GLC Analysis of Hydrochlorothiazide in Blood and Plasma

W. J. A. VANDENHEUVEL ˣ, V. F. GRUBER, R. W. WALKER, and F. J. WOLF

Abstract □ GLC with electron-capture detection was successfully applied to the analysis of hydrochlorothiazide in human blood and plasma with a sensitivity (0.05 μg/ml) suitable for use with persons on therapeutic dosage levels. On-column methylation with trimethylanilinium hydroxide in methanol was employed to convert hydrochlorothiazide and its bromo analog, which served as the internal standard, to their tetramethyl derivatives.

Keyphrases □ Hydrochlorothiazide—GLC analysis in human blood and plasma □ GLC, electron-capture detection—analysis, hydrochlorothiazide in human blood and plasma

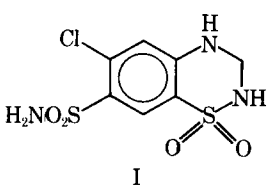
Hydrochlorothiazide[1] (I), 6-chloro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide, is a potent saluretic agent widely used in the treatment of hypertension, congestive heart failure, and other edematous conditions. Although a colorimetric method permits the estimation of urinary levels of this drug (1), it is not sufficiently sensitive for analysis of blood and plasma samples from patients receiving therapeutic doses.

GLC, especially with electron-capture detection, has proven to be a useful method for the determination of submicrogram quantities of biologically active compounds in small volumes of body fluids. Direct GLC analysis of polar compounds at such levels is usually not possible, and derivatization techniques are normally employed to convert these compounds to less polar derivatives with improved chromatographic properties. On-column methylation with trimethylanilinium hydroxide in methanol has been found to effect this conversion by transforming hydrochlorothiazide to its tetramethyl derivative.

This paper describes the successful application of GLC combined with electron-capture detection to the analysis of hydrochlorothiazide in human blood and plasma with a sensitivity (~0.05 μg/ml) suitable for use with persons on medication at normal dosage levels.

EXPERIMENTAL

GLC was carried out with an instrument[2] equipped with an automatic sampler[3] (fitted with a 10-μl syringe[4]) and an electron-capture detector (8 mCi ^{63}Ni, 50-μsec pulse width, 310°). Commercially available, acid-washed support[5] was sieved (80–100 mesh) and then acid washed and silanized (2). This material was coated, using the filtration coating technique (2), with a 1% solution [toluene–chloroform (1:1)] of a mixture of OV-1 and polycyclohexanedimethanol adipate (6:1).



I

The two-component stationary phase approach (3) was employed because the inclusion of a small proportion of polyester resulted in improved chromatographic behavior[6]. Because of the difference in bleed rates between the polyester and the less volatile OV-1 siloxane polymer, the column temperature was reduced to <190° when not in use, resulting in a useful column lifetime of approximately 150 hr. Column conditions were: 1.2-m (4-ft) × 2.5-mm i.d. glass U-tube; 235°; argon–methane (95:5) carrier gas, 60 ml/min; and injector temperature, 280°. The retention time for tetramethyl-I was ~15 min.

On-column conversion of I and the internal standard, 6-bromo-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide (II), to the tetramethyl derivatives at the nanogram level was effected by dissolution and injection in a solution of trimethylanilinium hydroxide[7] in methanol (0.005 *M*). Preparative scale methylation of these two benzothiadiazines was carried out independently by dissolving 1 mg of the compound in 0.2 ml of 0.5 *M* trimethylanilinium hydroxide, removing the methanol *in vacuo*, and heating the residue in a sealed tube under nitrogen at 250° for 30 min.

The total reaction mixture was partitioned between ethyl acetate and water, and the crude reaction product was obtained by evaporation of the ethyl acetate solution. Purification was achieved by preparative scale TLC (silica gel G) with benzene–ethyl acetate (90:10) (tetramethyl-I, R_f 0.50) or benzene–ethyl acetate (70:30) (tetramethyl-II, R_f 0.46) and elution of the appropriate zone with ethyl acetate. Purity and identity were established by GLC–mass spectrometry.

