Journal of Chromatography, 421 (1987) 401–406
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3813

Note

Determination of cyclopenthiazide in plasma by high-performance liquid chromatography

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(First received March 27th, 1987; revised manuscript received June 3rd, 1987)

Cyclopenthiazide, 6-chloro-3-cyclopentylmethyl-3,4-dihydro-2H-1,2,4-benzothiadiazide-7-sulphonamide 1,1-dioxide (CPT), is a potent diuretic agent, the pharmacological properties of which were first described in 1961 [1]. Since that time CPT has gained widespread acceptance as an affective agent in the treatment of hypertension [2].

Several methods have been reported for the qualitative detection of CPT in urine using paper chromatography [3] and thin-layer chromatography [4,5].The high-performance liquid chromatographic (HPLC) behaviour of CPT has been described [6] employing direct injections of standard solutions onto a reversed-phase column with a tetrahydrofuran–methanol–water eluent. The extraction of CPT from plasma has also been described [7] when the compound was employed as an internal standard in the analysis of a similar diuretic compound.

The recommended dose for CPT in the treatment of hypertension is 0.5–1 mg daily. At present, no methods have been reported for the measurement of CPT in plasma, following this recommended daily dose.

In order to support a pharmacokinetic and pharmacodynamic study, a quantitative assay for CPT was required for detecting and measuring low concentrations of the compound in plasma. The procedure reported here describes a method for the extraction and isolation of CPT and internal standard, bendrofluazide (BDF), onto a solid-phase extraction column. The eluted compounds are further extracted using organic solvent and subjected to sensitive assay using HPLC with UV detection.

0378-4347/87/$03.50

EXPERIMENTAL

Reagents and materials

CPT was obtained from Ciba-Geigy (Horsham, U.K.). The internal standard, BDF, was obtained from Boots (Nottingham, U.K.). Ethyl acetate (Analar grade) and diethyl ether (anaesthetic grade) were obtained from May and Baker (Dagenham, U.K.). Ethyl acetate was re-distilled before use. Methanol (HPLC grade) was obtained from Rathburn Chemicals (Walkerburn, U.K.). Bondelut C_{18} columns (Analytichem) were obtained from Jones Chromatography (Llanbradach, U.K.)

Chromatography

The HPLC system consisted of a Rheodyne 7125 injection valve (Anachem, Luton, U.K.), a Shimadzu Model LC-6A pump (Dyson Instruments, Tyne & Wear, U.K.) and a Shimadzu Model SPD-6A variable-wavelength detector. A prepacked column (15 cm×0.46 cm) packed with Spherisorb 3 ODS-2, 3 μm particle size (Phase Separations, Queensferry, U.K.) was used. A mobile phase, consisting of glass-distilled water–methanol (50:50) was used at a flow-rate of 1.0 ml/min. The column eluent was monitored at 272 nm and the signals were recorded on a Perkin-Elmer Model 056 recorder (Perkin-Elmer, Beaconsfield, U.K.).

Blood samples

Heparinised whole blood was obtained from normal subjects at various times before and after ingestion of CPT. The plasma was separated immediately and frozen before analysis.

Sample preparation

Bondelut columns were conditioned before use with 6 ml of methanol followed by 6 ml of water. Plasma (2 or 3 ml) containing 25 ng of BDF was passed completely through the column, slowly, under pressure using a 5-ml polypropylene syringe. The column was then quickly washed with 6 ml of 10% (v/v) methanol in water. After drying, the compounds were eluted with three 200-μl portions of 75% (v/v) methanol in water. The combined collected extracts were then diluted with 2 ml of water in a 12-ml screw-capped tube. After a brief vortex-mixing, 5.5 ml of ethyl acetate were added, and the tube was mixed for 5 min on a rotary mixer at approximately 30 rpm. The phases were separated by centrifugation, and at least 5 ml of the ethyl acetate phase were removed to a conical centrifuge tube. The ethyl acetate was evaporated to dryness at 45°C under a stream of oxygen-free nitrogen. Diethyl ether (1 ml) was added and the tube briefly vortexed to ensure concentration of the sample. The ether was evaporated as before. The residue was reconstituted in 75 μl of 40% (v/v) methanol in water, and a 50-μl aliquot was injected.

