# Chlorthalidone Analysis Using Carbonic Anhydrase Inhibition

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**Abstract** □ Chlorthalidone was analyzed in the concentration range of 0.1–3.0 μg/ml with a precision of ±0.05 μg/ml. Chlorthalidone inhibition of the enzymatic hydrolysis rate of *p*-nitrophenyl acetate by bovine erythrocyte carbonic anhydrase was used as a basis for the determination. The amount of *p*-nitrophenol formed was measured by monitoring the absorbance at 400 nm, and its formation rate was proportional to the chlorthalidone concentration. The mixing of the enzyme, substrate, and sample, the incubation of the reaction mixture, and the recording of the absorbance were automated. A survey of urine samples from 26 normal human subjects did not reveal any endogenous substances that interfered with the assay. Analyses of urine samples from six subjects after oral administration of 100 mg of chlorthalidone indicated rapid absorption and a biphasic elimination. The α-phase half-life was 1.5 hr, and the β-phase half-life was 35 hr.

**Keyphrases** □ Chlorthalidone—spectrophotometric analysis based on inhibition of carbonic anhydrase, urine □ Spectrophotometry—analysis, chlorthalidone, based on inhibition of carbonic anhydrase, urine □ Carbonic anhydrase—inhibition by chlorthalidone, spectrophotometric analysis, urine □ Diuretics—chlorthalidone, spectrophotometric analysis based on inhibition of carbonic anhydrase, urine □ Enzymes—carbonic anhydrase, inhibition by chlorthalidone, spectrophotometric analysis, urine

Two methods were reported for chlorthalidone analysis in biological fluids. One method involved the conversion of chlorthalidone to 2-(3′-sulfonamido-4′-chlorobenzoyl)benzoic acid and the measurement of the resulting change in UV absorption (1); the other method used GLC (2). The UV spectrophotometric method was not sensitive enough to detect the chlorthalidone levels in urine after a therapeutic dose of 50 or 100 mg, and the GLC method could not be reproduced since one reagent was not available commercially. Because of these difficulties, an alternative method of analysis was needed. Developmental work yielded two methods concurrently: one method was a GLC analysis utilizing on-column methylation (3), and the second was the enzymatic method described in this report.

Chlorthalidone inhibits carbonic anhydrase (EC 4.2.1.1) by decreasing the production rate of the product from the substrate by the catalytic action of the enzyme (4). Since the decrease in the catalytic rate is proportional to the chlorthalidone concentration, measurement of this decrease can be used to determine the chlorthalidone level in unknown samples. This principle was utilized previously for the assay of acetazolamide (5, 6) and furosemide (7). Carbonic anhydrase catalyzes the hydration of carbon dioxide, and the activity of the enzyme toward the physiological substrate, carbon dioxide, is usually measured by determining the rate of increase that the enzyme causes in pH during bubbling of carbon dioxide through a slightly alkaline buffer (5).

However, the enzyme also has estrolytic activity toward nonphysiological substrates (8). One substrate, *p*-nitrophenyl acetate, is hydrolyzed to *p*-nitrophenol; the hydrolysis rate is proportional to the change in absorbance at 400 nm. With this substrate, an automated analysis was developed for solutions containing 0.1–3.0 μg of chlorthalidone/ml.