The detailed procedure devised for isolation of the drug (and internal standard) in a form suitable for GLC analysis is presented here. The main steps are: (*a*) addition of the internal standard, (*b*) removal of extraneous substances with benzene extraction, (*c*) extraction with ethyl acetate, (*d*) back-extraction into ammonium hydroxide, (*e*) adjustment to pH 3.7 and extraction with ethyl acetate, (*f*) evaporation of the extract to dryness, (*g*) dissolution of the residue in trimethylanilinium hydroxide in methanol, and (*h*) GLC.

One-half milliliter of blood (heparinized) or plasma is placed in a 1.5-ml plastic centrifuge tube[8] to which previously has been added 0.15, 0.20, or 0.30 μg of the internal standard (II) in methanol (taken to dryness with nitrogen). The sample is thoroughly agitated to ensure dissolution of the internal standard. The blood or plasma is extracted with 0.5 ml of reagent grade benzene (a process blank should be run to ensure that none of the solvents or reagents cause interferences at the retention times of the drug and internal standard), the mixture is centrifuged[9], and the benzene layer is removed (disposable pipet) and discarded; this step is repeated. Then the sample is extracted with 0.5 ml of reagent grade ethyl acetate, the mixture is centrifuged, and the ethyl acetate layer is isolated (disposable pipet); this step is repeated.

The ethyl acetate extracts are combined in a second 1.5-ml plas-

[1] HydroDIURIL, Merck & Co.
[2] Hewlett-Packard model 7610A.
[3] Hewlett-Packard model 7671A.
[4] Hamilton 701N.
[5] Gas Chrom P, Applied Science Laboratories.
[6] Following submission of this manuscript, it was found that 3% OV-61 packing (Gas Chrom Q, 100–120 mesh, Applied Science Laboratories) also serves as a useful stationary phase; Dr. George O. Breault, personal communication.
[7] Fluka.
[8] Eppendorf Reaktionsgefässe microtube 3810.
[9] Brinkmann Eppendorf centrifuge 3200.

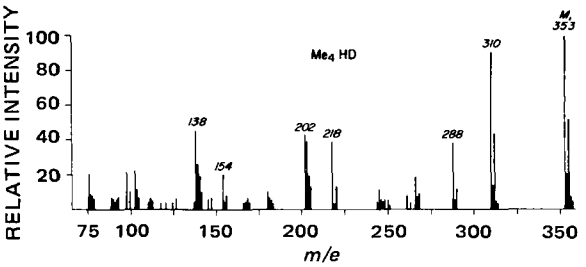


Figure 1—*Mass spectrum of tetramethyl-I as determined by combined GLC–mass spectrometry. Spectrometer conditions were: LKB model 9000; electron energy, 70 ev; source temperature, 270°; accelerating potential, 3.5 kv; and trap current, 60 μamp.*

tic centrifuge tube, and 0.5 ml of 0.1 *N* ammonium hydroxide is added. After thorough and immediate mixing and centrifugation, the ethyl acetate layer is discarded. The aqueous phase is taken to pH ~3.7 with 0.05 ml of acetic acid and extracted with 0.5 ml of ethyl acetate. Following centrifugation, the ethyl acetate layer is isolated; ethyl acetate extraction of the aqueous phase and isolation of the organic phase are repeated.

The ethyl acetate extracts are combined in a 5-ml glass centrifuge tube and taken to dryness with nitrogen (water bath, 45°). The residue is dissolved in 0.05 ml of 0.005 *M* trimethylanilinium hydroxide in methanol and transferred, using a disposable pipet, to a tapered 0.2-ml sampling vessel[10] for automated injection, GLC analysis (~1.8 μl of the final solution is injected).

A working curve for quantitation is obtained as follows. One-half-milliliter portions of control blood or plasma (preferably from the patient prior to medication) are added to 1.5-ml plastic centrifuge tubes containing 0.15, 0.20, or 0.30 μg of internal standard (added as methanol solution and taken to dryness) and sufficient I to result in levels of 0, 0.05, 0.20, 0.40, and 0.60 μg of drug/ml of blood or plasma. These samples are then carried through the isolation procedure and analysis, and the resulting peak height ratios (drug–internal standard) are plotted *versus* micrograms per milliliter of added I. The peak height ratio resulting from analysis of blood or plasma from a patient is then compared to a working curve (obtained as described) to give the concentration of drug in micrograms per milliliter.

