GLC Determination of Urinary Chlorthalidone Levels

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Abstract □ A sensitive and specific GLC method for the determination of urinary chlorthalidone levels was developed using on-column methylation. Chlorthalidone is converted to a tetramethylated derivative with trimethylanilinium hydroxide in methanol. This method, which permits the determination of as little as 0.1 μg of chlorthalidone/ml of dog urine, should be adequate for use with human subjects receiving a clinical dose.

Keyphrases □ Chlorthalidone—GLC analysis in urine □ GLC—analysis, chlorthalidone in urine □ Diuretics—chlorthalidone, GLC analysis in urine

Chlorthalidone[1] (I), 2-chloro-5-(1-hydroxy-3-oxo-1-isoindolinyl)benzenesulfonamide, is an oral diuretic agent for the treatment of edema and hypertension. The need to study the pharmacokinetics and bioequivalence of chlorthalidone formulations led to the development of two analytical methods: an enzymatic method based on the inhibition of carbonic anhydrase[2] and a GLC method, which is described in this paper.

One method (1) that used ^{14}C-chlorthalidone was not specific for chlorthalidone since the total radioactivity of the unchanged drug and the metabolites was measured. A spectrophotometric method was reported, based on the conversion of chlorthalidone to 2-(3′-sulfonamido-4′-chlorobenzoyl)benzoic acid (2, 3). This method also was not sufficiently specific or sensitive to determine chlorthalidone levels after a clinical dose.

GLC has been used widely in the quantification of substances isolated from biological materials when both sensitivity and specificity are required. However, underivatized chlorthalidone is not suitable for GLC because of polar active groups. In one GLC method (4), a suitable tetramethylated derivative of chlorthalidone was prepared successfully by extractive alkylation. However, the key reagent for the preparation, tetrahexylammonium hydrogen sulfate, is not commercially available. Attempts in this laboratory to use other quaternary ammonium reagents such as tetrabutylammonium hydroxide and tetrahexylammonium benzoate resulted in a low yield of the derivative. Other approaches to chlorthalidone derivatization such as silylation and acetylation also were explored but were not feasible.

Suitable methylated derivatives of barbiturates, phenolic alkaloids, and xanthine bases were prepared by on-column methylation (5). The procedure was simple and gave quantitative yields. This report describes the application of this technique to chlorthalidone analysis. On-column methylation of chlorthalidone under optimized conditions produced only one derivative peak as compared to the chromatogram obtained by injecting the methylating agent alone. This method is sensitive enough to detect 0.1 μg of chlorthalidone/ml in urine. Based on the reported (3) urinary chlorthalidone concentrations, the present method should be adequate for the determination of urinary chlorthalidone from human subjects after a 100-mg oral dose.

EXPERIMENTAL

Apparatus—A gas chromatograph[3] equipped with a flame-ionization detector was used. A 1.8-m × 0.63-cm i.d. glass column was packed with 3% JXR on 100–120-mesh Gas Chrom Q[4]. The injector and manifold were set at 300°. The column temperature was 190° initially and was programmed at 4°/min to 260°. For some experiments, an isothermal mode at 260° also was employed. The carrier gas was helium with a flow rate of 40 ml/min.

Reagents—Trimethylanilinium hydroxide[5] (0.2 *M*) in methanol was used as a methylating agent. Stock solutions of the following compounds[6] were made in acetone[7]: chlorthalidone, isopropyl 2-(3′-sulfonamido-4′-chlorobenzoyl)benzoate (II), 2-hydroxymethylphenyl-3′-sulfonamido-4′-chlorophenylcarbinol (III), and 3-isobutyloxy-3-(3′-sulfonamido-4′-chlorophenyl)phthalimidine (IV).

