warfarin and S(−)-warfarin with carbobenzyloxy-L-proline. The S(−)-warfarin carbobenzyloxy-L-prolyl ester elutes before the ester of R(+)-warfarin, with a separation factor of 3.0. A similar elution order is assumed for the internal standard, but could not be verified because this compound has never been resolved. Repeated analyses with time indicated that the derivatives were stable for at least 48 hr in the final reaction mixture.

The recovery of *RS*-warfarin investigated by taking *RS*-warfarin (0.8 μg) spiked with [^{14}C]*RS*-warfarin through the extraction, derivatization, and HPLC analysis was 70% (n = 3). The major losses occurred during evaporation of the sample, therefore the inside of the tube must be washed to concentrate the extracted material in the tip of the tube.

Linear calibration plots for either isomer ($r^2 > 0.99$) were obtained over the range 0.1–1.0 μg/isomer. As shown in Table II, based on either of the internal standard esters (*RS* or *SS*), the relative standard deviations of the peak height ratios were between 11.9 and 3.2% for 0.1 and 1.0 μg warfarin isomer, respectively. Table III summarizes the results of a similar assessment of between-assay variability for studies performed over a 6-week period using 0.1–1.0 μg of warfarin isomer. Satisfactory precision (<10%) is observed over a fivefold concentration range of warfarin isomers. The results indicate that the assay is accurate and reproducible.

The determination limit of the assay with a 10% coefficient of variation was computed from the expression (27):

$$X_0 = 10\sqrt{V(X_0)}$$

where X_0 is the determination limit at the preselected coefficient of variation, and $V(X_0)$ is the variance associated with X_0. Using the above procedure, the determination limits (10% *CV*) of the UV assay for *S*- and *R*-warfarin are 0.16 and 0.096 μg, respectively, using the first eluting standard. Similar values were obtained when using the second eluting standard. The difference in determination limits of the esters of warfarin may be ascribed to the greater potential for interference between the *SS*-ester of warfarin and the first internal standard, due to changes in retention as a result of column deterioration, or day-to-day variability in eluant composition.

Figure 2 shows the plasma concentration–time profiles for the *R*- and *S*-isomers (using 200-μl plasma samples) following administration of *RS*-warfarin (1.5 mg/kg po) to a normal volunteer. At this dose level the plasma concentration can be monitored for at least 5 days for the *S*-isomer or 6 days for the *R*-isomer. The more rapid elimination of the *S*-isomer confirms the findings using the stereospecific MS methods (7, 8) or following the administration of the separate enantiomers (2).

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Stability-Indicating Assay for Chlorthalidone Formulation: Evaluation of the USP Analysis and a High-Performance Liquid Chromatographic Analysis

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Abstract □ An investigation of the USP assay of chlorthalidone tablets showed that variable degradation of chlorthalidone occurred during assay preparation. The degradation products were isolated and identified. A stability-indicating high-performance liquid chromatographic (HPLC) assay which separates the degradation products from chlorthalidone was developed and used to examine the present USP preparation. The HPLC assay is suggested as an alternate.

Keyphrases □ Chlorthalidone—high-performance liquid chromatography, stability-indicating assay, comparison with the USP analysis □ Degradation products—of chlorthalidone, high-performance liquid chromatographic determination, comparison with the USP analysis □ USP analysis—for chlorthalidone and degradation products, comparison to a high-performance liquid chromatographic assay

Chlorthalidone (I) is a monosulfamyl diuretic used in the treatment of hypertension. The official analysis of chlorthalidone tablets, as prescribed in the United States Pharmacopeia, is a spectrophotometric assay (1). Chlorthalidone has been quantitated in various media using a variety of methodologies (2–8). The major techniques in

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Table I—Precision Study of USP Chlorthalidone Assay

Lot No.	Concentration	HPLC Assay, %	USP Assay, % Lab 1	Lab 2	Lab 3	Lab 4
16-910-AR	25 mg/tab	101.8	94.8	100.4	96.7	104.0
			95.2		95.6	102.3
Mean			98.43			
SD			±3.75			
RSD			±3.81%			
16-911-AR	50 mg/tab	100.5	96.4	101.4	104.8	106.8
			94.5		106.1	106.5
Mean			102.36			
SD			±2.65			
RSD			±2.58%			

