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Note

Simple, sensitive and selective high-performance liquid chromatographic method for analysis of chlorthalidone in whole blood

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Chlorthalidone, 2-chloro-5-(1-hydroxy-3-oxo-1-isoindolinyl)benzene sulphonamide (Fig. 1), is an oral diuretic used widely in anti-hypertensive therapy, both alone and in combination with other drugs [1–4]. Chlorthalidone differs from the thiazide diuretics by having an unusually long biological half-life (49–68 h) [5–8] due largely to its extensive distribution into erythrocytes where it has a strong affinity for carbonic anhydrase [6, 9–11]. Accurate plasma or serum level data are difficult to obtain due to leakage of the chlorthalidone from erythrocytes following blood collection [12]. This may be overcome by ensuring that time between sample collection and processing is always consistent or more simply by measuring chlorthalidone levels in whole blood.

Specific gas chromatography (GC) [12, 13] and high-performance liquid chromatography (HPLC) [8, 14–17] methods have been developed to quantify chlorthalidone in blood. The former require a derivatization step and cannot compete with HPLC for speed of analysis although some HPLC methods do involve a laborious clean-up procedure. The HPLC method described by Mcainsh et al. [8] requires a tedious triple extraction. Guelen et al. [14] described a simple HPLC method but chlorthalidone recoveries are poor (47.6%) and direct injection of protein-precipitated blood supernatant onto the analytical column column as is required can adversely affect column life. The HPLC assays described by Williams et al. [15] and by Rosenberg et al. [17] both employ an acetonitrile precipitation step. However, the former does not readily permit drug recovery estimation and the latter requires an internal standard which is not readily available. The assay described by MacGregor et al. [16] has probenecid as an internal standard but a laborious multistep sample preparation procedure is involved.

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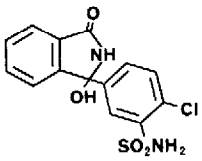

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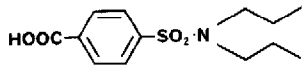

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Fig. 1. Structures of chlorthalidone (I) and probenicid, the internal standard (II).

The recent increase in the use of chlorthalidone in combination with other anti-hypertensive agents has resulted in oral doses as low as 12.5 mg once a day [4]. Correspondingly greater assay sensitivity is required for evaluation of pharmacokinetic studies at such low doses. However, such desirable accuracy and precision is not shown for previous HPLC methods below 0.2 μg/ml. The HPLC method presented here incorporates a readily available internal standard, probenecid (Fig. 1), involves a single extraction of perchloric acid-precipitated whole blood and recovers more than 90% of the chlorthalidone present. Excellent accuracy and precision are achieved throughout the range 2.5–0.062 μg/ml.

EXPERIMENTAL

Reagents

Chlorthalidone was obtained from Armour Medicamenta (Orriggio, Milan, Italy). Acetonitrile and methyl *tert.*-butyl ether, both HPLC grade, and perchloric acid (70%) were from Fisons (Loughborough, U.K.). Probenecid was obtained from Sigma (Poole, U.K.) and sodium acetate trihydrate from BDH (Poole, U.K.). Water was passed through a Milli-Q water purification system.

Phosphate buffer was prepared by mixing 39 ml of 0.2 *M* sodium dihydrogen orthophosphate, 61 ml of 0.2 *M* disodium hydrogen orthophosphate, 33.6 ml of 1 *M* sodium hydroxide and 66.4 ml of water.

Apparatus

The HPLC equipment consisted of an SP 8700 solvent delivery system (Spectra-Physics, St. Albans, U.K.), a WISP Model 710B autosampler (Waters Chromatography, Harrow, U.K.) and a Model LC75 UV detector (Perkin-Elmer, Beaconsfield, U.K.). The column was 25 cm × 5 mm, containing 10-μm LiChrosorb RP-18 (Hichrom, Reading, U.K.) with a 2 cm × 2 mm I.D. precolumn dry packed with CO:Pell ODS. The detector was connected to a 10-mV chart recorder and a Trivector (Sandy, U.K.) Trilab Model III computing integrator.