Procedure—A series of control human urine samples (5.0 ml) in 15-ml polytef-lined screw-capped tubes was spiked with 5, 10, 20, 30, 40, or 50 μl of chlorthalidone stock solution (1.03 μg/10 μl in acetone). These samples were used to generate a standard curve from which the chlorthalidone concentrations in unknown samples were calculated. Aliquots (5.0 ml) of the unknown urine samples were pipetted into similar tubes. Excess sodium bicarbonate was added to each tube, and saturation was obtained by vortexing. Five milliliters of ether[7] with 30 μl of *n*-docosane[4] (40 ng/μl in chloroform[7]) as an internal standard was added.

The samples were extracted for 10 min in a horizontal shaker[8]. After centrifugation at 5°, the ether phase was transferred to a conical tube. The extraction was repeated similarly for a second time with the omission of the internal standard, and the pooled ether phase was then evaporated to dryness by a gentle nitrogen stream. The residue was redissolved in 50 μl of 0.2 *M* trimethylanilinium hydroxide in methanol by vigorous vortexing. An aliquot (2.0 μl) was then injected into the gas chromatograph.

RESULTS AND DISCUSSION

Injector Temperature and Injector Geometry—The function of the injector was to act as a flash vaporizer and a reaction chamber. Initial studies showed that its effectiveness depended on the appropriate injector temperature and the integrity of the injector geometry. At an injector temperature of 250°, no methylated chlorthalidone peak was observed. However, at 300°, methylation took place instantaneously. A further increase in the injector temperature to 350° did not affect the reaction.

Experience in this laboratory also indicated that the injector geometry of some gas chromatographs gave more dependable results for the direct methylation of chlorthalidone. With a gas chromatograph[3] equipped with a removable glass injector liner in a heated injector assembly, reproducible derivatization was observed. However, with a different gas chromatograph[9] that employed an extension of the glass column proper as the injector instead of a removable glass injector liner, care had to be exercised to exclude any column packing materials in the heated zone of the injector. This precaution was critical for reproducible chlorthalidone derivatization.

Molar Ratio—Studies on the extent of chlorthalidone methylation showed that the products were dependent upon the methylating agent concentration (Table I). When the molar ratio of chlorthalidone to

[1] Hygroton, USV Pharmaceutical Corp., Tuckahoe, N.Y.

[2] Paper presented at the APhA Academy of Pharmaceutical Sciences, Atlanta meeting, November 1975.

[3] Model 900, Perkin-Elmer Corp., Norwalk, Conn.

[4] Applied Science Laboratories, State College, Pa.

[5] Pierce Chemical Co., Rockford, Ill.

[6] The Pilot Plant and Analytical Research & Development Department, USV Pharmaceutical Corp., Tuckahoe, N.Y.

[7] Mallinckrodt Chemical Works, St. Louis, Mo.

[8] Eberbach Corp., Ann Arbor, Mich.

[9] Model MT-550, Tracor Inc., Austin, Tex.

Table I—Qualitative Relationships between Molar Ratio of Chlorthalidone to Trimethylanilinium Hydroxide and Methylation of Chlorthalidone

Molar Ratio	Peak A (15.3 min)	Peak B (15.4–15.6 min)	Peak C (16.7–16.9 min)	Peak D (17.7 min)
1:20 or above	Very large	—	—	—
1:10	Large	Small	—	—
1:4	Medium	Small	Small	—
Chlorthalidone in excess[a]	—	Very small	Small	Large

[a] A saturated solution of chlorthalidone in 0.2 *M* trimethylanilinium hydroxide in methanol.

methylating agent was 1:20, one peak at the retention time of 15.3 min (*i.e.*, peak A) was observed. Further increases of the molar ratio did not cause any change. However, when the molar ratio was reduced stepwise, two or three additional peaks with tailing at higher retention times (*i.e.*, peaks B, C, and D) were observed. In the absence of the methylating agent, no derivative peak was observed.

The shorter retention time of peak A indicates that peak A has a higher degree of methylation than peaks B, C, and D. Furthermore, the absence of tailing in peak A as compared to the progressive severity of tailing in peaks B, C, and D suggests the total conversion of the active hydrogen atoms to methyl groups. Therefore, these results indicate that the derivative is tetramethylated chlorthalidone.