Calibration

CPT and BDF were dissolved in pure methanol at a concentration of 1 mg/ml. Stock standard solutions were prepared at a concentration of 500 ng/ml in methanol–water (50:50) for each compound. Calibration samples were prepared by adding known amounts of CPT to pooled human plasma samples in order to provide concentrations in a range of 1–10 ng/ml. Internal standard was added, and these samples were then treated in the manner described above.

Recovery of CPT and BDF from plasma

The recoveries of CPT and BDF, either individually or in combination, were determined by comparison of chromatographic peak heights obtained after extraction of CPT and BDF from spiked plasma samples with peak heights obtained after direct injection of equivalent amounts of CPT and BDF in 40% methanol in water. Recoveries of CPT and BDF from the initial Bondelut section of the assay were determined using aqueous samples.

Assay variability

Known amounts of CPT and a constant amount of BDF were added to blank human plasma. Aliquots from this pool were analysed several times over the course of one day and also several times over a period of weeks.

RESULTS

Fig. 1 shows representative chromatograms for blank plasma (I), plasma containing CPT and BDF (II) and plasma from a subject who had received CPT (III). Retention times were 5.1 and 7.6 min for BDF and CPT, respectively. Calibration curves were linear over the range 0–200 ng/ml, with a correlation coefficient (r) of 0.994. In the range 0–10 ng/ml there was a linear relationship between peak-height ratio (y) and CPT concentration (x) described by $y = 0.0824 + 0.205x$ ($r = 0.997$, $n = 6$).

Table I shows the recovery of CPT from 2-ml plasma samples used for the 1-mg dose and 3-ml plasma samples used for the 0.5-mg dose. The overall recovery of $96 \pm 5.5\%$ (mean $\pm$ S.D., $n = 6$) from plasma was not affected by the addition of 25 ng BDF. The overall recovery of BDF was not affected by the addition of CPT and was found to be $86 \pm 3.1\%$ (mean $\pm$ S.D., $n = 6$). The recovery of CPT at a concentration of 15 ng/ml in 2- or 3-ml volumes of water, when taken only through the Bondelut section of the assay, was $99 \pm 4.9\%$ ($n = 6$). Under identical conditions, the recovery of 25 ng BDF was $99 \pm 4.0\%$ ($n = 6$). The limit of detection of the assay under the conditions described was 0.4 ng/ml for 3-ml plasma samples. The intra-day precision and day-to-day reproducibility for the assay of 2-ml plasma samples showed mean coefficients of variation of 5.7 and 6.3%, respectively. For 3-ml plasma samples the mean intra-day precision was 5.7%, and day-to-day reproducibility was 6.4% (Tables II and III).

No interferences were observed when oxprenolol, metoprolol, propranolol, atenolol or betaxolol at a concentration of 1 μg/ml were taken through the assay procedure. The thiazide drugs, polythiazide, indapamide, chlorothiazide, meto-