Chromatographic conditions

Mobile phase (77% 0.01 *M* sodium acetate in acetonitrile) previously scintered-glass-filtered under vacuum, was pumped at a flow-rate of 1.5 ml/min. The detection wavelength was 214 nm with a range setting of 0.02 a.u.f.s. and the injection volume was 25 μl.

Calibration standards

A 5 μg/ml chlorthalidone solution in drug-free whole blood was prepared by a 200-fold dilution of a 1 mg/ml stock solution. Calibration standards in whole blood, containing 2.5, 1.25, 0.625 and 0.312 μg/ml, were obtained by a subsequent series of two-fold dilutions. A duplicate set of calibration standards was assayed with each batch of test samples.

Extraction procedure

Whole blood (200 μl) was vortex-mixed with 200 μl of 0.33 *M* perchloric acid in a 10-ml screw top glass blood tube (LIP Equipment and Services, Shipley, U.K.). Following 5 min sonication in an ultrasonic cleaner, the sample was neutralised by thorough mixing with 500 μl of phosphate buffer containing sodium hydroxide. Probenecid (12.5 μg/ml) was added and mixed prior to extraction with 10 ml of methyl *tert.*-butyl ether, facilitated by horizontal shaking on a Luckham R100/TW shaker (15 min, speed setting 8, approximately 120 cycles/min). Following centrifugation (10 min, 3000 *g*, Denley BS 400) the upper phase was transferred to a clean 10-ml glass tube by pouring and evaporated under a stream of compressed air in a water-bath at 45°C.The residue was redissolved in 200 μl of mobile phase for injection onto the HPLC column.

Calculations

For each sample, the peak heights were measured for chlorthalidone and the internal standard and the peak-height ratio chlorthalidone/probenecid was calculated. The peak-height ratio values for the calibration standards were plotted against chlorthalidone concentration and the standard curve was obtained by linear regression. Analyte concentrations for samples were calculated by fitting their peak-height ratio values to the standard curve.

RESULTS AND DISCUSSION

Chromatography

Using the HPLC conditions described, probenecid and chlorthalidone had retention times of 4.4 and 7.5 min, respectievly. Typical chromatograms for extracts of blank blood, chlorthalidone-spiked blood and a blood sample taken from a volunteer 6 h after receiving 12.5 mg of chlorthalidone orally are shown in Fig. 2.

Linearity

Standard curves were consistently linear. Table I shows standard curves obtained on five consecutive days during routine analysis of clinical samples.

Precision, accuracy and sensitivity

Blood samples containing chlorthalidone at each of six different concentration levels, 2,1, 0.5, 0.25, 0.125 and 0.0625 μg/ml, were assayed in quadruplicate and this was repeated on four separate days. The results (Table II) show excellent precision throughout the range with coefficients of variation (C.V.) of 3.3% at