GLC–Mass Spectrometry—Further structure elucidation of the postulated tetramethylated chlorthalidone was studied by GLC–mass spectrometry[10]. The optimized derivatization conditions and GLC conditions described were employed. The single derivative peak obtained was examined by electron-ionization and chemical-ionization mass spectrometry.

The molecular weight of tetramethylated chlorthalidone ($C_{18}H_{19}ClN_2O_4S$) is 394. With electron ionization, the expected molecular ion, M^+, was not observed at *m/e* 394, probably due to its low intensity. However, when chemical ionization with isobutane as the reactant gas was employed, an intense quasimolecular ion at *m/e* 395 and the corresponding chlorine isotope ion at 397 were observed. The intensity of these two peaks bore a ratio of 3:1, in accordance with the characteristic abundance of ^{35}Cl to ^{37}Cl.

The electron-ionization spectrum also showed strong fragment ions at *m/e* 363 and 365 with an intensity ratio of 3:1. These ions were probably formed by the loss of a methoxy radical. Furthermore, a similar doublet of the same intensity ratio at *m/e* 287 and 289 was observed. These peaks were probably due to the loss of a dimethylsulfonamide group. Based on these studies, the structure of the derivative formed by on-column methylation was assigned as 2-chloro-5-(2-methyl-1-methoxy-3-oxo-isoindolinyl)benzene-*N,N*-dimethylsulfonamide.

Standard Curves—With the temperature program mode as specified under *Apparatus*, the retention time and temperature of the *n*-docosane peak were 5.3 min and 211°; for the tetramethylated chlorthalidone peak, they were 15.3 min and 251°. The relative retention time of the tetramethylated chlorthalidone peak with respect to the *n*-docosane peak was, therefore, 2.9. Extracts of control urine samples from eight human subjects gave no interference with either the tetramethylated chlorthalidone or *n*-docosane peak.

The composite standard curves for the extracted and unextracted chlorthalidone for the range of 0.5–5 μg/5 ml of urine were obtained from two experiments. The values for chlorthalidone-spiked urine after extraction were compared with a similar series of unextracted reference standard solutions of chlorthalidone. The standard curves of the relative peak areas *versus* concentrations in micrograms per 5 ml of urine were determined by regression analysis. The equation for the extracted chlorthalidone was ($n_1 = 23$, $r_1 = 0.9740$) $y_1 = 0.0521 + 0.2040x_1$; that for the reference standard of chlorthalidone was ($n_2 = 9$, $r_2 = 0.9869$) $y_2 = 0.0271 + 0.2040x_2$. The standard curves for both the extracted and unextracted chlorthalidone were linear, as reflected by the values of the correlation coefficient.

Internal Standard—An ideal internal standard for GLC has similar chromatographic properties to the compound under investigation and can be quantitatively extracted under identical conditions from biological samples. Such an internal standard can be added to biological samples at the initial step of the procedure and is then subjected to the same extraction, derivatization, and chromatographic conditions as the compound of interest.

Initial testing for a suitable internal standard was carried out with chlorthalidone analogs. Among these, II and III showed both desirable retention times with reference to chlorthalidone and linear relative area–concentration relationships. However, these compounds were not quantitatively coextracted with chlorthalidone under the specified conditions. For this reason, *n*-docosane was used as an internal standard for the initial study.

Subsequent studies revealed that IV was a suitable internal standard and satisfied the aforementioned criteria. To establish the recovery after solvent extraction and the linearity of the standard curves using IV as an internal standard, a series of control human urine samples was spiked with different concentrations of IV and chlorthalidone. The concentration ratio between IV and chlorthalidone was 1.5. Extraction and sample analysis were performed as described under *Procedure*. For comparison, a similar series of unextracted reference standard solutions of chlorthalidone and IV also was determined.

