Improved Method for Estimating Chlorthalidone in Body Fluids

M. G. TWEEDDALE* and R. I. OGILVIE*

Abstract □ The reliable estimation of chlorthalidone in whole blood, plasma, urine, or bile depends upon solvent extraction followed by deamination in alkali at elevated temperature. The concentration of chlorthalidone is quantitatively determined from the difference in absorbance at 262 nm before and after deamination. The conditions required for quantitative extraction from the different body fluids are described. The assay permits detection of 1 mg/liter, which is 10 times better than the previously described assay of chlorthalidone. Mass spectroscopy showed the assay to be specific for unchanged chlorthalidone. The assay was applied to the measurement of chlorthalidone in human blood and urine, and results suggest that chlorthalidone has a long half-life and is extensively metabolized in humans. Red cell concentrations of chlorthalidone are 7–10 times greater than plasma concentrations.

Keyphrases □ Chlorthalidone—spectrophotometric determination in biological fluids □ Diuretics—improved assay of chlorthalidone in biological fluids □ Spectrophotometry—improved assay of chlorthalidone in biological fluids

While studying the antihypertensive actions of chlorthalidone[1] in humans, it became necessary to measure the concentrations of this drug in blood and urine. The only published assay for chlorthalidone in body fluids (1) was considered by its authors to be unsuitable for clinical purposes, because concentrations of chlorthalidone below 10 mg/liter could not be detected. The modifications described in this paper resulted in a 10-fold increase in sensitivity of the assay. The method described is used in this laboratory to study the pharmacokinetics of chlorthalidone in humans and to monitor diuretic and antihypertensive effects of this drug.

EXPERIMENTAL

Materials—All chemicals[2] used were of analytical purity. A concentrated stock solution (300 mg/liter) of chlorthalidone[3] in 0.1 *N* Na_2CO_3 is used to prepare the internal standards. Reference standards are prepared in bulk by adding known quantities of chlorthalidone to the appropriate biological fluid obtained from a "normal" subject. Both reference standards and biological samples are stored at −10° until required. Such storage for over 1 year does not result in deterioration of reference standards.

Procedure—All samples are analyzed for chlorthalidone in duplicate. Each 2-ml aliquot of a chlorthalidone-containing sample is added to 2 ml of buffer solution in a glass-stoppered bottle (125-ml capacity). (The buffer solution is described later.) Twenty-five milliliters of ether is added, and the mixture is shaken mechanically for 10 min. After settling, the ether layer is decanted into a graduated cylinder from which 20 ml is transferred to a glass separator (60-ml capacity, fitted with a Teflon stopcock) containing 2 ml 2 *N* NaOH. The mixture is agitated mechanically for 5 min and, after allowing time for separation, the aqueous layer is drawn off into a glass tube fitted with a plastic screw-cap.

Single aliquots of four reference standards (0, 5, 10, and 20 mg/liter chlorthalidone) and two reagent blanks (2 ml distilled water) are carried through with each run of the assay. One reagent blank serves as the spectrophotometric reference solution, while the other is converted to an internal standard by the addition of chlorthalidone to give a final concentration of 20 mg/liter. This internal standard is subjected to spectrophotometry and deamination along with the other extracts and is used to calculate the fractional recovery of chlorthalidone from the reference standards. In addition, this internal standard serves as a control for the deamination process.

The absorbance of the final alkaline extract from each sample is measured at 262 nm against the reagent blank, using silica cells of 5-mm width and 10-mm light path. Each extract is then returned to its tube and the cap is screwed down tightly. All tubes are immersed in a water bath at 97° for 2 hr, after which they are cooled to room temperature by standing in cold water. Finally, the absorbance of each incubated extract is measured at 262 nm against the incubated reagent blank. Two spectrophotometers[4] were used to measure absorbance, and identical results were obtained.