Table II—Standard Addition and Recovery of Chlorthalidone 25-mg and 50-mg Tablets

Chlorthalidone Added, mg	Placebo for 25-mg Tablets mg Found	Recovery,%	Placebo for 50-mg Tablets mg Found	Recovery,%
12.54	12.38	98.7	12.44	99.2
25.08	24.74	98.6	24.87	99.2
37.62	37.12	98.7	37.20	98.9
50.16	49.45	98.6	49.64	99.0
62.70	61.68	98.4	62.10	99.0

biological materials involve extractive alkylation and GLC (6–8) or deamination followed by UV spectrophotometric quantitation (2, 3, 5). Although a high-performance liquid chromatographic (HPLC) method using a polyamide column (9) has been published for chlorthalidone formulations, the USP still officially requires a UV spectrophotometric assay. An investigation of the precision of this analysis indicated erratic results (Table I). Two lots of chlorthalidone tablets were analyzed by the USP method in four separate testing laboratories with varying results.

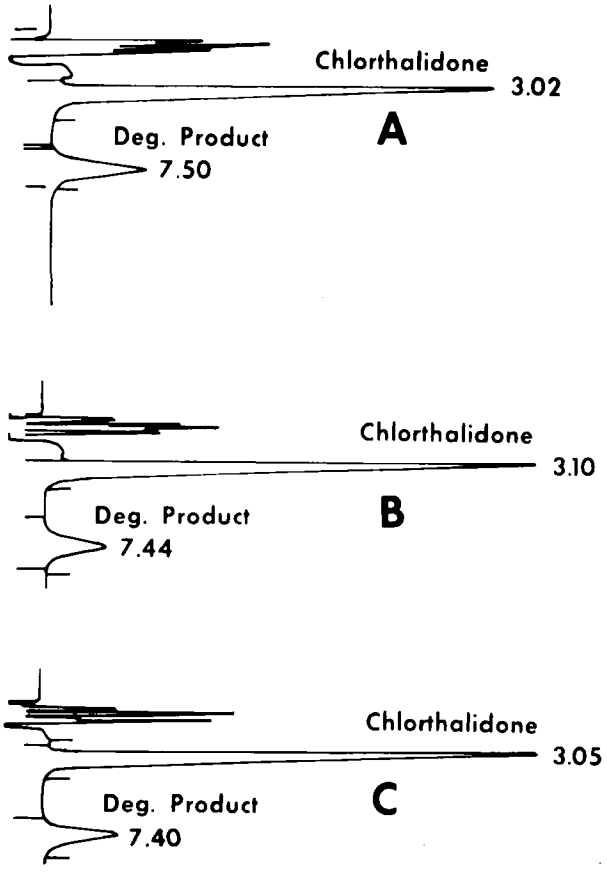
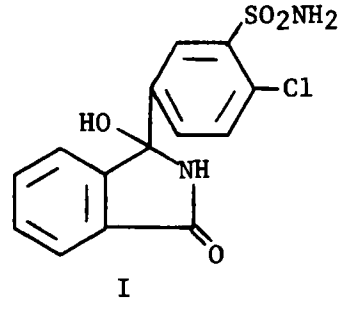


Figure 1—*Chromatograms of USP assay preparations. Key: (A) USP standard lot G immediately after acidification; (B) current marketed product immediately after acidification; (C) chlorthalidone (generic 25 mg) immediately after acidification.*



I

2-Chloro-5-(2,3-dihydro-1-hydroxy-3-oxo-1*H*-isoindol-1-yl)benzenesulfonamide

Since the UV absorbance of chlorthalidone is known to conform to Beer's law in the concentration region used for the assay, the possibility that chlorthalidone is unstable in the acidic assay preparation was investigated. The USP analysis is not stability indicating; therefore, development