The GLC analysis was performed on a similar model gas chromatograph[9] with a glass column (1.8 m × 0.63 cm i.d.) of 3% SP-2100 on 100–120-mesh Supelcoport[11]. At a column temperature of 260°, the retention times for the methylated chlorthalidone and IV were 4.8 and 6.3 min, respectively (Fig. 1). With the temperature programmed at 5°/min from 190 to 260°, the baseline disturbance was minimized and, therefore, was more suitable for the determination of low concentration samples.

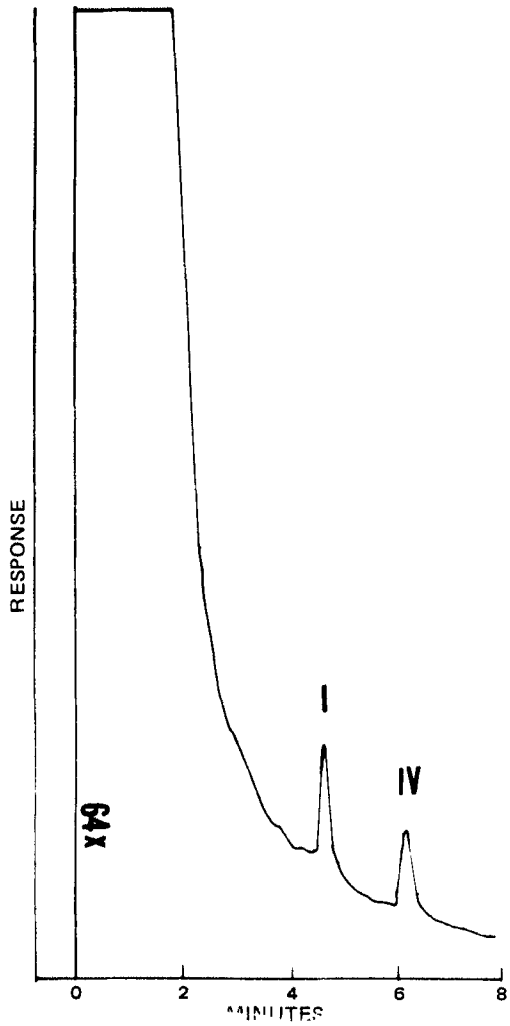

Figure 1—*Gas chromatogram showing the separation of chlorthalidone (I) and IV after on-column methylation. Compounds I and IV were coextracted after a control human urine sample was spiked with the compounds.*

[10] DuPont 492 gas chromatograph–mass spectrometer interfaced to an A.E.I. data processing system.

[11] Supelco Inc., Bellefonte, Pa.

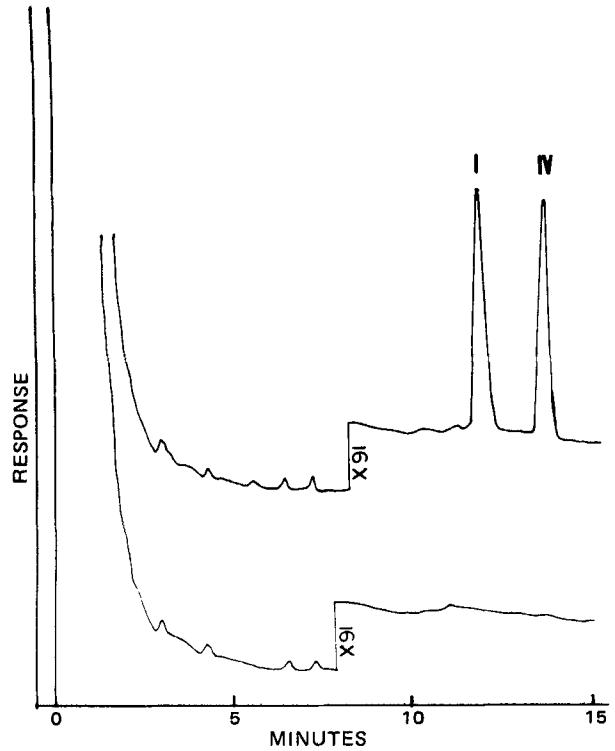

Figure 2—*Gas chromatogram from an extract of a control human urine sample spiked with I and IV. The peaks represent 67 ng of I and 100 ng of IV (upper tracing). The chromatogram of the corresponding blank urine extract is shown in the lower tracing.*

