Journal of Chromatography, 223 (1981) 466—472
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 832

Note

Determination of trichlormethiazide in human plasma and urine by high-performance liquid chromatography

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(First received October 27th, 1980; revised manuscript received December 31st, 1980)

Trichlormethiazide is a thiazide diuretic drug which is orally administered at relatively low doses of 4—8 mg. Studies of the time-course of the drug in biologic fluids of humans have not been published. However a colorimetric method, based on the Bratton and Marshall technique, has been utilized in quantitating the drug in plasma and urine specimens obtained from dogs [1]. Other methods, which have been employed in more recent studies involving other thiazide diuretics, include gas chromatography [2—4] and high-performance liquid chromatography (HPLC) [5—9]. In two HPLC assays for hydrochlorothiazide in serum [8] or serum and urine [9], trichlormethiazide has been used as an internal standard, although the trichlormethiazide was not added initially and carried through the entire assay procedure. A HPLC procedure has also recently been developed for the purpose of screening urine specimens for the presence of all currently used thiazide drugs [10].

The HPLC assays described below are simple, rapid, sensitive and specific for trichlormethiazide in plasma and in urine obtained from humans receiving the drug. The methods are suitable for single- or multiple-dose studies of the pharmacokinetics or bioavailability of this drug in man.

EXPERIMENTAL

Materials

Trichlormethiazide was provided by Schering (Kenilworth, NJ, U.S.A.) and bendroflumethiazide was provided by E.R. Squibb & Sons (Princeton, NJ, U.S.A.). The diethyl ether and acetonitrile were from Burdick & Jackson

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Labs. (Muskegon, MI, U.S.A.) and the 2-propanol and methanol were HPLC grade from Fisher Scientific (Fairlawn, NJ, U.S.A.). Water is deionized and all other chemicals were of reagent grade.

All glass tubes employed in the extraction and evaporation procedures were placed in a 500°C kiln for 1 h to eliminate any contaminants remaining after washing with detergent, rinsing with deionized water and drying at 100°C overnight. The 15-ml conical tubes (Pyrex No. 8060) were silanized with 4% trimethylchlorosilane (Alfa Products, Danvers, MA, U.S.A.) in dry toluene. After silanization, the tubes were rinsed with methanol and dried at 100°C overnight.

Extraction

Plasma samples. Standard curves for trichlormethiazide in plasma were prepared with pooled human plasma. Standard solutions of trichlormethiazide were made in methanol from an initial stock solution containing 1.2 mg/ml of drug in methanol. The aqueous spiking solutions were then prepared using 1 ml of the appropriate methanol standard, diluted to 100 ml with 0.01 *N* sodium hydroxide, to yield final concentrations of 60, 120, 240, 360 and 450 ng/ml of drug. A solution of bendroflumethiazide in 0.01 *N* sodium hydroxide was prepared at a concentration of 2 μg/ml, to be used as an internal standard. These stock solutions were stable for at least one week when stored at 4°C.

To 3 ml of plasma in a 50-ml PTFE-lined screw-cap centrifuge tube were added 1 ml of one of the trichlormethiazide standard solutions, 1 ml of the bendroflumethiazide solution and 0.2 ml of 0.01 *N* hydrochloric acid, to provide a final pH of 6.9—7.2. Blank plasma samples were prepared using 2 ml of 0.01 *N* sodium hydroxide in place of the drug and internal standard solutions. The assay of plasma samples obtained from a subject who had received the drug involved the addition of 1 ml of internal standard and 1 ml of 0.01 *N* sodium hydroxide to 3 ml of plasma.

The plasma was extracted with 10 ml of diethyl ether for 15 min, with gentle mixing on a platform shaker. Following centrifuging, 2200 *g* at —10°C for 15 min, the tubes were placed in a dry ice—acetone mixture until the aqueous phase was frozen. The ether was then decanted into a 15-ml conical tube. In some instances a small yellowish droplet formed at the bottom of the tube due to the transfer of a small portion of the aqueous phase. In such cases the tube was again frozen, and the ether decanted into a fresh 15-ml conical tube. The ether was evaporated under a stream of nitrogen at 70°C in a water bath for 45 min. The residue was reconstituted with 50 μl of 0.01 *N* sodium hydroxide, with vortexing for approximately 25 sec. A 20-μl aliquot was then injected into the chromatograph.

