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Spectrofluorometric Determination of Hydroflumethiazide in Plasma and Urine

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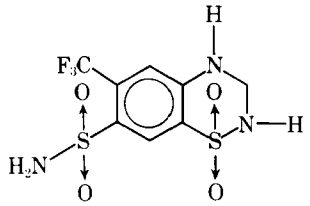
Abstract □ A rapid, accurate, sensitive, and reproducible assay for hydroflumethiazide in plasma and urine was developed after studies of its UV and fluorescence spectral properties and partitioning behavior. The assay is based on initial extraction from acidified plasma or urine into ether, back-extraction into basic solution followed by acidification to about pH 1, and measurement of the fluorescence derived from the unionized molecule. Analysis of variance indicated no significant differences in assays performed on the same day. The mean recovery was 98.8 ± 7.4% for plasma over a concentration range of 0.2–2.0 μg/ml. The method is convenient for routine clinical use and has sufficient sensitivity to quantify hydroflumethiazide levels after administration of therapeutic doses.

Keyphrases □ Hydroflumethiazide—spectrofluorometric analysis, plasma and urine □ Spectrofluorometry—analysis, hydroflumethiazide, in plasma and urine □ Diuretics—hydroflumethiazide, spectrofluorometric analysis, plasma and urine



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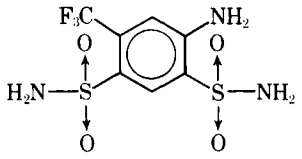

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Attempts to describe the comparative pharmacokinetics of diuretic agents in various species of animals and in humans have comprised a major research effort in these laboratories. The paucity of pharmacokinetic information on this class of compounds is due in part to the lack of sensitive analytical methods for their determination in biological fluids. Generally, diuretics are given at relatively low doses and have short half-lives; thus, the blood levels obtained are relatively low a short time after administration.

A proposed study of the pharmacokinetics of the diuretic hydroflumethiazide (I) prompted interest in analytical methodology for this compound. A fluorometric TLC procedure was reported for the quantitative analysis of hydroflumethiazide in urine and plasma (1). This method involved extraction and TLC followed by fluorometric determination of hydroflumethiazide utilizing a scanning spectrodensitometer; hydroflumethiazide fluoresced below 400 nm, but no specific emission or excitation maxima were given. Other quantitative or qualitative procedures for benzothiadiazine diuretics in urine or dosage forms principally involved hydrolysis and colorimetric determination of the diazotized amino degradation products (2, 3).

Since hydroflumethiazide fluoresces strongly, it was proposed that a direct fluorometric determination in biological fluids might prove possible. This technique would decrease assay time and markedly simplify routine clinical determination by eliminating the necessity for TLC and scanning spectrodensitometry. Based on the fluorescence and partitioning properties of hydroflumethiazide, the present work reports the development of a sensitive, accurate, precise, and convenient procedure for its determination in both plasma and urine.

EXPERIMENTAL

Equipment—A scanning spectrofluorometer[1] and a recording UV–visible spectrophotometer[2] were utilized for spectral measurements.

Materials—Hydroflumethiazide[3] powder was used in the analytical procedures without further purification. All chemicals and solvents were of analytical reagent grade. Standard solutions were prepared by dissolving hydroflumethiazide in enough methanol to solubilize the drug and then diluting to the desired volume with pH 7.0 phosphate buffer.

UV Spectral Measurements—The UV spectra of 2.53×10^{-5} *M* hydroflumethiazide solutions were recorded at various pH values in the 1–12 range. The following buffer solutions (4) were used: potassium chloride–hydrochloric acid (pH 1.0–2.0), glycine buffers (pH 2.4–3.4), acetate buffers (pH 4.0–5.4), phosphate buffers (pH 6.0–7.2), borate buffers (pH 8.0–9.0), carbonate buffers (pH 9.4–10.4), and potassium chloride–sodium hydroxide (pH 11.0–12.0).

Fluorescence Spectral Measurements—The fluorescence spectra of 2.53×10^{-6} *M* hydroflumethiazide solutions were recorded

[1] Turner model 430, G. K. Turner Associates, Palo Alto, Calif.
[2] Coleman model 124, Perkin-Elmer Corp., Maywood, Ill.
[3] Bristol Laboratories, Syracuse, N.Y.