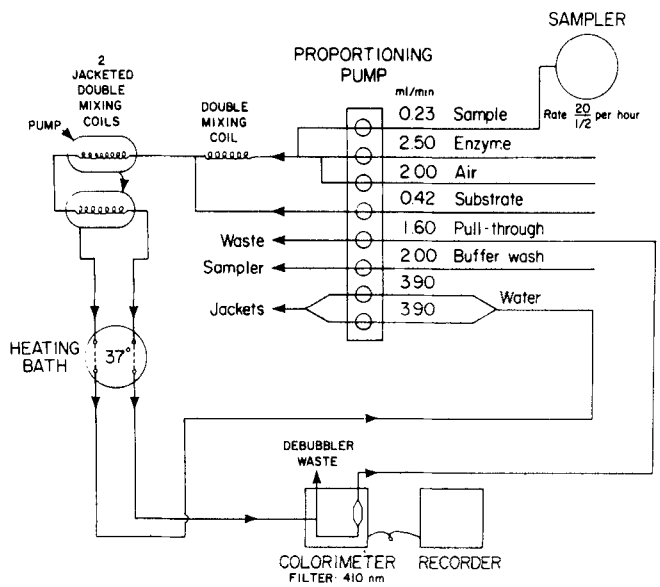



**Figure 1**—*Diagram of automated analysis system. For the substrate line, a silicone pump tube connected to polyethylene tubing was used. All other pump tubes and other tubing were polyvinyl chloride. Either 400- or 410-nm filters may be used for the colorimeter.*

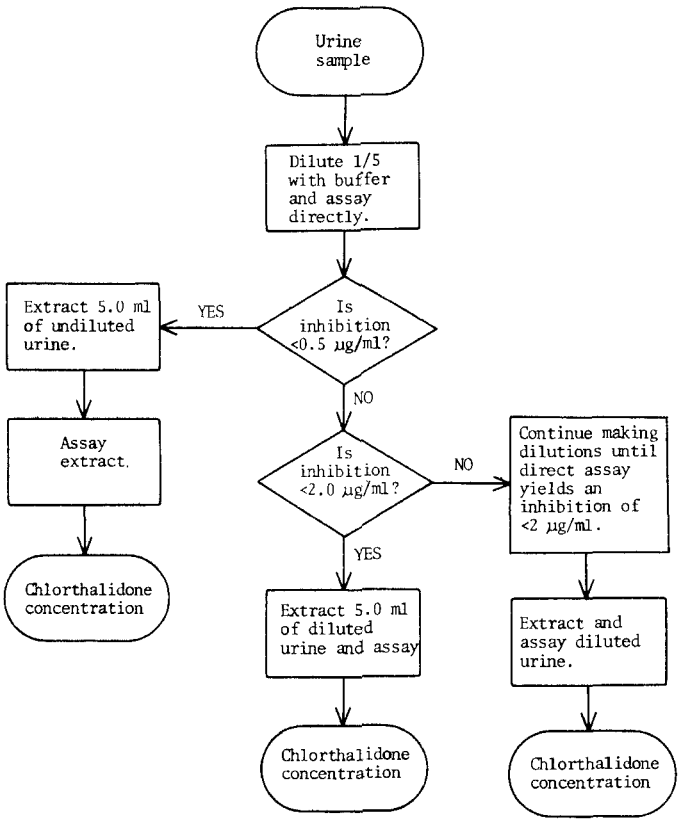
The drug in urine can be analyzed by this method either directly or after chlorthalidone has been extracted selectively. The direct assay yields only an estimate of chlorthalidone since it is influenced by the yellow pigments in the urine and the urine pH. However, an estimate is useful, particularly for samples with a high chlorthalidone concentration, so that the the urine can be diluted appropriately before the extraction.

Scheme I shows a flowchart for the analysis of chlorthalidone in urine samples. First, the sample is assayed directly; then it is diluted and reassayed until the inhibition is less than that caused by 2 μg of chlorthalidone/ml. With the use of this information, the urine sample is diluted, extracted, and assayed. The methods for the direct assay, the extraction, and the assay of the extracts are described here.

## EXPERIMENTAL

**Reagents for Automated Analysis System**[1]—The buffer was 0.1 *M* tromethamine[2], pH 7.5, plus 0.1% surfactant[3]. The enzyme solution contained 30 mg of bovine erythrocyte carbonic anhydrase[4]/liter of buffer. The substrate solution contained 1.0 m*M* *p*-nitrophenyl acetate[5] in polyethylene glycol 400[6].

[1] AutoAnalyzer I, Technicon Industrial Systems, Tarrytown, N.Y.
[2] Nutritional Biochemical Corp., Cleveland, Ohio.
[3] Triton X-405, Rohm and Haas Co., Philadelphia, Pa.
[4] Sigma Chemical Co., St. Louis, Mo.
[5] Schwarz/Mann, Orangeberg, N.Y.
[6] J. T. Baker Chemical Co., Phillipsburg, N.J.