The retention times of the methylated chlorthalidone and IV were 12 and 13.8 min, respectively. The upper tracing in Fig. 2 was obtained from an extract of a control human urine sample spiked with the drug and the internal standard. The lower tracing represents the extract of the same urine sample without spiking and shows no interfering peaks with either chlorthalidone or IV.

With the temperature program mode, the regression equation for the unextracted samples ($n = 16, r = 0.9922$) was $y_3 = 0.1245 + 1.3440x_3$; that for the unextracted samples ($n = 16, r = 0.9927$) was $y_4 = 0.0993 + 1.2931x_4$. These results demonstrated good agreement and linearity of the standard curves for the extracted and unextracted chlorthalidone when IV was used as an internal standard.

The recovery of chlorthalidone from urine by solvent extraction is shown in Table II. As compared to a similar series of unextracted reference standards of chlorthalidone samples, the average recovery was 96% for the range of 0.6–5.8 μg/5 ml of urine. Therefore, these results showed that IV was coextracted with chlorthalidone under the same conditions and demonstrated similar reactivity in the derivatization and chromatographic conditions. For these reasons, IV can be added to the urine samples as an internal standard at the initial step of the procedure and is recommended for use in future studies.

Determination of Chlorthalidone in Dog Urine Samples—The suitability of this method for the determination of urinary chlorthalidone was tested with urine from a male 18-kg mongrel dog. A 200-mg oral dose of chlorthalidone dissolved in 2 ml of polyethylene glycol 200 was given to the dog, and the urine was collected in a metabolic cage. The results (Table III) indicated rather rapid chlorthalidone excretion; 62% of the administered dose was found in the urine after 30 hr.

Conclusions—The present procedure is simple to use as compared to the extractive alkylation employed by Ervik and Gustavii (4). Furthermore, the feasibility of using an electron-capture detector in conjunction with on-column methylation was demonstrated by VandenHeuvel *et al.* (6) in the determination of hydrochlorothiazide. Since the same derivative of chlorthalidone was formed in both the extractive alkylation and the on-column methylation, the use of an electron-capture detector would increase the sensitivity limit of the present method.

Table II—Coextraction of Chlorthalidone and IV (Internal Standard) from Urine as Compared to the Unextracted Reference Standards

Chlorthalidone Concentration, μg/5 ml of Urine	Corrected Relative Area[a]		Recovery, %
	Unextracted	Extracted	
5.82	7.693 ± 0.002	7.542 ± 0.147	98
4.99	7.010 ± 0.383	6.432 ± 0.397	92
4.16	5.489 ± 0.017	5.529 ± 0.390	101
3.33	4.959 ± 0.195	4.560 ± 0.204	92
2.50	3.576 ± 0.331	3.453 ± 0.286	97
1.66	2.501 ± 0.158	2.406 ± 0.006	96
0.83	1.226 ± 0.014	1.192 ± 0.030	97
0.62	0.660 ± 0.009	0.614 ± 0.045	93

[a] Corrected relative area = (peak area of chlorthalidone/peak area of IV) × μg of IV. Each value represents the mean of duplicate determinations.

Table III—Urinary Excretion of Chlorthalidone from a Dog[a]

Sample, hr	Chlorthalidone Excretion, mg	Cumulative Excretion, % of Dose	Excretion Rate, mg/hr
4	48	24	12
6	31	40	15.5
23.5	37	58	2.1
30	7	62	1.1

[a] Oral dose: 200 mg of chlorthalidone dissolved in 2 ml of polyethylene glycol 200.

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