RESULTS AND DISCUSSION

Preliminary experiments indicated, not unexpectedly, that I itself is not detected by GLC using column conditions suitable for compounds of similar molecular weight but possessing less polarity. Since derivatization techniques are often employed to allow or improve GLC of polar compounds of moderate molecular weight, this approach was investigated. Trimethylsilylation was found not to be fruitful, nor was methylation with diazomethane.

MacGee (4) reported that a number of polar drugs can be successfully analyzed by GLC by employing an on-column methylation, using a solution of tetramethylammonium hydroxide in methanol as both solvent and reagent. Similarly, Brochmann-Hanssen and Oke (5) described an on-column methylation method for the GLC of polar compounds, using trimethylanilinium hydroxide in methanol. VandenHeuvel *et al.* (6) reported the use of this reagent in a heavy isotope tracer study involving GLC–mass spectrometry. An on-column methylation approach for the determination of the drug bumetanide in urine also was reported (7).

Initial experiments at the microgram level using this reagent with flame-ionization detection were highly encouraging. Compound I was converted to a single product with satisfactory chromatographic properties; this product was shown to be the tetramethyl derivative (mol. wt. 353) by combined GLC–mass spectrometry (Fig. 1). The requirements of an assay for I in human blood and plasma are such that a sensitivity of at least 0.1 μg/ml is needed. Successful derivatization, chromatography, and detection of submicrogram quantities of drug are needed in a reproducible procedure.

The presence of a chlorine atom in I would be expected to lead to moderately good electron-capture sensitivity for the tetramethyl derivative of this drug, and a value of 1.75×10^3 coulombs/mole (8) was found under the described conditions. This sensitivity is sufficient to allow detection and quantification at the nanogram (injected) level; linearity of detector response is observed up to 12 ng with the instrument conditions employed. On-column conversion of I to its tetramethyl derivative is quantitative, since injection of 4 ng of drug in trimethylanilinium hydroxide in methanol results in the same electron-capture detector response as injection of an equivalent amount of authentic tetramethyl-I dissolved in methanol.

Analogous experiments with ^{14}C-labeled I and authentic tetramethyl-I-^{14}C, involving collection and counting of the carbon dioxide resulting from combustion of the appropriate GLC component, using the method of Trenner *et al.* (9), demonstrated that equivalent amounts of radioactivity were eluted from the column in both cases. Only 75% of the theoretically available radioactivity was collected from the tetramethyl-I peaks, however, indicating some loss on the column during chromatography. Experiments with mass standards and I-^{14}C indicated that the yield of drug carried through the entire isolation procedure is ~50%.

The molarity of the methanolic trimethylanilinium hydroxide solution used for on-column methylation is important (10). At reagent concentration of <0.0005 *M* and I concentrations of 1–5 ng/μl, methylation drops off sharply and no peak is observed for tetramethyl-I (or even trimethylhydrochlorothiazide). Maximal tetramethyl-I formation is observed at reagent molarities of ~0.005–0.01 *M*; at higher molarity, the size of the tetramethyl-I peak diminishes until essentially no derivative peak is observed at 0.5 *M*.

At the optimal reagent molarity, the flash heater temperature of the instrument used in this study can be varied from 240 to 320°