*Scheme I*

**Chlorthalidone Standards**—To prepare a stock solution, 10 mg of chlorthalidone[7] was dissolved in 10 ml of dioxane[8] and diluted to 100 ml with 0.1 *M* tromethamine, pH 7.5. For the direct assay method, chlorthalidone standards of 0.5, 1.0, 2.0, 3.0, and 4.0 μg/ml were made by dilution of the stock solution with buffer. For the assay of extracted urine samples, chlorthalidone standards were prepared by adding 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, and 20.0 μg of chlorthalidone to 4.0-ml aliquots of normal human urine. Sufficient water was added to bring the sample volumes to 5.0 ml. These standards were extracted with the unknown urine samples.

**Automated Procedure**—Figure 1 is a diagram of the automated analysis system. The colorimeter was calibrated by adjusting the absorbance to zero, with buffer being pumped through the enzyme and sample lines and substrate being pumped through the substrate line. This baseline corrected for the nonenzymatic hydrolysis of the substrate during the reaction. The enzyme pump line was then switched from the buffer solution to the enzyme solution, which caused the increase in absorbance, $A_0$, shown in Fig. 2. This increase represents the uninhibited enzymatic velocity. Five minutes later, the samples were introduced, and the degree of inhibition of carbonic anhydrase by chlorthalidone was reflected by the reduction in absorbance, $\Delta A$.

Standards were reassayed repeatedly during the automated procedure, with no more than 15 unknowns between each set of standards. The chlorthalidone concentrations of each set of unknowns were calculated by using a standard curve of $\Delta A$ *versus* chlorthalidone concentration values from the sets of standards run immediately before and after the unknowns.

**Direct Assay Procedure**—The urine samples were diluted 1:5 with buffer, as indicated in Scheme I, and assayed automatically. To correct for the effect of the yellow urine pigments, the samples were run through the automated system using buffer instead of enzyme. The differences in absorbance caused by the urine samples were subtracted from the $\Delta A$ values obtained when the enzyme was introduced. The chlorthalidone concentration was calculated from this corrected $\Delta A$ value. Unextracted chlorthalidone standards were used for the standard curve.

**Extraction Procedure**—The results of the direct assay were used to estimate the amount of dilution necessary to bring the chlorthalidone concentration of the sample to between 1.0 and 2.0 μg/ml. The urine dilutions were made with water, and 5.0 ml of the diluted sample was placed in a polytef-lined screw-capped culture tube. Sodium bicarbonate (0.6–1.0 g) was added to saturate each sample, 5.0 ml of anhydrous ether[8] also was added, and the tubes were shaken for 10 min at 140 oscillations/min and centrifuged for 5 min at 500×*g* and 5° to separate the phases. Then the ethereal layer was transferred to conical sample cups[9].

A second portion of 5.0 ml of ether was added to the aqueous phase, the extraction was repeated, and the second ether extract was added to the first. The samples were dried under nitrogen at 40°, and the residues were dissolved in 0.5 ml of polyethylene glycol 400. The samples were diluted with 4.5 ml of buffer and assayed.

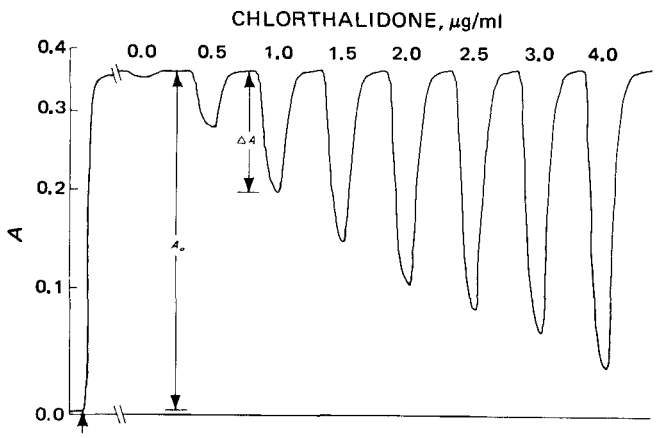



**Figure 2**—*Typical recording obtained using chlorthalidone standards extracted from urine. The initial portion of the tracing shows the baseline obtained when only substrate and buffer were pumped through the system. The arrow indicates the addition of enzyme, and* $A_0$ *represents the velocity of the uninhibited enzymatic reaction. The inhibition of the enzyme by the samples caused the decreases in absorbance,* $\Delta A$. *The samples were prepared by adding chlorthalidone to normal human urine and extracting as described in the text.*