Determination of Chlorthalidone Concentration—The absorption spectra of chlorthalidone before and after incubation in alkali are shown in Fig. 1. The difference between the absorbance readings $(A_2 - A_1)$ is proportional to the concentration of chlorthalidone. When chlorthalidone is extracted from a biological fluid, this absorbance difference must be corrected for background activity by subtraction of the absorbance difference $(A_2^B - A_1^B)$ of a "blank" sample taken prior to the administration of chlorthalidone. (Experience has shown that this blank value ranges from zero to ±2 mg/liter and is usually negative when urine or bile samples are assayed.) The difference $(A_2 - A_1) - (A_2^B - A_1^B)$ is referred to as the final absorbance, A_F, of a sample. Values of A_F are linearly related to the concentration of chlorthalidone present in the original sample (Fig. 2).

The chlorthalidone content of a sample is determined either by reading off the A_F values from a standard curve or by use of Eq. 1:

$$\text{chlorthalidone concentration (mg/liter)} = \frac{A_F}{b \times f \times e} \quad \text{(Eq. 1)}$$

where b is the slope of the standard curve for chlorthalidone in 2 *N* NaOH, f is the fraction of the ether layer used for back-extraction of chlorthalidone into alkali, and e is the fractional recovery of chlorthalidone from the reference standards.

Assay Conditions with Differing Biological Fluids—Consistent extraction of chlorthalidone from whole blood or from packed red cells requires prior hemolysis of the cells, which was accomplished by slow freezing and thawing. The buffer system giving optimal recovery of chlorthalidone from whole blood or packed red cells is a citric acid–disodium hydrogen phosphate mixture at pH 5.0 and of approximately 1 *M* ionic strength (60 g citric acid plus 108.8 g disodium hydrogen phosphate dissolved in water and diluted to 1 liter). This buffer system also proved satisfactory for the recovery of chlorthalidone from plasma and serum; but when used with samples of urine or bile, it resulted in such high background activity (due to extraction of pigments) that chlorthalidone was undetectable. Milder, alkaline extraction conditions are necessary to recover chlorthalidone from these body fluids. A suitable buffer system (0.25 *M* at pH 8.5) contains 6.2 g boric

[1] Hygroton, Geigy Pharmaceutical Co.
[2] Obtained from either J. T. Baker Chemical Co. or British Drug Houses.
[3] A pure sample of chlorthalidone (lot 538) was obtained through Ciba-Geigy (Canada) Ltd.
[4] Hitachi Perkin-Elmer model 139 and Heath-Schlumberger model EU-701.

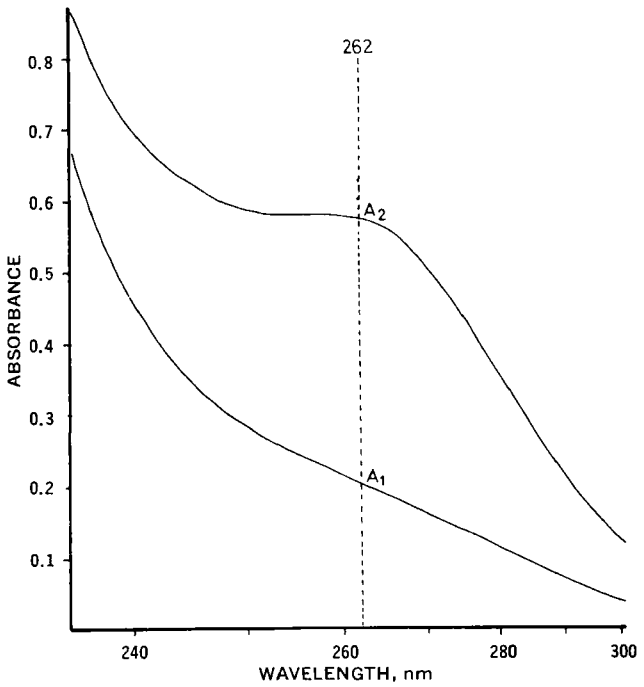

Figure 1—*Absorption spectra of chlorthalidone in 2 N NaOH before (lower curve) and after (upper curve) incubation at 98° for 2 hr. The difference between the readings at 262 nm* ($A_2 - A_1$) *is proportional to the concentration of chlorthalidone in the solution.*

