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Note

Analysis of chlorthalidone in whole blood by high-performance liquid chromatography

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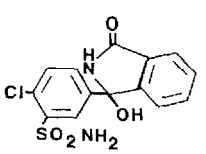
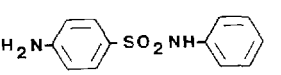
Chlorthalidone, 2-chloro-5-(1-hydroxy-3-oxo-1-isoindolinyl)benzenesulfonamide (I in Fig. 1), is an orally effective diuretic agent used alone and in combination for the treatment of hypertension and edema [1—4] Chlorthalidone has a prolonged duration of action which may be attributable to its long biological half-life (40—65 h) [5—11] Chlorthalidone has a high affinity for red blood cells [7, 8, 12, 13] where it is estimated to be between 95 and 99% bound [5—7] The red blood cell binding may be related in part to chlorthalidone's affinity for the membrane-bound enzyme, erythrocyte carbonic anhydrase [6, 13, 14] In light of chlorthalidone's association with red blood cells, it is desirable to measure its whole blood concentrations to obtain relevant pharmacokinetic information

Initial assays for determining chlorthalidone in blood relied on gas chromatographic procedures based on the extractive alkylation of the drug to its tetramethyl derivative [15, 16] These methods are time-consuming and not ideal for processing large numbers of samples More recently, high-performance liquid chromatographic (HPLC) procedures for the measurement of chlor-



I



II

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

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thalidone in biological fluids have been reported [17, 18]. The technique described for blood samples by Guelen et al. [17], however, did not use an internal standard and reported only 47 6% recovery of chlorthalidone from whole blood The HPLC assay described by Williams et al [18], while employing an acetonitrile precipitation technique and an internal standard, does not readily allow for the determination of complete drug recovery The presently described HPLC method incorporates an internal standard (sulfanilanilide, II in Fig. 1) to improve quantitation and recovers 86 1% of chlorthalidone from whole blood

In addition, this procedure allows for the quantitation of at least 0 20 μg/ml chlorthalidone which provides adequate sensitivity to measure chlorthalidone blood concentrations to at least 96 h following a single 25-mg dose administered to an adult of approx 70 kg This sensitivity limit compares well to an apparent quantitation limit of 0 75 μg/ml reported by Guelen et al [17] and 0.20 μg/ml reported by Williams et al [18]

EXPERIMENTAL

Apparatus

A Waters Assoc (Milford, MA, U S A) modular chromatography system comprised of a Model 6000A pump, a WISP Model 710B autosampler, a Model 730 data module, a Model 720 system controller and a Model 441 UV absorbance detector was used to perform the chromatography A μBondapak CN, reversed-phase column, 30 cm × 3.9 mm, 10 μm particle size (Waters) was used.

Reagents

Chlorthalidone and the internal standard, sulfanilanilide, were produced by Revlon Health Care (Tuckahoe, NY, U S A) Water was deionized and glass-distilled Acetonitrile and tetrahydrofuran were HPLC grade (Fisher Scientific, Fairlawn, NJ, U S A) Dibutylamine phosphate (D-4 reagent) was obtained from Waters Assoc All other reagents were analytical grade

Chromatographic conditions

The mobile phase was tetrahydrofuran—acetonitrile—water (2 0 0 5·97.5) containing 10 m*M* dibutylamine phosphate adjusted to pH 5.0 with 2 0 *M* sodium hydroxide The flow-rate was 2 5 ml/min and the detection wavelength was 214 nm

Preparation of standards

A 5.27 μg/ml standard of chlorthalidone in blood was prepared by diluting an appropriate volume of a methanolic solution (5%) of 105 4 μg/ml chlorthalidone with heparinized whole blood (Plasma Alliance, Knoxville, TN, U.S.A). Standards of 0.20, 0 66, 1 32 and 2 64 μg/ml were made by sequential dilution of the 5.27 μg/ml standard with whole blood.

Extraction

A 160-μl aliquot of a thoroughly mixed whole blood sample was added to

480 μl of an aqueous solution containing sulfanilanilide (1 33 μg/ml). The mixture was stirred on a Vortex Genie mixer (Scientific Products, Evanston, IL, U.S A.) and sonicated for 15 min in an ultrasonic cleaner (Model 42, Branson Sonic Power, Danbury, CT, U.S.A.). Acetonitrile (6 ml) was added to the mixture followed by vortexing for 30 s and further sonication for 15 min.