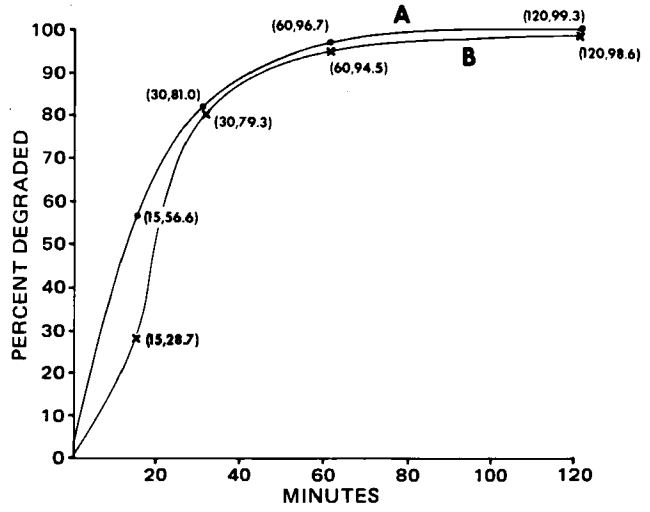


Figure 2—*Rate of degradation of chlorthalidone in USP assay medium. Key: (A) standard preparation; (B) sample preparation.*

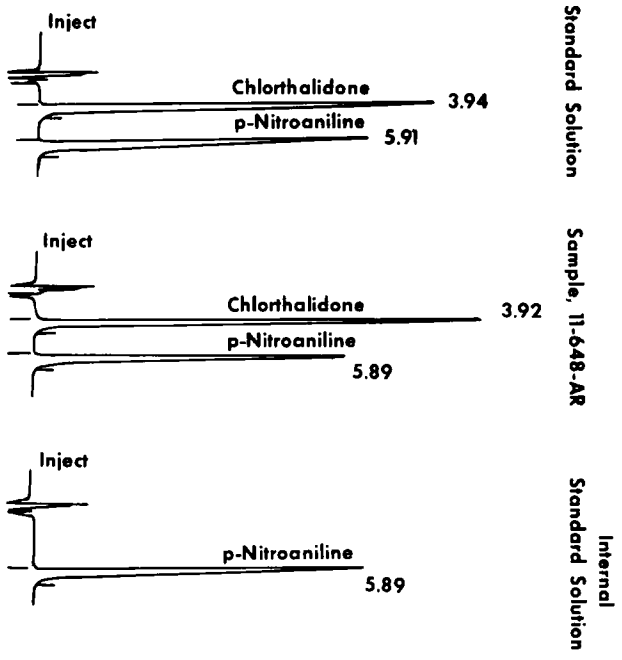


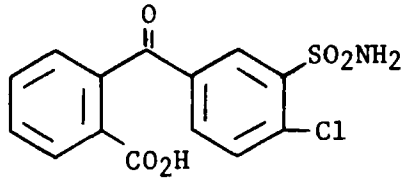
Figure 3—*Typical chlorthalidone chromatograms.*

of an HPLC assay using a less specialized and sensitive column than previously reported (9) was undertaken. The HPLC system was used to evaluate the current USP methodology and is suggested as an alternate assay.

BACKGROUND

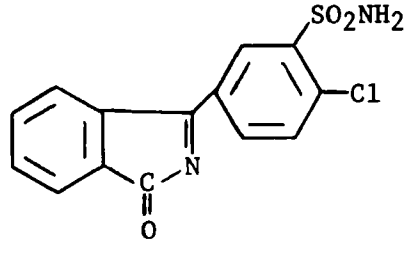
The USP assay for chlorthalidone consists of an acetone extraction followed by filtration and evaporation of the extract. The residue is then dissolved in acidic methanol and quantitated by UV spectrophotometry. The analytical wavelength is 275 nm.