Urine samples. Standard curves were prepared for trichlormethiazide using freshly voided human urine. Standard solutions of drug were prepared similarly to those for the plasma assay, beginning with a stock solution of 1 mg/ml in methanol. The final spiking solutions contained 0.2, 0.6, 1.0 and 2.0 μg/ml of drug in 0.01 *N* sodium hydroxide. The same internal standard solution was used for the urine and plasma assays. Standard curves were prepared by adding 1 ml of drug solution and 1 ml of internal standard solution to 2 ml of

freshly voided human urine. Urine samples obtained from subjects receiving the drug were assayed after the addition of 1 ml of internal standard solution and 1 ml of 0.01 *N* sodium hydroxide. Blank urine samples were assayed after the addition of 2 ml of 0.01 *N* sodium hydroxide to 2 ml of urine. The urine samples were extracted by adding 2 ml of urine to a 50-ml PTFE-lined screw-cap centrifuge tube containing 500 mg of sodium bicarbonate. Following the addition of the trichlormethiazide, internal standard and/or 0.01 *N* sodium hydroxide solution, 10 ml of ether was added, and the samples were extracted and treated as given in the plasma assay. The residue remaining after evaporation of the ether was dissolved in 100 μl of methanol. A 20-μl aliquot was then injected into the chromatograph.

Chromatography

The chromatograph consisted of a M6000 pump fitted to a μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm, particle size 10 μm) from Waters Assoc. (Milford, MA, U.S.A.). The urine samples were injected with an automatic sampler (WISP) and the plasma samples utilized a U6K injector from Waters Assoc. A Schoeffel SF 770 Spectroflow variable-wavelength UV detector, set at 269 nm, at a range of 0.02 a.u.f.s. was used for the plasma assays. A Waters Assoc. 440 fixed-wavelength detector, set at 280 nm, at a range of 0.02 a.u.f.s., was employed for the urine assays. The mobile phase for the plasma assays consisted of 17% 2-propanol and 1% acetic acid in deionized water. A mobile phase of 35% methanol, 5% acetonitrile and 1% acetic acid in deionized water was used for the urine assays. The flow-rate was 2 ml/min (pressure approximately 138 bar) for both assays.

Quantitation

Standard curves were prepared from pooled human plasma and urine. An unweighted least-squares regression was employed to fit plots of peak height ratio (drug/internal standard) versus drug concentration in plasma and urine.

Stability studies

Plasma samples (3 ml) were spiked with 1 ml of trichlormethiazide solutions containing 0, 60, 120, 240, 360 and 450 ng/ml of drug. Similarly 2-ml urine specimens were spiked with 1 ml of trichlormethiazide solutions containing 0.1, 0.4, 0.8 and 1.6 μg/ml of drug. A portion of the plasma and urine samples was assayed immediately, and the remaining portion was frozen for five weeks until the time of assay.

Sample collection

A healthy human subject received two 4-mg tablets of trichlormethiazide (Schering). Urine samples were collected before dosing and over the time intervals 0–2, 2–4, 4–6, 6–8, 8–12, 12–16, 16–24, 24–36 and 36–48 h after dosing. Also, 15-ml blood samples were taken just before dosing and 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h following drug administration. The resulting plasma and urine specimens were stored frozen until assay.

RESULTS AND DISCUSSION

The detection wavelength and composition of the mobile phase were not the same for the urine and plasma assays. The changes were required because common parameters for both assays could not be found which permitted resolution of drug and internal standard from interfering constituents of the plasma or urine samples.

The recovery of internal standard added to ten plasma and eight urine samples averaged (± S.D.) 85.7 ± 4.7% and 89.1 ± 7.0%, respectively. The recovery of trichlormethiazide from ten plasma samples prepared in duplicate over a concentration range of 20—150 ng/ml averaged 70.9 ± 4.1%. The recovery of drug from eight urine samples prepared in duplicate over a concentration range of 0.05—0.8 μg/ml averaged 82.8 ± 4.6%. The recoveries were essentially the same at both extremes of the plasma and urine concentration ranges. The precision of the plasma assay was evaluated by assaying ten samples containing 20 ng/ml of drug and ten others containing 80 ng/ml. The relative standard deviations for the 20 ng/ml and 80 ng/ml samples were 9.1% and 3.9%, respectively.