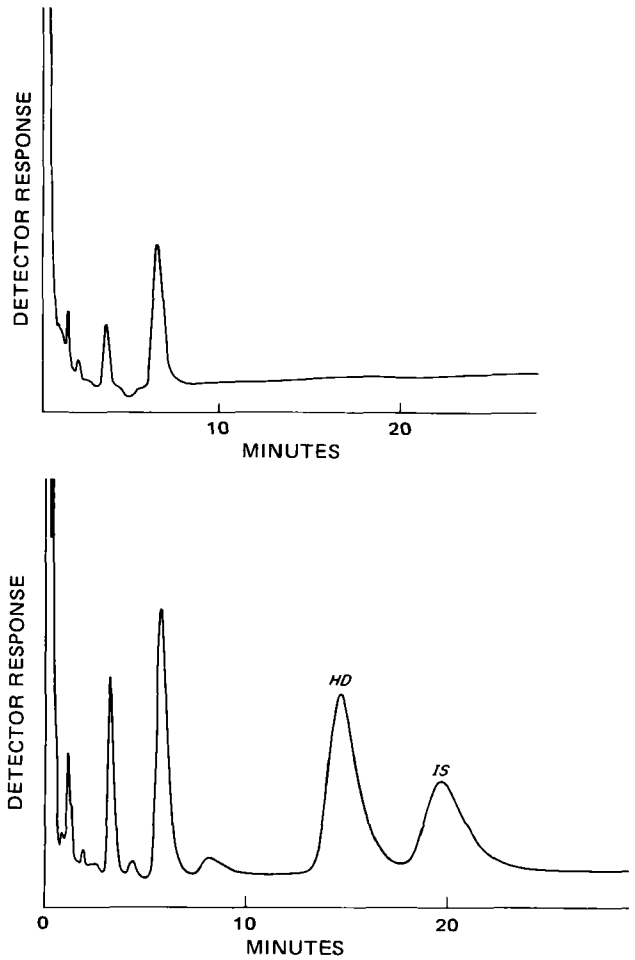


Figure 2—*Gas chromatograms resulting from analysis of control blood (top) and of control blood spiked with I and the internal standard (0.40 μg/1.0 ml each) (bottom). Isolation and chromatographic conditions are as given in the* Experimental *section.*

[10] Hewlett-Packard glass vials, 4330-0540.

Table I—Hydrochlorothiazide Levels in Blood and Plasma

Subject	Dose, mg	Hours Postdose	Blood Level, μg/ml	Plasma Level, μg/ml
W	50	3	0.62	—
		24	0.11	—
W	25/50	2	0.22/0.51	0.10/0.31
W[a]	50	1.5	0.36	—
		4	0.64	—
		6	0.44	—
		24	0.09	—
K	50/100	1.5	—	0.23/0.23
		2	—	0.35/0.36
		3	—	0.28/0.43
		4	—	0.24/0.29
L	50/100	1.5	—	0.05/0.15
		2	—	0.11/0.34
		3	—	0.29/0.33
		4	—	0.18/0.22
G	100	1.5	—	0.71
		2	—	0.51
		3	—	0.35
		4	—	0.35

[a] These values were obtained without use of the internal standard.

with little effect upon the size of the tetramethyl-I peak. The temperature normally employed, 280°, is similar to that (270–275°) reported for on-column methylation of uric acid with trimethylanilinium hydroxide in methanol (11).

Automated injection is of obvious value in GLC assays. The samples are usually sealed in sampling vials, but occasionally a vial may not be sealed tightly enough to prevent the evaporation of a relatively volatile solvent such as methanol at the temperature (40–45°) of the automatic injector and an increase in sample concentration results. This should not be a problem when an internal standard is employed for the assay; but if the concentration of reagent increases sufficiently, this effect of molarity may be encountered. It is desirable to keep the length of time I is exposed to the basic reagent to a minimum.

The bromo analog of I, II, was chosen as an internal standard. The bromo compound is converted upon injection in methanolic trimethylanilinium hydroxide to its tetramethyl derivative (mol. wt. 397). The retention time of this compound is sufficiently greater than that of the tetramethylated drug to allow its use as an internal standard, and no interfering peaks (for drug or internal standard) are observed when control blood or plasma is carried through the isolation procedure (Fig. 2, top). The chromatogram resulting from analysis of control blood spiked with I and II is illustrated in Fig. 2 (bottom).

Linear relationships are observed when mixtures of these two compounds, containing constant amounts of the internal standard and varying amounts of I, are spiked into control blood or plasma and carried through the assay and the resulting peak height ratios are plotted against micrograms of spiked I per milliliter blood or plasma. Such plots are established each day and are used as working curves in the quantification of blood and plasma levels (see *Experimental*).

A sensitivity limit of ~0.05 μg/ml of blood and plasma is found using the isolation procedure and instrument conditions described in the *Experimental* section. Satisfactory replica of the entire assay results and precision of the GLC analysis were obtained. For example, quadruplicate assay of a spiked plasma sample gave a value of 0.66 ± 0.02, and quintuplicate GLC analysis of one of these isolates gave a value of 0.67 ± 0.01 μg/ml.