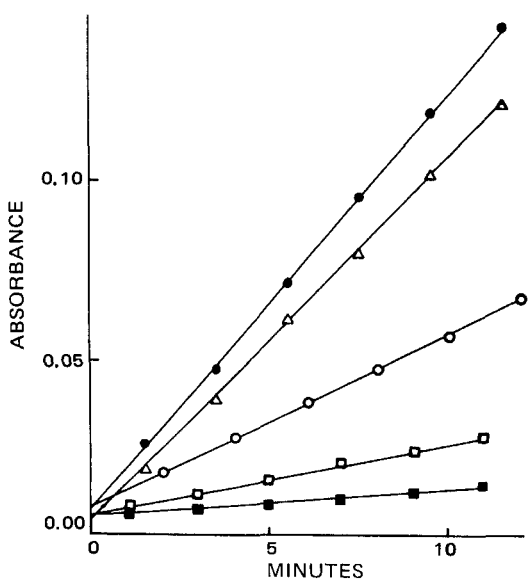



**Figure 3**—*Inhibition of carbonic anhydrase by chlorthalidone. The reaction mixtures contained 0.09 mg of carbonic anhydrase and 1.0 μg (□), 0.5 μg (○), 0.1 μg (△), or no (●) chlorthalidone in 2.7 ml of 0.05* M *tromethamine, pH 7.5. The reaction was started at zero time by adding the substrate, 0.3 ml of 1.0 m*M *p-nitrophenyl acetate in acetonitrile. The absorbance was measured at 400 nm at the times indicated. The rate of nonenzymatic hydrolysis of* p*-nitrophenyl acetate was measured in an acetonitrile–tromethamine control (■).*

[7] USV Pharmaceutical Corp., Tuckahoe, N.Y.
[8] Mallinckrodt, St. Louis, Mo.
[9] Brinkmann Instruments, Westbury, N.Y.

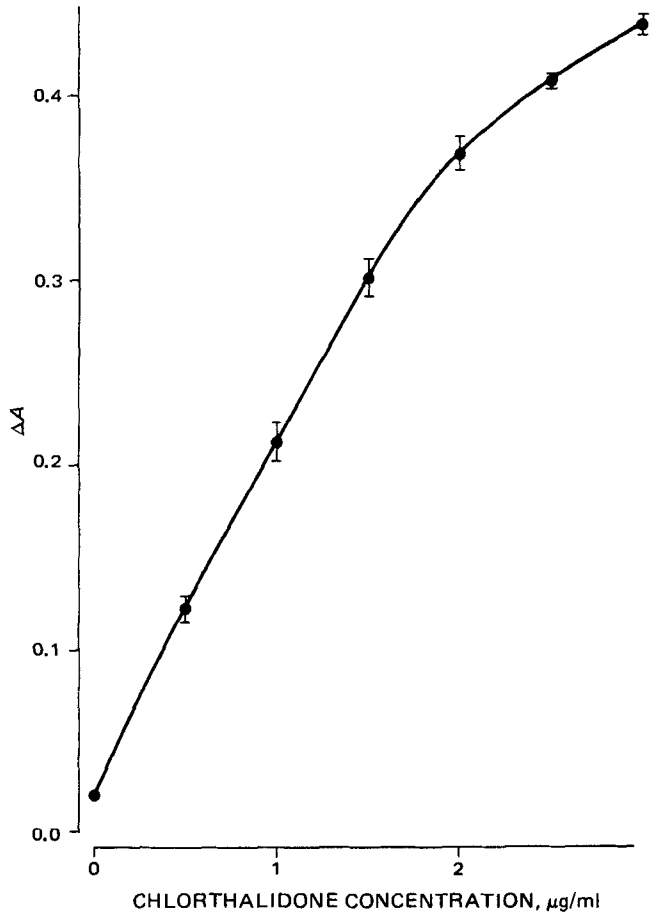



**Figure 4**—*Typical standard curve for chlorthalidone extracted from urine. Standards of chlorthalidone in normal human urine were prepared, and three or four samples of each standard were extracted and analyzed. The error bars represent the standard error of the* ΔA *values obtained at each concentration.*