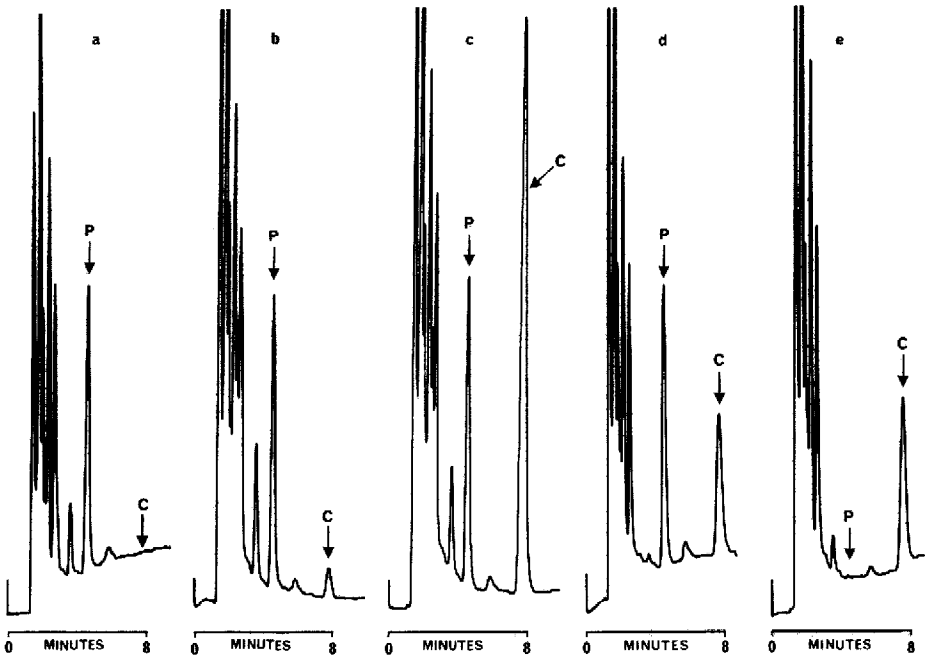

Fig. 2. Chromatograms of whole blood extracts. (a) Drug-free whole blood; (b) whole blood containing 0.125 μg/ml chlorthalidone; (c) whole blood containing 2.5 μg/ml chlorthalidone; (d, e) whole blood taken from a volunteer 6 h after receiving 12.5 mg (single dose) of chlorthalidone orally, with and without added internal standard. Peaks C and P indicate expected retention times of chlorthalidone and probenicid, the internal standard, respectively.

2.0 μg/ml and 7.6% at 0.0625 μg/ml. Between-day variation was not found to be significantly greater than within-day variation.

The accuracy of the method, indicated by the proximity of the mean measured concentrations to the theoretical concentrations, was shown (Table II) to be within 3%.

Accurate and precise quantification was permitted as shown (Table II) at chlorthalidone concentrations as low as 0.0625 μg/ml, indicating improved sensitivity over previous HPLC methods. MacGregor et al. [16] reported a C.V. of 30% with an accuracy of only 22% at 0.075 μg/ml. Other published HPLC methods have not included assay performance parameters for chlorthalidone levels below 0.2 μg/ml.

TABLE I

LINEARITY OF THE METHOD

Day	Standard curve	r
1	$y=1.01476x+0.0338$	0.999
2	$y=1.0350x-0.0147$	0.999
3	$y=1.0086x-0.0106$	0.999
4	$y=1.0084x-0.0092$	0.999
5	$y=0.9951x+0.0146$	0.999

TABLE II

PRECISION AND ACCURACY FOR WHOLE BLOOD SPIKED WITH CHLORTHALIDONE

Theoretical concentration (μg/ml)	Mean ($n=16$) measured concentration (μg/ml)	Coefficient of variation (%)	Difference of mean from theoretical (%)
2.0	2.043	3.3	2.2
1.0	1.008	4.2	0.8
0.5	0.498	4.4	−0.4
0.25	0.243	5.0	−2.8
0.125	0.128	8.4	2.4
0.0625	0.064	7.6	2.4

The limit of detection, defined as the amount of chlorthalidone corresponding to three times the noise level is approximately 25 ng/ml.

Recovery of chlorthalidone from whole blood

Recovery was estimated by comparing peak heights for chlorthalidone standards in mobile phase and for chlorthalidone-spiked blood extracts. These were found to be 93.3, 94.5, 90.4 and 91.8% at 2.5, 1.25, 0.625 and 0.312 μg/ml, respectively, and represent an improvement over previous extraction procedures [14, 16, 17].

Selectivity

As shown in Fig. 2 there is no inteference from endogenous components in whole blood. Additionally, a range of compounds was tested for the possibility of chromatographic interference. However, none of these interfered with the chlorthalidone or probenecid peaks (Table III).

TABLE III

COMPOUNDS TESTED FOR CHROMATOGRAPHIC INTERFERENCE

Substance tested	Possibility of interference
Acetylsalicylic acid	None
Caffeine	None
9-Hydroxytheophylline	None
Theophylline	None
4-Hydroxyacetamilide	None
Nitrazepam	Retention time 42 min, may interfere with later samples
Indomethacin	None
Amitriptyline hydrochloride	None
Verapamil hydrochloride	None
Atenolol	None
Celiprolol hydrochloride	None

CONCLUSION

A sensitive and highly selective HPLC assay for chlorthalidone in whole blood is described. The overall performance and practical simplicity of the method supercede the requirements, e.g., for application to a pharmacokinetic study involving low oral doses (25 or 12.5 mg) of chlorthalidone. This method has now been used in our laboratory routinely over a three-month period with excellent results and the original HPLC column is showing no sign of deterioration despite elution of more than 2000 extracts.

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