When the final acidic methanol solutions from the USP assay were examined by HPLC (Fig. 1) they were found to contain mixtures of chlorthalidone and one or both of the degradation products (II and III), which coelute, shown below:



II

2-(3-aminosulfonyl-4-chlorobenzoyl)benzoic acid



III

3-[(4-chloro-3-aminosulfonyl)phenyl]-1*H*-isoindol-1-one

Both compounds II and III have larger extinction coefficients at the analytical wavelength than does chlorthalidone. This study reports the occurrence of a noncontrolled degradation of chlorthalidone in the USP

Table III—Precision Study for the Chlorthalidone HPLC Assay

~25 mg/tablet		~50 mg/tablet	
mg Found	mg Found	mg Found	mg Found
23.77	23.97	47.91	47.26
23.92	23.74	48.04	47.04
23.88	23.49	47.92	47.04
23.87	23.98	47.54	47.39
23.80	23.58	47.81	47.03
Mean	23.80 mg/tablet		47.50 mg/tablet
SD	±0.16 mg/tablet		±0.40 mg/tablet
RSD	±0.68%		±0.84%

sample and standard preparations and details an HPLC analysis for chlorthalidone, which is stability indicating in so far as the degradation products are separated from the parent drug. These products can be quantitated independently if desired. Kinetic studies of the standard preparation and the tablet preparation during the USP assay show that, although similar, the rates of degradation of the sample and standard are different (Fig. 2). Since the degradation is not quantitative, the samples assayed are heterogeneous mixtures and errors result in both directions.

The use of a mineral acid in the USP assay causes chlorthalidone to degrade. The HPLC analysis presented here does not use strong acid during sample preparation.

EXPERIMENTAL

Materials—Acetonitrile[1], acetic acid[1], methanol[2], and *p*-nitroaniline[3] were used as received. The chlorthalidone used was USP reference standard lot G.

A high-performance liquid chromatograph[4], a scanning spectrophotometer[5], a UV-visible spectrophotometer[6], and a mechanical shaker[7] were used. A microparticulate octadecylsilane column[8] was used. The

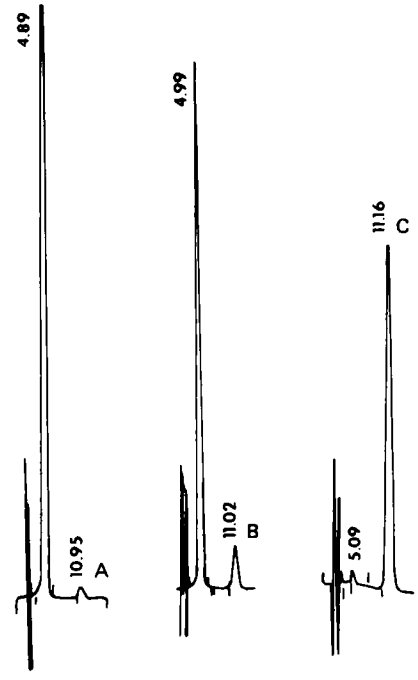


Figure 4—*Time study of USP assay using HPLC. Key: (A) immediately after preparation; (B) 10 min after; (C) 30 min after.*

[1] Glass Distilled, Burdick and Jackson.
[2] Mallinckrodt.
[3] Aldrich Chemical Co.
[4] Waters Model 6000A liquid chromatographic pump; Spectra-Physics Model 4100 computing integrator; DuPont variable-wavelength UV detector; Rheodyne Model 7120 injector with a 20 μl loop.
[5] Cary/Varian 219 recording spectrophotometer.
[6] Beckman DU spectrophotometer with Gilford Model 222 photometer update attachment.
[7] Shaker in the round Model S-500, Kraft Apparatus Inc.
[8] Waters Associates C_{18} μ-Bondapak.