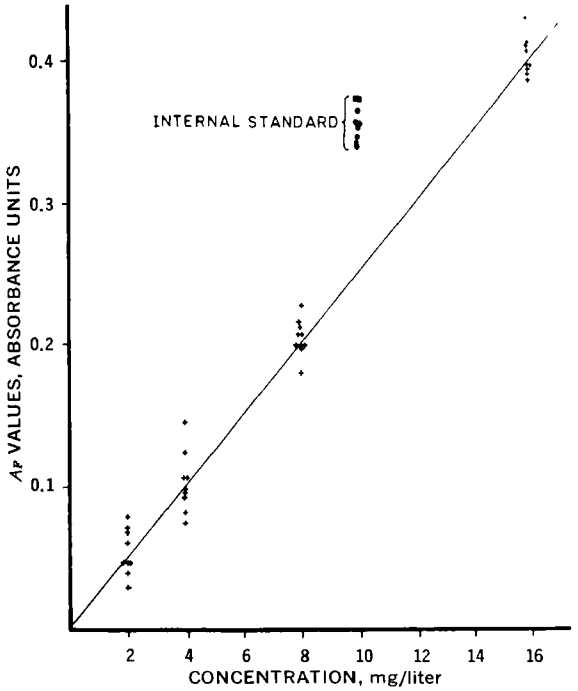

Figure 2—*Assay of reference standards of chlorthalidone in normal human plasma. The results of nine completed assays are shown (+) together with the simultaneous values for the internal standards (●). The least-squares line of best fit is shown. All samples were assayed singly; duplicate assays would reduce the scatter by about one-third.*

acid, 7.26 g KCl, and 1 g NaOH in each liter of solution. Because of the relatively low buffering capacity of this system, acid urines are made alkaline prior to extraction by the addition of a few drops of 5 *N* NaOH. Despite the use of this buffer system, pigment extraction from some very concentrated samples of bile is still great enough to impart a marked color to the ether layer. When this occurs the ether layer is "washed" by transferring it to another bottle containing 4 ml fresh buffer solution. Mechanical agitation for 10 min is followed by separation of the ether layer and back-extraction into 2 *N* NaOH in the usual manner. Although this additional extraction procedure makes the assay unreliable at concentrations of chlorthalidone below 3 mg/liter, concentrations greater than this can be estimated with an error comparable to that for chlorthalidone in urine or blood.

RESULTS

Standard Curves—The A_F values obtained from the first nine runs using reference standards of chlorthalidone in normal human plasma are shown in Fig. 2. This figure also shows the values obtained from the internal standard during the same runs. It can be seen that the best straight line obtained by least-squares linear regression analysis passes through zero and that the relationship between A_F and the concentration of chlorthalidone is linear over the experimental range. Other experiments showed that this relationship remains linear over the 1-40-mg/liter range. With concentrations of chlorthalidone greater than 40 mg/liter, the absorbance after incubation with alkali is so great that the readings are unreliable. Such samples can only be measured after dilution to bring the concentration into the usual range. Apart from minor differences in slope, reference standards in packed red cells, plasma, and bile provided standard curves similar to that shown (Fig. 2).

Recovery and Reproducibility—The mean recovery of chlorthalidone from reference standards in packed red cells was 86.3 ± 1.1% (mean ± *SE*, $n = 36$). The recovery from reference standards in urine was slightly less at 83.8 ± 0.70% (mean ± *SE*, $n = 47$). The reproducibility of the assay was assessed from the day-to-day variations in *(a)* the slope of the standard curve, and *(b)* the A_F value of the internal standard. For each of these parameters, the percent deviation of individual values from the grand mean was determined. Thirty-six complete runs were made using reference standards in packed red cells. Twenty-two (60%) of the values for the slope of the standard curve lay within ±5% of the mean, while only three values (12.5%) varied from the mean by more than ±10%. Similarly, 47 runs were completed with reference standards in urine. Of these, 36 (76%) lay within ±5% of the mean, and no value differed from the mean by more than ±10%. The results for the internal standard were more variable (45% of the values were within ±5% of the mean, and 15% differed from the mean by more than ±10%, $n = 83$), but this was not surprising since preparation of the internal standards involved the repeated accurate measurement of small quantities of fluid.