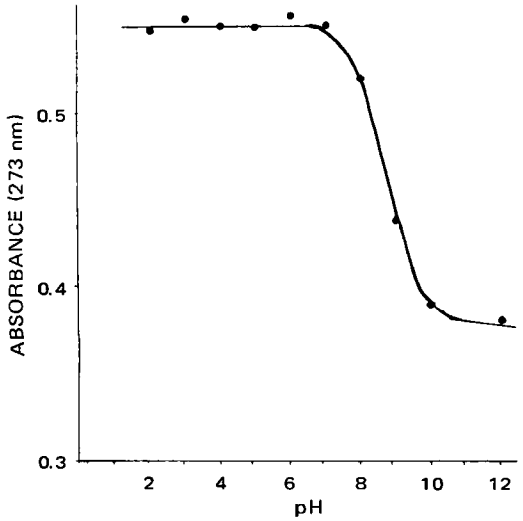

Figure 1—*Absorbance (273 nm) of hydroflumethiazide (2.53×10^{-5} M) as a function of pH. The curve is generated from the equation $pKa = pH - log\ [(A - A_u)/(A_I - A)]$, where A_I and A_u are the asymptotic absorbances in basic and acid solutions, respectively. The points are the experimentally measured values.*

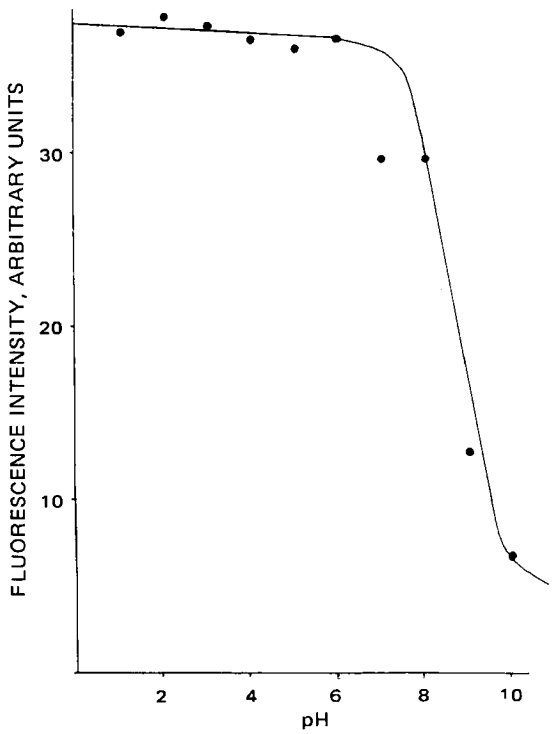

Figure 2—*Fluorescence intensity of hydroflumethiazide (2.53×10^{-6} M) as a function of pH at 333 and 393 nm.*

at various pH values in the 1–12 range using the described buffers. The fluorescence spectra in the Hammett acidity region were also obtained by preparing 2.53×10^{-6} *M* solutions in 10, 20, 30, 40, 50, 70, 80, 90, and 100% (v/v) sulfuric acid.

Partitioning Studies—Hydroflumethiazide (2.53×10^{-6} *M*) in pH 4 acetate buffer was analyzed spectrofluorometrically before and after extraction with an organic solvent. The following solvents were screened for use: ether, ethyl acetate, methylene chloride, chloroform, hexane, and heptane.

Procedure for Assay of Plasma—A stock plasma solution of hydroflumethiazide (2 μg/ml) was prepared by adding a concentrated aqueous solution of hydroflumethiazide to fresh human plasma. This stock plasma solution was further diluted with plasma to prepare hydroflumethiazide solutions containing 0.05, 0.5, 1.0, 1.5, and 2.0 μg/ml. One milliliter of pH 4 acetate buffer was added to 1 ml of diluted plasma solutions in 115 × 13-mm screw-capped culture tubes. Five-milliliter portions of ether were added, and the contents were agitated for 30 sec[4].

The samples then were centrifuged (3000 rpm) for 2 min, and 4 ml of the ether layers was transferred to a second similar screw-capped culture tube. One milliliter of 0.01 *N* sodium hydroxide ($\mu = 0.2$, adjusted with potassium chloride) was added to the ether, and the mixture was agitated for 30 sec. After centrifugation (1–2 min, 3000 rpm), the ether layer was removed and discarded. Residual ether in the aqueous phase was evaporated under a stream of nitrogen. One milliliter of 1 *N* hydrochloric acid was added and the fluorescence was measured at λ_{exc} of 333 nm and λ_{em} of 393 nm. The instrument settings were: blank, high; sensitivity, low; meter damp, −1; range, minimum sensitivity; and attenuation, ×3 or ×10.

Determination of Hydroflumethiazide in Urine—Fresh human urine was collected, and a stock urine solution containing 37.5 μg/ml of hydroflumethiazide was prepared. The stock urine solution was diluted with blank urine to prepare solutions containing 37.5, 28.1, 18.8, and 9.4 μg/ml. Then 0.1-ml aliquots of these solutions were diluted with 3 ml of distilled water. One milliliter of the diluted solutions and 1 ml of pH 4 acetate buffer were placed in a 115 × 13-mm culture tube, and 5 ml of ether was added. The same procedure as for plasma was then followed.

Hydrolysis of Hydroflumethiazide—Hydroflumethiazide solutions (10 μg/ml) were prepared in 0.01 *N* sodium hydroxide, 10% (v/v) sulfuric acid, and 1 *N* hydrochloric acid. These solutions were heated in a boiling water bath for 8 hr. The basic solution was made acidic by the addition of 1 *N* hydrochloric acid, and all three solutions were extracted twice with 10 ml of ether. The combined ether extracts from each solution were evaporated to dryness at 50° in 115 × 13-mm culture tubes. The sides of the tubes were washed with 1 ml of ether and again evaporated to dryness.

