system of methanol—0.01 *M* potassium dihydrogen orthophosphate (1 : 1, v/v), at a flow-rate of 2 ml/min. Under these conditions, bemetizide and cyclopenthiazide were eluted with retention times of 4 and 5 min, respectively (Fig. 2). As used in therapy, bemetizide is a mixture of stereoisomers in a 5 : 1 ratio, which are capable of being resolved by HPLC, but the conditions were adjusted so that the isomers were eluted from the column as a single peak, and total unchanged drug concentrations were measured.

Collection of samples

The method of analysis was applied to samples taken from six male

volunteer subjects who were participating in pharmacokinetic studies of bemetizide. The subjects were screened clinically before and after the study and remained under medical supervision in a clinical pharmacology unit throughout its duration. The studies were carried out following consent from the volunteers and from the appropriate ethics committees. After an overnight fast, each subject received a dose of 25 mg of bemetizide which was finely suspended in 150 ml of water. Fasting was continued for 3 h after dosing. Blood samples were withdrawn into heparinised tubes before dosing and at several times thereafter. Plasma was separated by centrifugation. Urine samples were collected from each subject before dosing and during the subsequent 12 h after dosing.

RESULTS AND DISCUSSION

Concentrations of bemetizide were calculated from calibration curves constructed by plotting the peak height ratios of drug to internal standard over the concentration range 10 to 100 ng/ml in plasma and 200 to 1000 ng/ml in urine. The recovery of internal standard from plasma was 95% ± 0.9 S.D. (n = 5) and from urine was 99% ± 0.0 S.D. (n = 5). The recoveries of bemetizide from plasma (means of 94% at 10 ng/ml and 88% at 100 ng/ml) and urine (means of 98% at 200 ng/ml and 99% at 1000 ng/ml) were calculated by comparing peak height ratios of standards to those of standards extracted from plasma and corrected for any losses of internal standard (Table I). The overall recovery of bemetizide from plasma in the concentration range 10 to 100 ng/ml was 90% ± 3.4 S.D. and from urine was 98% ± 0.7 S.D. in the concentration range 200 to 1000 ng/ml.

The calibration curves were constructed from five replicate measurements at five concentrations over the ranges, and plots of peak height ratios against concentration were linear ($y = a + bx$; where a = 0.0172 ± 0.0110 S.D. and b = 0.0117 ± 0.0002 S.D. for plasma, 10 to 100 ng/ml, and a = 0.0304 ± 0.0073 S.D. and b = 0.0024 ± 0.00001 S.D. for urine, 200 to 1000 ng/ml). The intercept of the plasma calibration curve on the y axis was not significantly different from zero. The 95% confidence limits of the least-squares regression line for plasma forced through the origin were ± 36% at 10 ng/ml, ± 7% at 50 ng/ml and ± 4% at 100 ng/ml. The precision of the method for measurement of bemetizide in plasma ranged from ± 9.3% at 10 ng/ml and ± 1.5% at 100 ng/ml (Table I). The 95% confidence limits of the regression line for urine were ± 8% at 200 ng/ml, ± 2% at 600 ng/ml and ± 1% at 1000

TABLE I

RECOVERIES OF BEMETIZIDE FROM PLASMA AND URINE

Concentration added to plasma (ng/ml)	Recovery (%)	Coefficient of variation (%)	Concentration added to urine (ng/ml)	Recovery (%)	Coefficient of variation (%)
10	94	9.3	200	98	2.5
25	93	8.9	400	98	1.8
50	89	2.2	600	98	1.6
75	86	2.7	800	97	1.0
100	88	1.5	1000	99	0.5