Accuracy—A series of 16 "unknown" samples containing 1–20 mg/liter chlorthalidone in plasma were assayed in duplicate. The mean error of estimation was 0.5 mg/liter with a range of from −1.1 to +0.8 mg/liter. Similar results were obtained with unknown samples dissolved in urine, but the determination of unknown samples in whole blood or packed red cells was not so accurate, the mean error being 0.8 mg/liter with a range of from −1.6 to +1.2 mg/liter ($n = 12$).

Specificity—A normal adult male volunteer received a single oral dose of 400 mg of chlorthalidone (as 100-mg tablets), and all urine was collected for 3 days. The urine was made alkaline and then extracted repeatedly with ether until assay of the remaining aqueous material revealed that all chlorthalidone had been removed. The ether extracts were pooled and evaporated to dryness under a stream of warm air. The residue was reconstituted in 2 ml 99% ethanol and diluted with distilled water to 50 ml. This reconstituted solution was again extracted with ether, and the process was repeated until material assayable as chlorthalidone accounted for 70% of the weight of the residue left after evaporation of the ether. This final residue was recrystallized from 99% ethanol and the crystals were submitted to mass spectrometry[5]. The mass spectra of the material extracted from urine were compared with that of chlorthalidone extracted from chlorthalidone tablets by solution in, and crystallization from, 99% ethanol. The mass spectra obtained were identical.

Assay Control—Statistical control of the assay was main-

[5] Mass spectra were obtained and interpreted by Morgan-Schaffer Enterprises, Montreal, Quebec, Canada.

tained by day-to-day plotting of six variables. These were the slope, the Y-intercept, and the standard error of Y on X (obtained by the method of least squares from A_F values for the reference standards), the value of the internal standard, the percent recovery from the reference standards, and the within-assay variation. The latter parameter was defined as the mean difference between the duplicate values of the unknown samples. On the basis of the results from the first 10 runs of the assay, means and 95% confidence intervals for each parameter were determined. The assay remained in acceptable control throughout these experiments.

Interfering Substances—Whole blood and urine samples from patients receiving a number of drugs in addition to chlorthalidone were assayed. These drugs included digitalis, chlorpropamide, benzodiazepines, phenobarbital, aspirin, allopurinol, methyldopa, and an unidentified sulfonamide preparation (for a urinary tract infection). Only the latter compound interfered with the assay of chlorthalidone.

Blood Concentrations and Urinary Excretion of Chlorthalidone in Humans—A normal, fasting, adult male subject received two 100-mg tablets of chlorthalidone. Blood samples were collected at intervals over the subsequent 5 days, and the cumulative urinary excretion of chlorthalidone was measured over the same period. Chlorthalidone became detectable in the whole blood in less than 1 hr, and levels reached a peak of 6.3 mg/liter at 6 hr. Thereafter, the concentrations declined steadily to reach 1.5 mg/liter at 5 days. Cumulative urinary excretion of chlorthalidone during the experimental period amounted to 52 mg.

The 24-hr urinary excretion of chlorthalidone was assessed in three hospitalized male patients who had received stable doses of chlorthalidone (100 mg daily) for at least 1 month. In these subjects, the daily excretion of chlorthalidone ranged from 36 to 64 mg. Heparinized blood samples were obtained from 15 subjects receiving chlorthalidone for the control of hypertension in doses ranging from 25 to 200 mg/day. The chlorthalidone content of these samples ranged from 4 to 34 mg/liter. When the serum from these blood samples was assayed separately, it was observed that the serum concentrations were markedly lower than those obtained from whole blood. In only three of the subjects was there any agreement between the whole blood and serum concentrations, and each of these serum samples showed evidence of hemolysis.

These results led to an investigation of the distribution of chlorthalidone in whole blood. Heparinized blood was obtained by venipuncture from a normal volunteer. Chlorthalidone was added to portions of the blood to give final concentrations of 0, 3, 6, 12, and 24 mg/liter. Aliquots were withdrawn from each lot and were assayed to confirm the accuracy of the initial dilution. After separation of the red cells and serum, aliquots of each layer were assayed separately. The results are shown in Fig. 3 where the nominal concentration in whole blood is plotted against the actual concentrations in the red cell and plasma layers. It can be seen that the concentration of chlorthalidone in the red cell layer is approximately 7 times that of the serum. In this experiment, 90% of the chlorthalidone originally added to the whole blood was recovered. Similar experiments were repeated on two other occasions using outdated blood from the blood bank. The results were again similar, the concentration in the red cells exceeding that of the plasma by 8 and 10 times, respectively. It would appear from these experiments that chlorthalidone is extensively bound in or on the red cell.