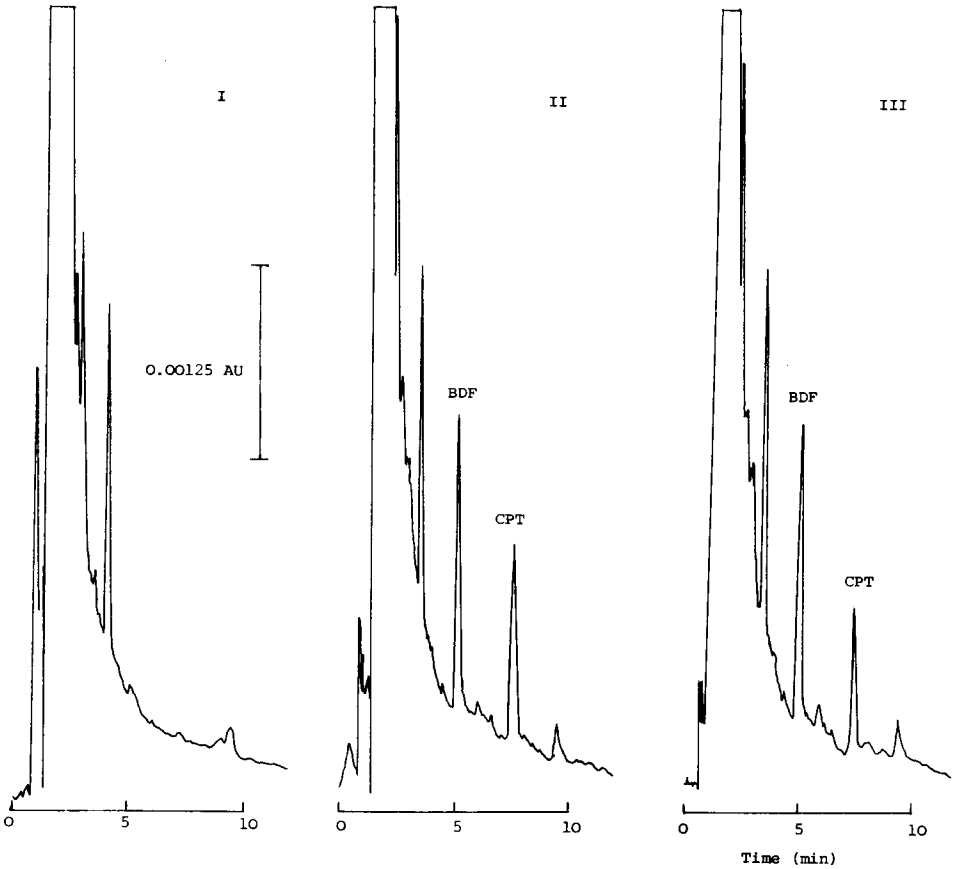

Fig. 1. Chromatograms obtained from assay of 2 ml of pre-dose plasma (I), pre-dose plasma to which were added 6 ng/ml CPT and 25 ng BDF (II) and post-dose plasma sample determined to contain 4.3 ng/ml CPT (III).

lazone, xipamide and mefruside, as well as the commonly used loop diuretics, frusemide and bumetanide, also failed to produce interference in the assay, when tested at a concentration of 1 μg/ml.

Fig. 2 shows the mean plasma concentrations of CPT achieved after oral administration of 1 or 0.5 mg to three normal subjects. Peak plasma levels were

TABLE I

RECOVERY OF CPT FROM PLASMA SAMPLES

Plasma volume (ml)	Concentration (ng/ml)	Recovery (mean ± S.D., $n=5$) (%)
2	2	97.8 ± 5.1
	8	94.5 ± 4.8
3	2	96.3 ± 6.1
	4	95.0 ± 5.9

TABLE II

INTRA-DAY PRECISION OF THE HPLC ASSAY FOR CPT IN PLASMA

Plasma volume (ml)	Actual concentration (ng/ml)	Measured concentration (ng/ml)	Coefficient of variation ($n=6$) (%)
2	2	1.98	7.6
	8	7.93	3.7
3	2	2.03	6.0
	4	3.95	5.3

TABLE III

DAY-TO-DAY REPRODUCIBILITY OF THE HPLC ASSAY FOR CPT IN PLASMA

Plasma volume (ml)	Actual concentration (ng/ml)	Measured concentration (ng/ml)	Coefficient of variation ($n=6$) (%)
2	2	1.93	7.8
	8	7.98	4.7
3	2	2.00	7.4
	4	4.03	5.4