The chromatograms resulting from the assay of blood samples from a patient (W) receiving hydrochlorothiazide therapy, but who had ceased medication 4 days prior to resumption, are presented in Fig. 3. Blood drawn immediately after resumption of medication (single dose, 50 mg) gave an insignificant peak at the retention time of tetramethyl-I (Fig. 3, top; referred to as 0 hr but actually 96 hr postdose). The tetramethyl-I peak in the 3-hr postdose chromatogram is greatly enhanced (Fig. 3, middle), whereas this peak is markedly reduced in intensity with the 24-hr sample (Fig. 3, bottom). The concentrations calculated for these two samples are 0.62 and 0.11 μg/ml, respectively. The corresponding 3- and 24-hr urinary concentrations of I, as determined with the GLC method

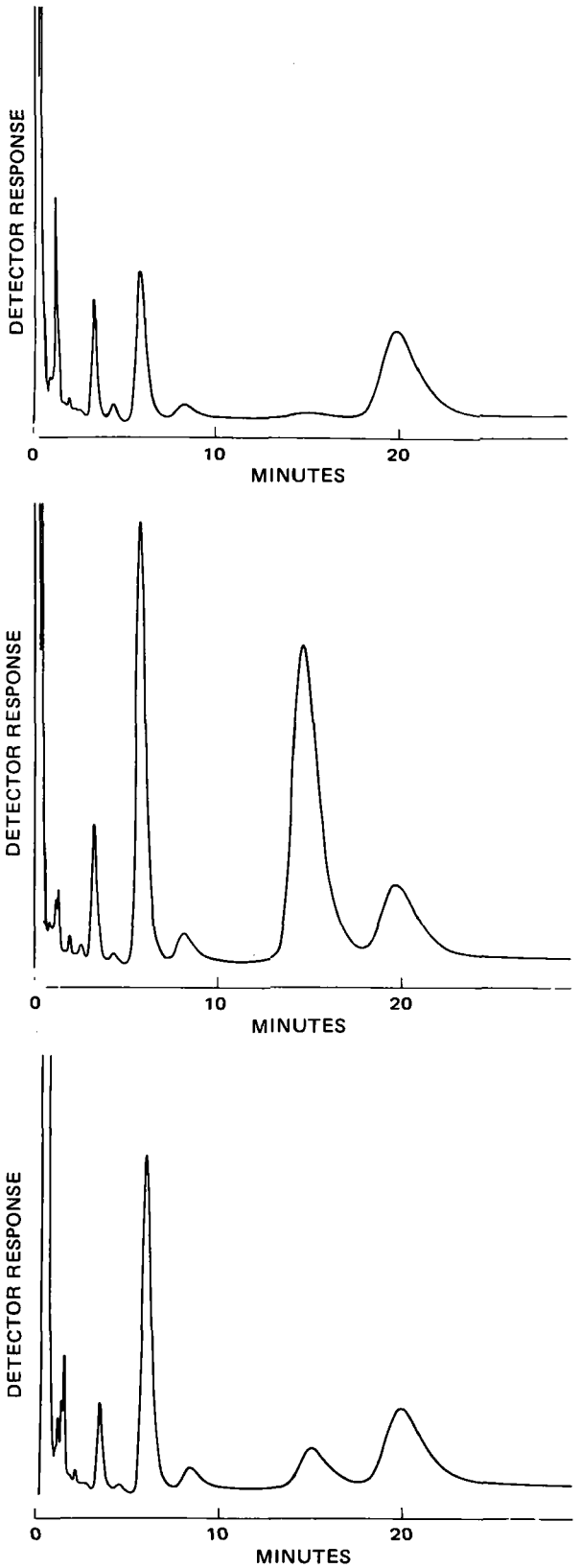


Figure 3—*Gas chromatograms resulting from analysis of blood from Subject W (0 hr but actually 96 hr postdose) (top), blood from Subject W 3 hr postdose (50 mg I) (middle), and blood from Subject W 24 hr postdose (bottom).*

(simply substituting urine for blood), were 19.8 and 6.4 μg/ml, respectively.