The mixture was centrifuged at 1500 *g* for 10 min at 4° C The supernatant was decanted into a clean tube and evaporated to dryness under nitrogen in a 40° C water bath. The residue was redissolved in 80 μl of mobile phase and 25 μl were injected onto the HPLC column

Calculation of results

The areas of the chlorthalidone and sulfanilanilide peaks were determined by electronic integration and the peak-area ratio of chlorthalidone/sulfanilanilide was calculated. Calibration curves were constructed by plotting the peak-area ratio versus the chlorthalidone concentration for the standards and the linear regression line was determined Chlorthalidone sample concentrations were calculated from their peak-area ratio relative to the calibration curve

RESULTS AND DISCUSSION

Chromatographic properties

Chromatograms of extracted blank and whole blood standards are depicted in Figs. 2 and 3, respectively The retention times of chlorthalidone and sulfanilanilide were 8.7 and 13.9 min, respectively There were no interfering peaks in control blood with retention times the same as chlorthalidone and the internal standard

Linearity and sensitivity

The peak-area ratio versus the chlorthalidone blood concentration exhibited a linear relationship between 0 20 and 5 27 μg/ml (Table I). The correlation

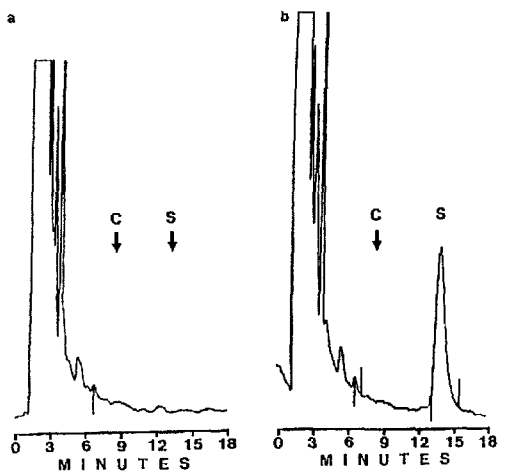

Fig 2 Chromatograms of extracted blank whole blood (a) without internal standard, (b) with internal standard C and S indicate expected retention times of chlorthalidone and sulfanilanilide, respectively

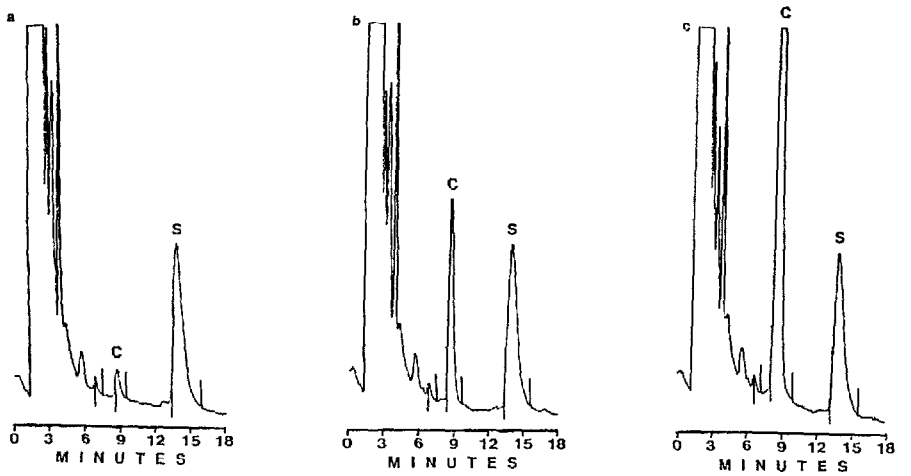

Fig 3 Chromatograms of extracted whole blood containing chlorthalidone (C) and internal standard sulfanilanilide (S) (a) 0 2 μg/ml chlorthalidone, (b) 1 32 μg/ml chlorthalidone, (c) 5 27 μg/ml chlorthalidone

TABLE I

REPRODUCIBILITY OF THE METHOD

Concentration (μg/ml)	Peak-area ratio (mean* ± S D)	Coefficient of variation (%)
0 20	0 0953 ± 0 0058	6 1
0 66	0 332 ± 0 030	9 0
1 32	0 660 ± 0 029	4 4
2 64	1 40 ± 0 08	5 7
5 27	2 80 ± 0 11	3 9
Mean		5 8

*Average of six replicate determinations at each concentration

coefficient of the composite curve (n = 36) was 0 998 The limit of quantitation, based on the lowest concentration point of the standard curve, was 0 20 μg/ml which is more than a three-fold increase in the sensitivity reported by Guelen et al. [17] This quantitation limit is adequate for current applications and may be extended further, if necessary.