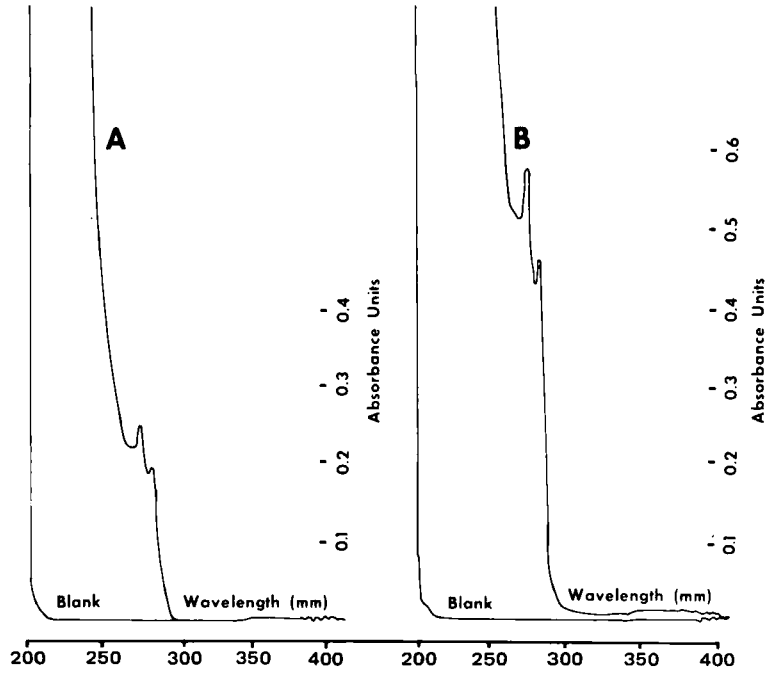


Figure 5—*UV scans of acidified* versus *neutral solution. Key: (A) chlorthalidone in methanol; (B) USP assay preparation.*

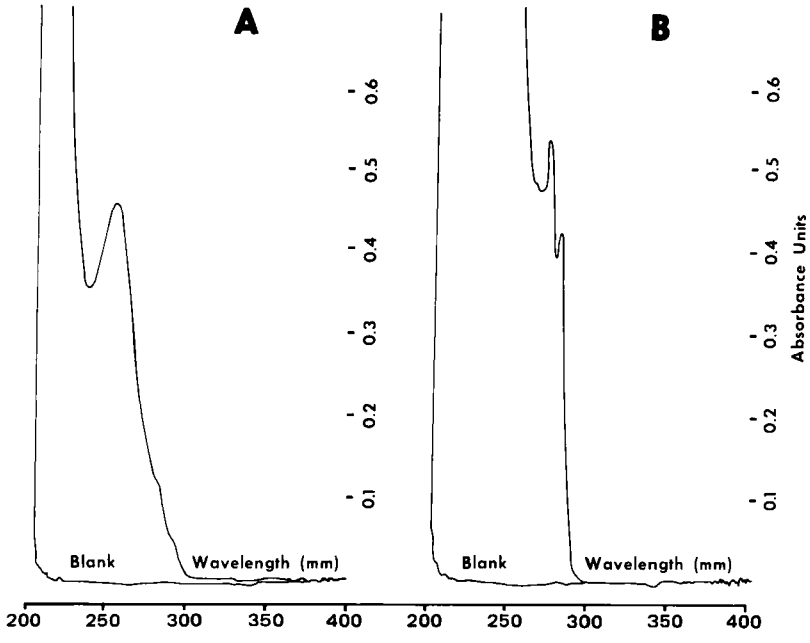


Figure 6—*UV scans of chlorthalidone degradation products. Key: (A) 2-(3-aminosulfonyl-2-chlorobenzoyl)benzoic acid 9.9 μg/ml in acidified MeOH; (B) 3-[(4-chloro-3-aminosulfonyl)-phenyl]-1H-isoindol-1-one 10 μg/ml in acidified MeOH.*

mobile phase was acetonitrile–2% acetic acid (30:70) at a flow rate of 1.5 ml/min. The analytical wavelength was 280 nm.

Reagent Preparation—The internal standard solution was 5 mg/ml of *p*-nitroaniline in methanol.

Approximately 40 mg of chlorthalidone USP standard was accurately weighed and dissolved in 100.0 ml of methanol containing 5.0 ml of the internal standard solution. The solution was diluted 1:1 with water before injection into the liquid chromatograph.

Sample Preparation—Twenty intact tablets were weighed to determine an average tablet weight, finely ground, and a quantity equivalent of 40 mg of chlorthalidone was weighed into a 100-ml volumetric flask. Approximately 50 ml of methanol was added, and the mixture was shaken mechanically for 1 hr. After shaking, 5.0 ml of the internal standard solution was added; the solution was diluted to volume with methanol. A portion of this solution was filtered and diluted 1:1 with water before injection into the liquid chromatograph.