The level of drug in the blood of Subject W 2 hr postdose (50

mg) on a single day during an extended medication period was 0.51 μg/ml, in line with earlier findings. The corresponding value during a period in which the dosage was only 25 mg/day was 0.22 μg/ml. Plasma levels corresponding to these two values were 0.31 and 0.10 μg/ml, respectively. These values are presented in Table I, along with plasma levels for three individuals (Subjects G, K, and L) at dosage levels of 50 and 100 mg. Blood levels for Subject W (50 mg) at 1.5, 4, 6, and 24 hr, determined prior to introduction of the internal standard approach, are also presented in the table.

This paper presents a method for the determination of hydrochlorothiazide in small volumes of blood and plasma from persons receiving therapeutic levels of this drug. Drug levels are reported for a limited number of patients, demonstrating the adequacy of the method for measuring the drug in blood and plasma at clinical doses. The method described in this paper should provide the means for carrying out pharmacokinetic and bioavailability studies.

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ACKNOWLEDGMENTS AND ADDRESSES

Received October 25, 1974, from *Merck Sharp & Dohme Research Laboratories, Rahway, NJ 07065*

Accepted for publication December 19, 1974.

The authors thank Dr. John E. Baer for his continuing interest in this work.

[x] To whom inquiries should be directed.

Ocular Evaluation of Polyvinyl Alcohol Vehicle in Rabbits

THOMAS F. PATTON and JOSEPH R. ROBINSON [x]

Abstract □ The flow properties and viscosity of the vehicle into which drugs are incorporated can be determining factors in the bioavailability of topically applied ophthalmic drugs. It is shown, in rabbits, that when polyvinyl alcohol and methylcellulose are compared on a viscosity basis, there is essentially no difference in the two vehicles with regard to their influence on ocular drug bioavailability. Moreover, the rate of drainage loss for polyvinyl alcohol solutions, as determined by the radioactive technetium technique, compares favorably to methylcellulose solutions of similar viscosity. The relationship between viscosity and contact time or drainage loss of a drug is not a direct one, but an optimum viscosity range exists for polyvinyl alcohol solutions. This optimum range of 12–15 cps in rabbits is identical to that found for methylcellulose and differs considerably from the commonly employed viscosity in commercial preparations. Based on the methylcellulose–polyvinyl alcohol comparison, it appears that vehicles exhibiting or approximating Newtonian flow properties show comparable effects as ophthalmic vehicles. Finally, a discussion of non-Newtonian vehicles and their expected behavior in the eye is presented.

Keyphrases □ Polyvinyl alcohol—effect of vehicle viscosity on ocular drug bioavailability in rabbits, compared to methylcellulose □ Ocular bioavailability—effect of vehicle viscosity, polyvinyl alcohol and methylcellulose solutions, rabbits □ Ophthalmic vehicles—evaluation of polyvinyl alcohol, compared to methylcellulose, optimum viscosity range, rabbits

Great time and effort have been expended on determining the optimum liquid vehicle in which to incorporate drugs for instillation into the eye. Most efforts have been based on the premise that a highly viscous solution would prolong contact time of the drug with eye tissues, thus enabling a greater amount of drug to be absorbed into the desired area.

Recently, Adler *et al.* (1) made the important observation that high viscosity solutions do not greatly increase corneal contact time in humans and have only a small effect on bioavailability. An extensive study on methylcellulose solutions (2), however, has shown that there is considerable prolongation of contact time in rabbits and a two- to threefold improvement in bioavailability. This inconsistency apparently is due to differences between humans and rabbits with respect to the effect of medium viscosity solutions in the eye.

The present study explores the influence on ocular drug bioavailability of various polyvinyl alcohol solutions. Methylcellulose and polyvinyl alcohol are both used extensively as vehicles for ophthalmic drugs and the literature is confusing as to which polymer is best for ocular use. The aim of this report is to resolve that confusion as well as to provide a rational basis for predicting ophthalmic vehicle viscosity effects in general.

EXPERIMENTAL

Materials—Water was double distilled from alkaline permanganate in an all-glass distillation apparatus. Technetium solutions