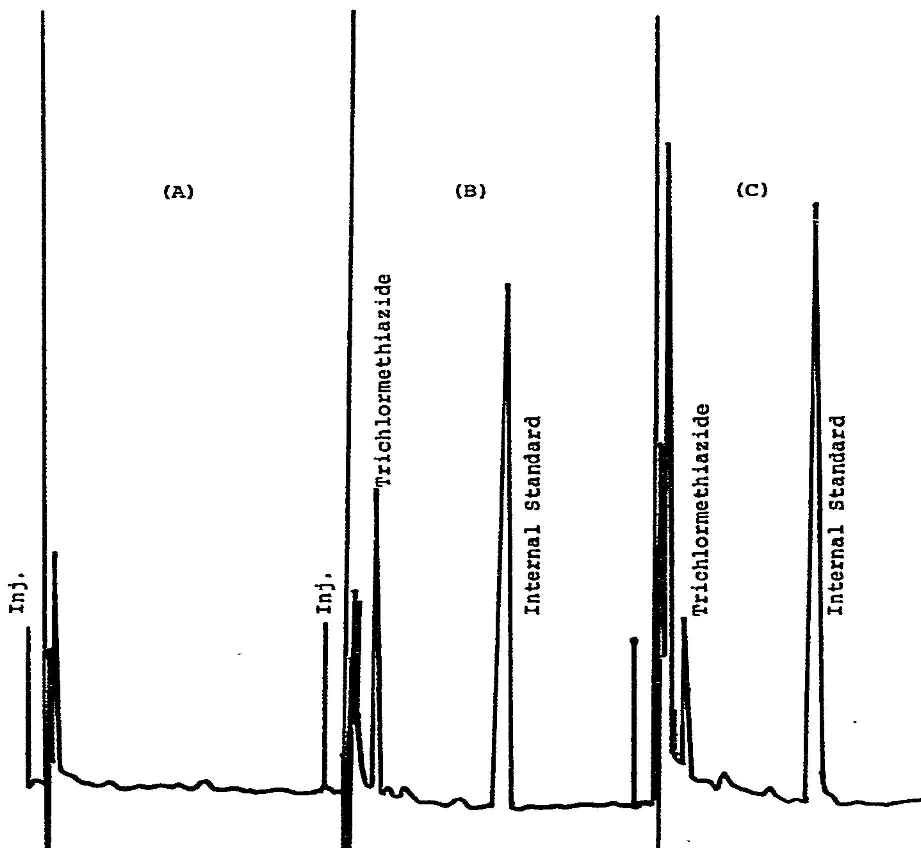

Fig. 1. Chromatograms of (A) predose control plasma, (B) plasma obtained from a subject 1 h after receiving 8 mg of trichlormethiazide and containing internal standard and (C) pooled plasma containing internal standard and 40 ng/ml of trichlormethiazide.

Standard curves for duplicate plasma concentrations, over a concentration range of 20—150 ng/ml, prepared on five different days, exhibited excellent linearity, with correlation coefficients of at least 0.995 for each curve. The intercepts did not differ significantly from zero and the mean slope forced through zero was 0.00491 ± 0.00007. The mean relative standard deviation for the concentration-normalized peak height ratios was 7.5 ± 1.2%. Similarly, five standard curves for duplicate urine concentrations, over a concentration range of 0.05—0.5 μg/ml were linear, with correlation coefficients of at least 0.998 for each curve. The intercepts were not significantly different from zero and the mean slope forced through zero was 0.6461 ± 0.0114. The mean relative standard deviation for the concentration-normalized peak height ratios was 3.6 ± 2.6%. The frozen plasma and urine specimens did not exhibit any evidence of degradation after five weeks of storage. Based on a minimum detectable peak in the chromatogram of 5 mm, the lower limit of sensitivity for the plasma and urine assays was 10 ng/ml and 50 ng/ml, respectively. Attempts were not made to increase the sensitivity of the urine assay, since it was anticipated that the drug levels in urine would be higher than those in plasma. Fig. 1 illustrates the chromatograms obtained from plasma