Precision and accuracy

Standards of 0.20, 0 66, 1 32, 2 64 and 5 27 μg/ml chlorthalidone concentration were assayed in triplicate on two different days to determine the reproducibility (precision) of the assay The results (Table I) show that the precision of the assay, expressed as the coefficient of variation, ranged between 9.0% at 0.66 μg/ml and 3.9% at 5 27 μg/ml.

The accuracy of the method was determined by assaying whole blood chlorthalidone standards at 0.20, 0.66, 1.32, 2 64 and 5 27 μg/ml Table II shows that the assay was accurate to within 5% or better for the concentration range

TABLE II

ACCURACY OF MEASUREMENT OF CHLORTHALIDONE ADDED TO WHOLE BLOOD

Theoretical concentration (μg/ml)	Calculated concentration (mean* ± S D) (μg/ml)	Coefficient of variation (%)	Difference of mean from theoretical (%)
0 20	0 21 ± 0 01	4 8	+5 0
0 66	0 65 ± 0 05	7 7	−1 5
1 32	1 27 ± 0 05	3 9	−3 8
2 64	2 65 ± 0 16	6 0	+0 4
5 27	5 28 ± 0 21	4 0	+0 2

*Average of six replicate determinations at each concentration

studied. The coefficient of variation of the calculated concentrations varied from 7.7% at 0.66 μg/ml to 3 9% at 1 32 μg/ml

Recovery

The recoveries of chlorthalidone and the internal standard were determined by comparison of the peak areas of extracted standards to the peak areas of aqueous standards directly injected onto the HPLC column The recoveries of chlorthalidone and sulfanilanilide averaged 86 1 ± 5% and 74.4 ± 2 3%, respectively, over the concentration range of the standard curve The recovery is 81% higher than reported previously [17] for the extraction of chlorthalidone from whole blood. The increased recovery is apparently a result of sonication before and after hemolysis, allowing for a more complete release of the bound drug from the red blood cell.

Drug interferences

Analysis of blood samples from human subjects administered chlorthalidone

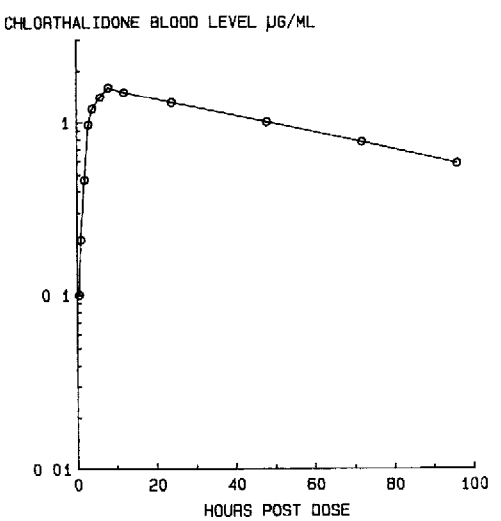

Fig 4 Whole blood concentrations of chlorthalidone in a human subject who received an oral dose of Hygroton®, 25 mg

concomitantly with the β-adrenergic blocking agents propranolol or celiprolol showed no interfering chromatographic peaks

Clinical samples

The HPLC procedure described has been used for the analysis of whole blood samples from human bioavailability and bioequivalency studies Fig 4 shows the time course profile of chlorthalidone whole blood concentration in a human subject, following a single oral dose of Hygroton®, 25 mg

CONCLUSION

An HPLC assay for the determination of chlorthalidone in whole blood has been developed. The method is an improvement over the previously published method of Guelen et al [17] in that it provides for greater sensitivity The presently described method incorporates an internal standard to insure accuracy This assay has been used for the analysis of samples from human pharmacokinetic studies

ACKNOWLEDGEMENTS

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