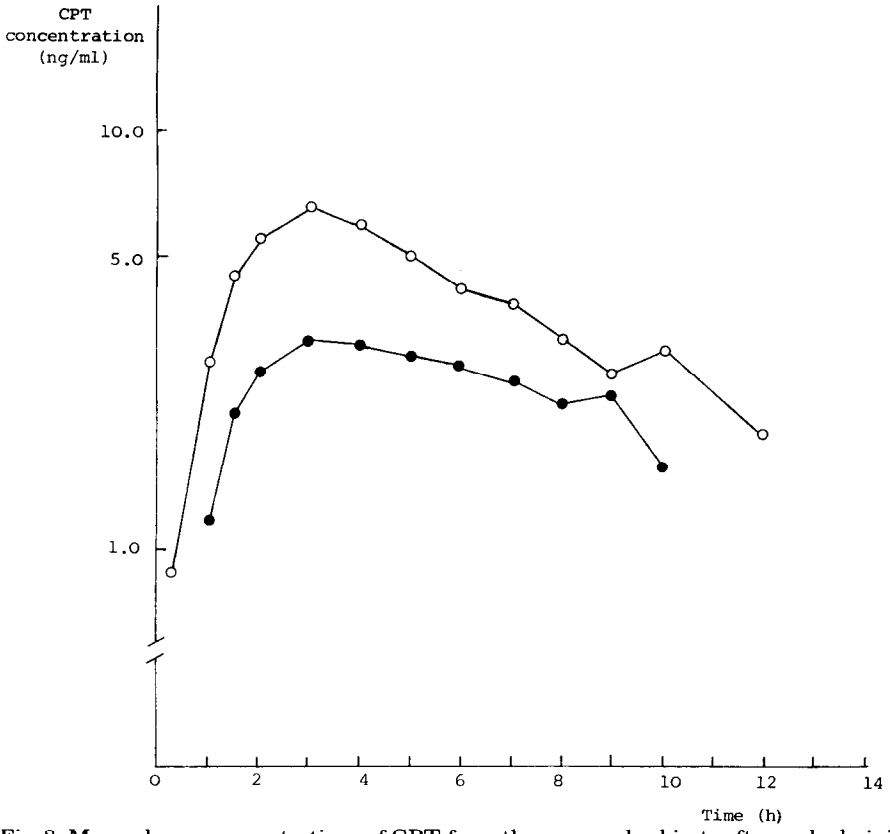

Fig. 2. Mean plasma concentrations of CPT from three normal subjects after oral administration of 1 mg (○) or 0.5 mg (●) CPT.

achieved 3–4 h after administration. In all subjects there was a slight rise in plasma levels between 8 and 10 h, which may be due to some form of recirculation or discontinuous absorption of the drug.

DISCUSSION

Administration of cyclopenthiazide in a dose of 1 or 0.5 mg would be expected to produce low plasma concentrations. The sensitivity of the assay described relies on the efficient and specific extraction of CPT from plasma. The solid-phase Bondelut procedure selectively extracts and concentrates both CPT and internal standard from plasma samples. However, further sample purification was required to produce chromatograms which were free from interfering peaks. This was achieved using a further extraction with ethyl acetate. Further concentration of the sample was achieved by washing the sides of the tubes with ether after evaporation of the ethyl acetate. Washing of the columns with 10% methanol removed more interferences than washing with water alone. Both CPT and BDF were lost from the column if methanol concentrations in excess of 10% were used. Elution of retained compounds from the Bondelut columns was optimised using 75% methanol in water. Concentration of this elution solvent by evaporation was difficult, and elution with methanol alone resulted in interferences. For these reasons, the extracts obtained using 75% methanol in water were further concentrated and purified using extraction with ethyl acetate. Addition of water at this stage was necessary to provide adequate partitioning of the aqueous and organic phases. As shown, recovery of BDF by ethyl acetate extraction was lower than for CPT. Reversal of the Bondelut and organic solvent extraction steps was possible, but resulted in interfering chromatographic peaks.

The dried ethyl acetate extracts were reconsituted in 75 μl of 40% methanol in water as this appeared to be the optimum volume necessary for dissolution, without undue dilution of the sample. Injection of volumes much larger than 50 μl onto the 15-cm column produced slight broadening of the peaks.

In conclusion, the method presented is a selective, sensitive assay for the measurement of CPT levels which may be found in plasma after a normal therapeutic dose of 1 or 0.5 mg. In the past, lack of such an assay has precluded any pharmacokinetic studies with cyclopenthiazide.

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