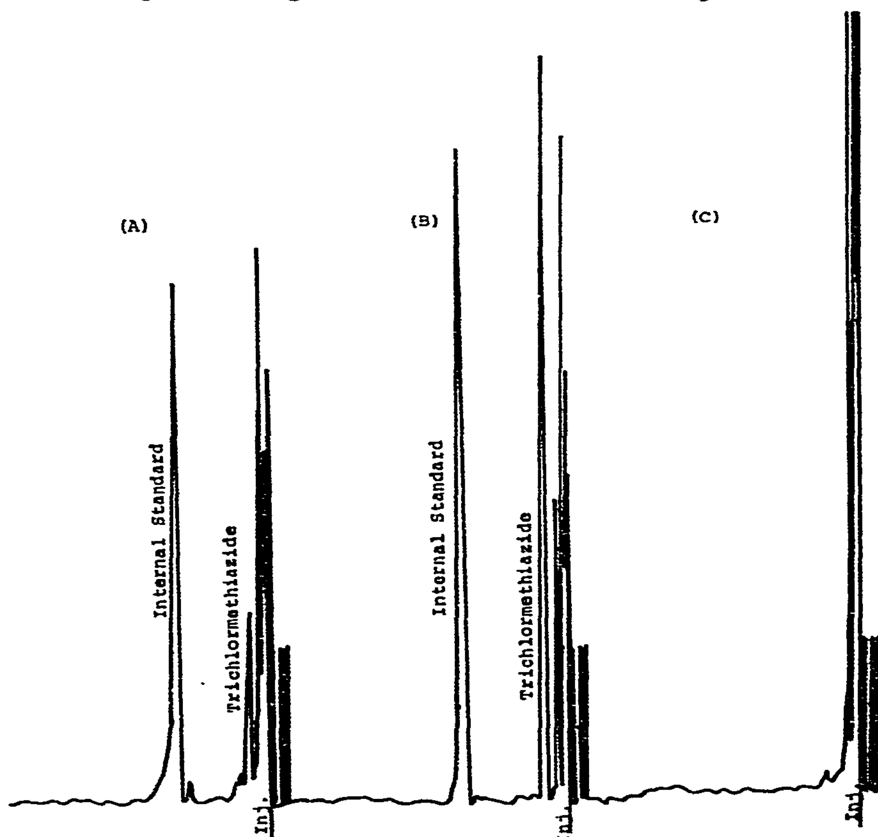

Fig. 2. Chromatograms of (A) pooled urine containing internal standard and 0.25 μg/ml of trichlormethiazide, (B) urine obtained from a human subject 4 h after an 8-mg dose of trichlormethiazide and containing internal standard and (C) predose urine obtained from the human subject.

extracts from the subject who received an 8-mg dose of trichlormethiazide. Also illustrated is a plasma sample spiked with 40 ng/ml of drug. The retention times for the drug and internal standard were 3.7 and 14.4 min, respectively. Fig. 2 illustrates the chromatograms for urine extracts from the same subject, as well as a pooled urine sample spiked with 0.25 μg/ml of drug. The retention times for the drug and internal standard were 3.7 and 10.7 min, respectively. These procedures have several advantages over the recently published HPLC urine screening method for thiazide drugs [10]. The screening method was not developed as a quantitative assay. Further, the present approach, which is applicable to both urine and plasma, requires fewer extraction steps.

No interferences were noted in the chromatograms for pre-dose plasma or urine extracts. However, preliminary studies did indicate the potential for a preservative in heparin solution to interfere with the quantitation of trichlormethiazide. When plasma samples were assayed which were obtained from whole blood exposed to heparin solution, a large peak appeared in the chromatogram which overlapped the drug peak. This interference was traced to benzyl alcohol which was present in a concentration of 1% in the heparin solution. The interference was eliminated by evaporating the ether extracts

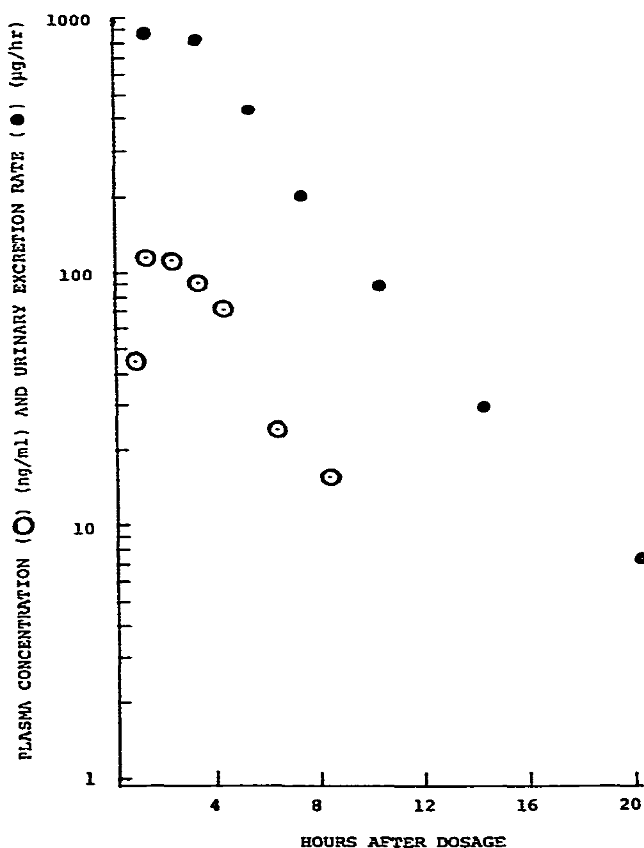

Fig. 3. Semi-logarithmic plot of plasma trichlormethiazide concentrations (○) and urinary excretion rates (●) for a human subject who received an 8-mg dose of trichlormethiazide.

and any residual benzyl alcohol for at least 45 min, at 70°C, under a stream of nitrogen.

Fig. 3 illustrates a semi-log plot of the plasma concentration and urinary excretion rate profiles for trichlormethiazide in the subject who received an 8-mg dose. There was no drug detectable in the plasma samples obtained after 12 h. The total urinary recovery of trichlormethiazide was 4.97 mg (62% of the dose) after 48 h, with 4.66 mg being excreted within the first 12 h. There are no known metabolites of trichlormethiazide, and no evidence for the presence of a metabolite was observed in the chromatograms for samples obtained from this subject.

ACKNOWLEDGEMENTS

The work was supported in part by the U.S. Food and Drug Administration (Contract No. FDA 223-77-3011) and the Tennessee Department of Public Health.

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