Calculations—The percent of label claim was calculated on the basis of peak height ratios as follows.

$$\frac{(\text{peak height chlorthalidone})_{spl}}{(\text{peak height internal standard})_{spl}} \times \frac{(\text{peak height internal standard})_{std}}{(\text{peak height chlorthalidone})_{std}} \times \frac{\text{Conc. of std.}}{(\text{mg/ml})} \times \frac{100}{\text{Sample Wt}} \times 2 \times \frac{\text{av. tablet wt.}}{(\text{mg/tablet})_{theory}} = \% \text{ Label Claim}$$

RESULTS AND DISCUSSION

The precision data for the HPLC analysis and the precision study conducted on the USP assay (Tables I and III) demonstrate that the HPLC analysis is significantly better than the current compendial method.

When spiked samples were analyzed, recoveries ranged from 98.4 to

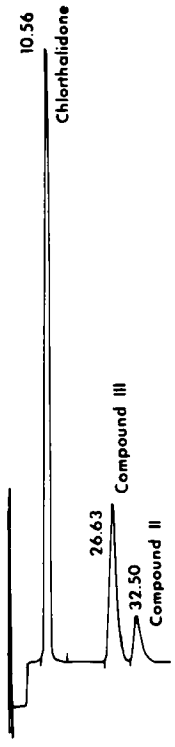


Figure 7—*HPLC system for both degradation products.*

99.2% (Table II). The HPLC method had a relative standard deviation of 0.68% for the analysis of 25 mg of chlorthalidone and 0.84% for 50-mg tablets (Table III). These data were obtained by separate analysts in three laboratories. *p*-Nitroaniline was used as the internal standard. Typical chromatograms for the analysis are shown in Fig. 3.

HPLC analysis of the USP preparation indicated that the solutions being analyzed were mixtures of compounds I, II, and III. The degradation products were isolated from the USP preparation using HPLC. Compound II was identified by comparison with an authentic sample[9] and compound III was identified by spectroscopic techniques[10].

The extent to which chlorthalidone degrades in the acidic methanol appears to depend on pH, water content, and time, although these factors were not all studied in depth. Both of the degradation compounds (II and III) have larger extinction coefficients at the wavelength used for the USP assay than does chlorthalidone. Consequently, the extent of degradation has a marked effect on the assay results and can cause errors in either direction. Figure 4 shows the decrease in chlorthalidone and increase in III with time. Since the degradation is not quantitative and no restraints are prescribed in the USP for pH or time, the absorbance recorded cannot be attributable to any single component.

Comparison of the UV curve for the USP assay preparation with that of chlorthalidone (Figs. 5 and 6) shows substantially the same maxima and minima with slightly greater absorbances. The UV spectrum of compound III is strikingly similar to that of chlorthalidone except for the increased absorptivity for compound III (Fig. 6). The large absorption of chlorthalidone below 260 nm could easily mask the maxima of compound II. This juxtaposition explains how the degradation of chlorthalidone under the USP assay conditions could have been misinterpreted simply as an increase in absorbance due to pH.

The HPLC method described here was designed to quantitate chlorthalidone only. It is stability indicating in so far as chlorthalidone is separated from its degradation products. If a quantitation of the degradation products is desired, a modification of the mobile phase to 85% of 2% acetic acid and 15% acetonitrile flowing at 2 ml/min will allow analysis of I, II, and III concomitantly (Fig. 7).

Close examination of the current compendial assay for chlorthalidone tablets reveals that the drug degrades during analysis. The extent of the degradation is neither quantitative nor reproducible, making the USP assay unreliable. The HPLC method developed in our laboratory is stability indicating and provides a rapid, reliable assay of chlorthalidone. Slight changes in the mobile phase also allow quantitation of the individual degradation products.

[9] Two authentic samples were used: one prepared by Abbott Laboratories, characterized by MS, NMR, IR, CHN; a second obtained as a USP standard. Comparison was done by HPLC and MS to the impurities found in the USP assay preparation.

[10] Compound III was identified by high-resolution mass spectroscopy.

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