## RESULTS

**Initial Development**—The feasibility of the assay was first determined using a spectrophotometer[10] to measure the absorbance changes. Figure 3 shows the rate of enzymatic hydrolysis, the rate of nonenzymatic hydrolysis, the slight inhibition by 0.03 μg of chlorthalidone/ml, and the nearly complete inhibition by 0.33 μg of chlorthalidone/ml. The small variations in the $y$-intercepts were due to variations in the elapsed time for the manual addition and mixing of the substrate before the initial absorbance reading was made. However, the slope, which is proportional to the enzymatic velocity, was constant for repetitive samples. The amount of product formed in 12 min by enzymatic hydrolysis was 10% of the available substrate.

Since some inhibitors of carbonic anhydrase cause a time-dependent inhibition (9), chlorthalidone was preincubated with the enzyme for various time intervals before substrate addition. The percent of inhibition did not depend on the preincubation time for intervals between 1 and 120 min.

**Assay Automation**—Figure 1 is a diagram of the manifold designed for the assay. The transit time of the first mixing coil was greater than 1 min, eliminating any possible effect due to time dependency of the chlorthalidone–enzyme interaction. Acetonitrile, used as a solvent for $p$-nitrophenyl acetate in the manual procedure, was changed to polyethylene glycol 400 to avoid degradation of the tubing.

Figure 2 is a typical recording of the chlorthalidone standards extracted from urine. The small $\Delta A$ value obtained with the urine without chlorthalidone was due to the polyethylene glycol 400 used to reconstitute the sample. The system was sensitive to small changes in chlorthalidone concentration between 0.5 and 3.0 μg/ml, indicating the useful range of the automated system. Above 3.0 μg/ml, there was little change in the amount of inhibition as the drug concentration was increased. Thus, samples with a high drug concentration were diluted before analysis. Some batches of the commercial carbonic anhydrase exhibited different sensitivities to chlorthalidone inhibition than the batch used for the data in Fig. 2. This variation in sensitivity may have been due to the purity of the enzyme preparation.

To test the variability of the automated analysis system, solutions containing 1.0–4.0 μg of chlorthalidone/ml in buffer were assayed six times each, and $\Delta A$ was calculated. The standard deviation at each chlorthalidone concentration was ±0.003.

**Sensitivity of Direct Assay**—Control urine samples from 26 normal human males were diluted fivefold with buffer and assayed directly. For 25 subjects, the range of apparent chlorthalidone values was from −0.03 to +0.57 μg/ml. The value of the 26th subject was 7.8 μg/ml. Additional control samples taken at a later time from the same subjects were also assayed. The apparent chlorthalidone values ranged from −0.08 to +0.71 μg/ml. The value of the sample from the 26th subject was zero.

Some samples were assayed after diluting 10-fold with buffer, and the apparent chlorthalidone values were the same as those obtained with a fivefold dilution, indicating that the positive values were not due to the buffering capacity of the urine. The positive values are possibly due to inhibition of the carbonic anhydrase by anions, a phenomenon reported previously (8).

**Sensitivity of Assay after Extraction**—The urine samples from the 26 normal subjects that were assayed directly were also assayed after extraction. The range of the apparent chlorthalidone values after extraction was from −0.07 to +0.09 μg/ml, indicating that the sensitivity limit of the assay was approximately 0.1 μg/ml.

**Standard Curve**—Figure 4 shows a standard curve obtained after the extraction of normal human urine spiked with known amounts of chlorthalidone. The variation in the $\Delta A$ values indicates that chlorthalidone concentrations can be determined with a precision of ±0.05 μg/ml. The curvature in the standard curve at the higher chlorthalidone concentrations could be reduced by plotting log $(A_0/A_0 - \Delta A)$ *versus* chlorthalidone concentration, but a reverse curvature became evident at the lower levels. If dilutions are made before extraction, as indicated in Scheme I, then the values of unknown samples should be <2 μg/ml and thus be in the most linear portion of the standard curve.