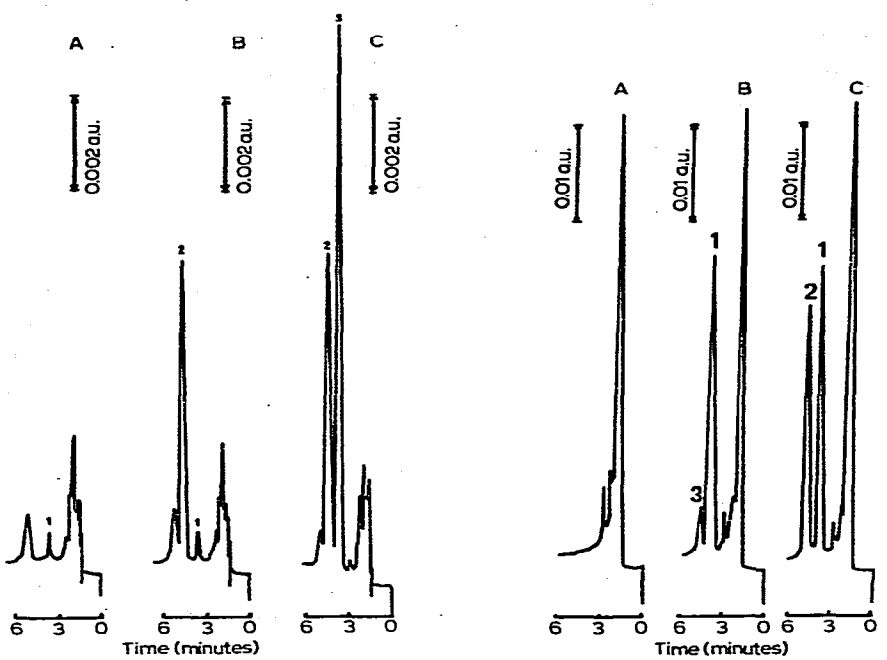

Fig. 2. Chromatograms of (A) predose control plasma, (B) predose control plasma containing internal standard (50 ng/ml) and (C) plasma sample at 4 h after dosing containing 94 ng/ml of bemetizide. Column (30 cm × 4 mm I.D.) prepared with μBondapak C_{18}; flow-rate, 2 ml/min; attenuation, 0.02 a.u.f.s.; solvent system, methanol—0.01 *M* potassium dihydrogen orthophosphate (1 : 1, v/v); wavelength, 271 nm. Peaks: 1 = plasma component (equivalent to 6 ng/ml of bemetizide); 2 = internal standard (cyclopenthiazide); 3 = bemetizide.

Fig. 3. Chromatograms of (A) predose control urine, (B) 0—12 h urine sample without internal standard and (C) 0—12 h urine sample with internal standard (400 ng/ml) containing 590 ng/ml of bemetizide. Conditions as for Fig. 1, except attenuation, 0.1 a.u.f.s. Peaks: 1 = bemetizide; 2 = internal standard (cyclopenthiazide); 3 = possible urinary metabolite with same retention time as internal standard.

ng/ml. The precision of the method for measurement of bemetizide in urine ranged from ± 2.5% at 200 ng/ml to ± 0.5% at 1000 ng/ml (Table I).

An interfering peak with the retention time of bemetizide was present in extracts of predose plasma (Fig. 2) equivalent to 6 ng/ml ± 1.5 S.D. (n = 6). This "blank" value was subtracted from measured concentrations in postdose plasma and the upper 95% confidence limit of the "blank" (10 ng/ml) was considered to be the limit of detection. No interfering peak was present with the retention time of bemetizide in extracts of urine (Fig. 3), but a peak was present in urine extracts of postdose urine (possibly a metabolite of bemetizide as it did not occur in extracts of predose urine) with the retention time of the internal standard. To determine the contribution of this material to the final peak height of the internal standard, urine was assayed with and without addition of the internal standard. The limit of detection of bemetizide in urine was not determined since measured concentrations exceeded 200 ng/ml.

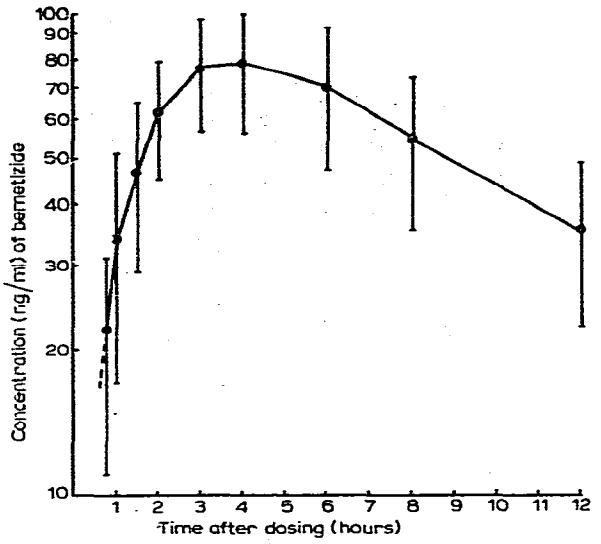

Fig. 4. Semi-logarithmic plot of mean plasma concentrations of bemetizide with time after an oral dose of 25 mg to six human subjects. Standard deviations at each time interval are shown.

When applied to the collected samples, the method showed that the peak of mean concentrations of bemetizide occurred at 4 h after dosing (78 ng/ml ± 22 S.D.) and declined to 35 ng/ml ± 13 S.D. at 12 h after dosing (Fig. 4). In urine, concentrations of bemetizide of 325—590 ng/ml were measured in samples collected during 0—12 h after dosing. During this period, a mean of 3% of the dose was excreted in the urine as unchanged drug [9].

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