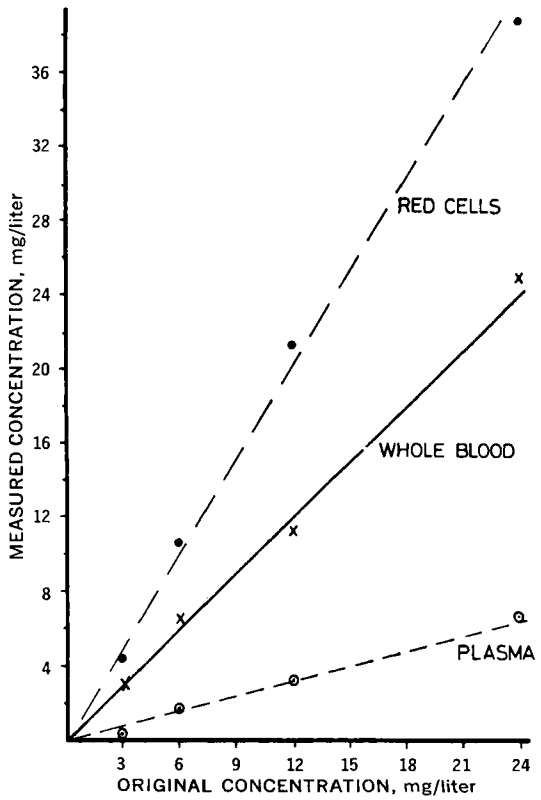

Figure 3—*Distribution of chlorthalidone in blood. Chlorthalidone was added to whole blood and aliquots were assayed for chlorthalidone content* (×). *Red cells* (●) *and plasma* (○) *from the chlorthalidone-containing blood were separated and assayed independently. High concentrations of chlorthalidone were found in the red cells and correspondingly low concentrations in the plasma. (In the experiment illustrated, the hematocrit was 45%.)*

DISCUSSION

The present paper describes modifications of the only published assay for chlorthalidone in biological materials (1). As a result of these modifications, the assay sensitivity was increased 10-fold, so that concentrations of chlorthalidone occurring with normal therapeutic regimens can now be readily measured. The modified assay for chlorthalidone appears to be specific for the unchanged compound and has an acceptable degree of reproducibility and accuracy. Although no formal search was made for interfering compounds, it appears that the assay is valid in the presence of various drugs commonly used with chlorthalidone.

There are two main reasons for the increased sensitivity of the current assay over that described by Pulver *et al.* (1). These are the initial extraction conditions and the volume of 2 N NaOH into which the ether layer is back-extracted. These experiments showed that the extraction conditions described by Pulver *et al.* will not quantitatively remove chlorthalidone from whole blood or packed red cells. Furthermore, the original extraction technique yielded high background activity when used with samples of urine, thereby obscuring the presence of small quantities of chlorthalidone. Although the modified extraction conditions failed to extract 14–18% of the chlorthalidone present, they nonetheless extracted chlorthalidone both quantitatively and reliably from different biological fluids and provided low background activity. The original assay used sample sizes from 2 to 5 ml which, after ether extraction, were back-extracted into 10 ml 2 N NaOH, thereby diluting considerably any chlorthalidone present in the original sample. In contrast, the use of small volume cells with a 1-cm light path allows the transfer of chlorthalidone from a 2-ml sample to an equal volume of alkali, thereby avoiding this diluting effect. Furthermore, Pulver *et al.* calculated the concentration of chlorthalidone by a standard formula which took no account of either differing recovery rates of chlorthalidone from various biological fluids or of the sample blank values. The methods described in the present paper allow for both of these variables.