The slope of the standard curve varied slightly from day to day because of small variations in the concentration of the enzyme solution, which was made fresh daily. Use of a different batch of enzyme from the supplier would sometimes cause a change in the slope. The standard curve was not dependent on the composition of the aqueous phase from which the chlorthalidone was extracted since the extraction efficiency was the same for various normal urine samples and buffer. The data in Fig. 4 are a composite of standard chlorthalidone samples extracted from two different normal human urines.

**Selectivity**—Although these results indicate that endogenous urinary substances have little or no effect on the assay, particularly after extraction, other drugs that contain an unsubstituted aryl sulfonamide group do inhibit carbonic anhydrase (9). Aqueous solutions of several such drugs were tested in the automated analysis system, and the drug concentration necessary to cause the same inhibition as 1.0 μg of chlorthalidone/ml was determined. The results were (per milliliter) 0.5 μg of acetazolamide[4], 1.1 μg of benzthiazide[11], 3.5 μg of trichlormethiazide[12], 10 μg of sulfanilamide[4], 11 μg of hydrochlorothiazide[13], 12 μg of polythiazide[14], 14 μg of methyclothiazide[15], 18 μg of cyclothiazide[16], 36 μg of bendroflumethiazide[17], and 58 μg of hydroflumethiazide[18]. The substituted aryl sulfonamides, sulfisoxazole[4], sulfacetamide[4], and sulfadiazine[4], did not inhibit the carbonic anhydrase at concentrations up to 100 μg/ml.

Since the only urinary metabolite reported for chlorthalidone is an acid (10), it would not be extracted into the ether at pH 8.3 and would not affect the chlorthalidone assay.

**Extraction Efficiency**—The extraction efficiencies from normal human urine at various concentrations of chlorthalidone are shown in Table I. The trend toward lower efficiency at higher concentrations meant that samples had to be diluted into an assayable range before extraction to be compared accurately to the extracted standards. Thus, the process

[10] DU spectrophotometer, Beckman Instruments, Fullerton, Calif.

[11] A. H. Robins Co., Richmond, Va.
[12] Schering Corp., Kenilworth, N.J.
[13] Merck Sharp & Dohme, Rahway, N.J.
[14] Pfizer Inc., Brooklyn, N.Y.
[15] Abbott Laboratories, North Chicago, Ill.
[16] Eli Lilly and Co., Indianapolis, Ind.
[17] E. R. Squibb & Sons, Princeton, N.J.
[18] Bristol Laboratories, Syracuse, N.Y.

**Table I—Extraction Efficiency for Chlorthalidone from Urine**

| Chlorthalidone Concentration, μg/ml | Recovery[a], % |
|---|---|
| 0.5 | 99 |
| 1.0 | 88 |
| 1.5 | 89 |
| 2.0 | 89 |
| 2.5 | 84 |
| 3.0 | 83 |
| 4.0 | 84 |
| 10.0 | 74 |
| 50.0 | 42 |

[a] Average of duplicates.

for handling unknown samples shown in Scheme I was developed. The decrease in extraction efficiency may be due to the limited chlorthalidone solubility in ether, since increasing the number of ether extractions from two to four yielded a greater extraction efficiency.

**Stability**—There was no detectable loss of chlorthalidone from urine samples during 16 weeks of frozen storage. In addition, no substances were produced in the urine that interfered with the assay. After extraction and reconstitution, chlorthalidone samples were stable for 24 hr at room temperature or at least 3 weeks at −15°. The polystyrene sample cups used for the automated system were inert to the solution containing polyethylene glycol 400, which was used to reconstitute the extracted samples.

**Human Urinary Excretion**—Six male subjects each received a 100-mg chlorthalidone tablet, and urine samples were collected for 72 hr. Figure 5 shows the mean excretion rate *versus* time. The peak excretion rate occurred between 2 and 3 hr after drug administration, indicating rapid absorption. After absorption, the elimination was biexponential, indicating a pharmacokinetic model with a minimum of two compartments. The half-life values obtained by the method of residuals were 1.5 hr for the α-phase and 35 hr for the β-phase. Analysis of urine samples from a subject given orally 100 mg of chlorthalidone as a solution showed that the chlorthalidone concentration could be determined up to 11 days after drug administration.