Pulver *et al.* (1) reported that, after a single oral dose of chlorthalidone (600 mg) administered to a normal subject, only 31% was recovered in the urine during 24 hr. In the present experiment, only 26% was recovered in the urine during the 5 days following a single oral dose of 200 mg. Twenty-four-hour collections from three patients on a stable dose of chlorthalidone (in which daily input should equal daily output) showed that the daily excretion of chlorthalidone ranged from 30 to 60% of the daily dose. Since the mass spectrometry results show the present assay to be specific for unchanged chlorthalidone, it appears that another significant route of elimination of this drug exists in the human as compared with the dog (1) and rat (2). In the latter paper (2), the

authors showed that 6 times as much chlorthalidone was present in the red cell fraction of rat blood as in the plasma. The present study reports similar findings in humans where from 8 to 10 times as much chlorthalidone is found in the red cells as in the plasma. On the basis of the results described in this paper, approximately 90% of a given dose of chlorthalidone is bound in the tissues. It is this tissue binding that probably accounts for the long half-life (54 hr) noted in the normal human subject and for the prolonged diuretic action of chlorthalidone in humans (3).

REFERENCES

(1) R. Pulver, H. Wirz, and E. G. Stenger, *Schweiz. Med. Wochenschr.*, **89**, 1130(1959).

(2) G. Beisenherz, F. W. Koss, L. Klatt, and B. Binder, *Arch. Int. Pharmacodyn. Ther.*, **161**, 76(1966).

(3) R. V. Ford, *Tex. Med.*, **56**, 343(1960).

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* Present address: Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland, Canada.

ˣ To whom inquiries should be directed.

Dependence of Toxicity on Molecular Structure: Group Theory Analysis

J. L. COHEN*, W. LEE, and E. J. LIEN ˣ

Abstract □ Group theory was applied to a wide variety of toxic substances, drugs, and endogenous organic compounds to test the apparent empirical relationship between toxicity of a molecule and its degree of molecular symmetry. Many compounds in each classification were analyzed and separated into individual symmetry groups using "space-filling" molecular models. With few exceptions, an apparent relationship was noted between each symmetry group and the relative toxicities. A brief introduction to group theory is presented as well as the rationale and implications of these observations.

Keyphrases □ Structure-activity relationships—group theory analysis, toxicity-molecular symmetry □ Toxicity—dependence on molecular structure, group theory analysis of various substances □ Molecular structure—role in toxicity, group theory analysis of various substances

Numerous reports in the literature attempt to correlate the activities or toxicities of drugs and chemicals with various structural or physical-chemical parameters of the molecules (1-3). These approaches, in general, have shown the greatest applicability for structurally related series of compounds, but there has been no satisfactory general correlation which explains the activities of molecules with significantly different structures and physical-chemical properties. The reason for this may be that fundamental parameters, such as molecular symmetry, or other relevant properties of molecules have not been included among the physical-chemical parameters used in existing quantitative structure-activity correlations. Because the overall objective of all correlations of this type is to design drugs with very specific activities and limited toxicities, the approach along specific drug classification lines or specific disease lines is clearly appropriate. Therefore, the implication is that a fundamental explanation of the general type stated may direct future research to include additional specific parameters required to design drugs of specific pharmacological activity.

Table I—Mean LD_{50} Values for Oral Doses in Rats of Different Symmetry Groups

Group	Number of Compounds	LD_{50}[a]	*df*	*t*	Significance Level, %
C_1	29	17.60 ± 2.59	46	4.26	99.5
C_{2v}	19	3.57 ± 0.91	—	—	—
C_{1h}	5	5.21 ± 2.58	32	1.95	95
D_{2h}	5	3.21 ± 1.54	32	2.27	97.5

[a] LD_{50} values in rats, millimoles per kilogram, mean ± standard error ($S\bar{x}$, where $S\bar{x} = S/\sqrt{n}$).

In this paper the empirical relationship between molecular symmetry and toxicity is explored with various drugs, toxic chemicals, and endogenous organic compounds.

GROUP THEORY

Group theory invariably differentiates between all possible geometric isomers and places them into their respective symmetry groups. Once the compounds are classified into their respective symmetry groups and subgroups, the similarities and contrasts within the groups can be compared. An extensive mathematical discussion of group theory will not be presented here; however, several references (5, 6) discuss the theoretical details of group theory analysis. Most appropriate to this discussion is the actual application of some principles to the compounds analyzed in the present study.

Group symmetry operations are mathematical operators which perform linear transformations on molecular orbital wave func-