## DISCUSSION

The described method is sensitive enough to determine urinary levels after the administration of therapeutic doses of chlorthalidone to humans. The only compounds that would interfere with the analysis of urinary extracts would be other drugs containing unsubstituted aryl sulfonamide groups. In a controlled clinical trial, these drugs would not be present.

The two-compartment pharmacokinetic model for chlorthalidone indicated in Fig. 5 is consistent with previous studies on its pharmacokinetics. Two studies (10, 11) used $^{14}$C-chlorthalidone, and a third study in a single subject (12) used a GLC assay. The α-phase shown by the urinary data may reflect the distribution rate of chlorthalidone from the plasma to the erythrocytes since the half-life of this phase is consistent with the report that it takes several hours for the distribution of $^{14}$C-chlorthalidone between the plasma and the erythrocytes to reach a steady state (10).

Assay automation is a major advantage for analysis of a large number of samples. However, this method can be performed manually with a colorimeter or spectrophotometer, as shown in Fig. 3, if an automated system is not available.

The underlying principle of this assay for chlorthalidone is that the drug inhibits the catalytic activity of the carbonic anhydrase. Because of the catalytic nature of the enzyme, a low concentration of carbonic anhydrase can catalyze the formation of a large amount of product in a short time. Chlorthalidone, present at a low concentration, can interact directly with the enzyme, inhibiting the catalytic rate of product formation. The effect of the drug is determined by measuring the difference in the amount of product formed during the transit time in the automated analysis system before and after addition of the sample. Because of the catalytic nature of the product formation, the effect of a low concentration of chlorthalidone in the reaction mixture (about 0.2 μg/ml) is magnified so that the difference in the concentration of product formed (about 2 μg of *p*-nitrophenol/ml) is large enough to be easily measured spectrophotometrically.

This principle could be applied to the analysis of other drugs. Carbonic anhydrase and the automated system described could be used for other drugs containing unsubstituted aryl sulfonamides such as hydrochlorothiazide. Methods could be developed for inhibitors or activators of other enzymes. Because of the magnification effect of the enzymatic catalysis, these methods, which could be spectrophotometric, would have sensitivity limits similar to fluorometric assays or to GLC assays using a flame-ionization detector. In addition, the enzymatic assays would be amenable to automation.

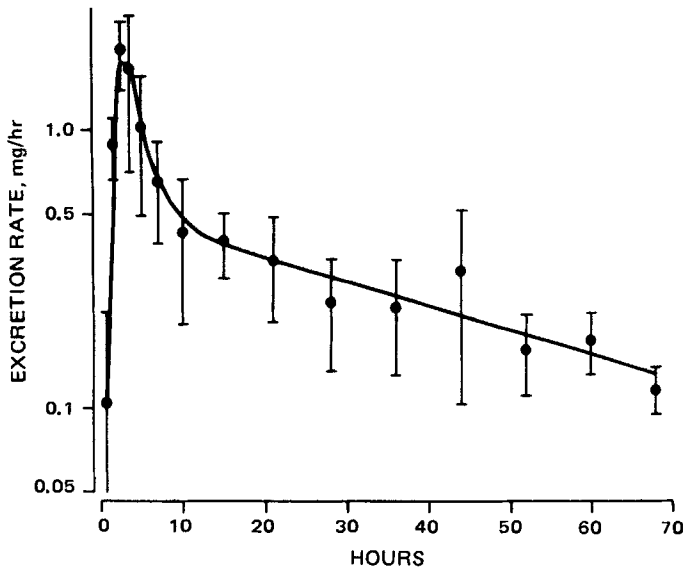



**Figure 5**—*Urinary excretion rate of chlorthalidone by normal human subjects. Six male subjects, 19–30 years old, each received a 100-mg chlorthalidone tablet. The mean excretion rate and standard error for the six subjects during each collection period were calculated.*

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