The residue in each tube was dissolved in 100 μl of methanol and subjected to TLC using a system described previously (1). The bands on the TLC plate were visualized under a short wavelength UV lamp. The band corresponding to the known degradation product, 2,4-disulfamyl-5-(trifluoromethyl)aniline (II), was scraped from the plate and shaken with two 5-ml aliquots of methanol. The mixture was centrifuged (4 min, 3000 rpm), and the supernate was transferred to a second culture tube.

After evaporation of the methanol, the residue was taken up in 1 ml of 0.01 *N* sodium hydroxide and made acidic by the addition of 2 ml of hydrochloric acid. Then the UV spectra were determined. After a 1:10 dilution with 1 *N* hydrochloric acid, the fluorescence spectrum also was determined.

Instrument Calibration—Prior to initiating a series of readings, the spectrofluorometer was adjusted to maximum sensitivity and a quinine bisulfate standard solution (0.05 μg/ml in 0.1 *N* sulfuric acid) was utilized to standardize to a meter reading of 35 at range ×10 at 333 and 393 nm. The standard was checked periodically thereafter to adjust for any drift.

Validation of Method—A beagle dog was given 15 mg of hydroflumethiazide intravenously in the same manner as described by Garceau *et al.* (1). Heparinized blood samples were collected prior to and at 5, 10, 15, 20, 30, 45, 60, and 90 min after injection. The samples were centrifuged, and the plasma was separated and assayed as already described.

RESULTS AND DISCUSSION

Hydroflumethiazide is a weak acid with reported pKa's of 8.9 and 10.5 (5). The UV spectrum of hydroflumethiazide contained two maxima. In solutions with a pH less than the first pKa, the maxima occurred at 273 ($\epsilon = 22{,}750$) and 325 ($\epsilon = 4340$) nm. When the pH of the solution was greater than the first pKa, a hypochromic and bathochromic shift of the latter maximum to 333 nm was observed. There was no change in the spectra of solutions with pH's greater than the reported second pKa. The absorbance of hydroflumethiazide at 273 nm, as a function of pH, is shown in Fig. 1. The pKa calculated from these data (pKa = 8.73) is in good agreement with the appropriate literature value (5).

Fluorometric spectra of hydroflumethiazide demonstrated an excitation maximum at 333 nm and an emission maximum at 393 nm

[4] Micro-mixer, Lab-lines Inc.

Table I—Results[a] of Repetitive Assays of Drug Spiked in Extraction Blanks and Extracted from Plasma and Urine

Concentration, μg/ml	Assay 1	2	3	4	Mean	±SD
			Drug Added to Acidic Extraction Blanks			
0.00	0.2	0.4	0.1	0.6	0.325	0.221
0.05	1.6	1.85	1.5	1.9	1.713	0.193
0.5	10.2	10.5	9.6	9.3	9.90	0.548
1.0	19.0	19.4	19.0	20.0	19.35	0.472
1.5	28.5	29.0	27.9	28.8	28.55	0.479
2.0	37.5	38.0	37.8	40.9	38.55	1.58
			Drug Extracted from Plasma			
0.00	1.1	0.9	1.5	1.0	1.13	0.263
0.05	1.6	1.4	1.9	1.7	1.65	0.208
0.50	6.8	5.4	6.2	6.1	6.13	0.574
1.00	11.5	11.5	12.5	10.5	11.5	0.816
1.5	17.6	17.5	17.5	16.8	17.35	0.369
2.0	22.0	22.0	23.0	21.0	22.0	0.816
			Drug Extracted from Urine[b]			
0.0	1.1	1.51	2.1	1.0	1.427	0.499
0.31	4.5	4.33	4.3	3.55	4.17	0.422
0.615	6.9	6.83	7.66	7.00	7.097	0.381
0.915	9.7	9.63	10.33	9.35	9.752	0.413
1.219	13.8	12.0	12.6	11.7	12.525	0.919

[a] Results are given in the arbitrary fluorescence units of the spectrofluorometer. [b] Concentration after dilution.

when determined at pH's between 1 and 12. The fluorescence intensity of hydroflumethiazide as a function of pH (333 and 393 nm) is shown in Fig. 2. The apparent pKa calculated from these data is 8.79, which is in very close agreement to the value calculated from the absorbance data and indicates that the fluorescence is a function of the ground-state pKa.

The fluorometric titration in the Hammett acidity region (333 and 393 nm) is depicted in Fig. 3 and shows that fluorescence intensity decreases with increasing sulfuric acid concentration. The apparent pKa is approximately −4.3, which probably corresponds to the protonation of the anilino nitrogen. The data from the Hammett acidity region and pH 1–12 indicate that the observed fluorescence of hydroflumethiazide in aqueous solution is due to the unionized molecule.

Of the solvents screened for extraction of hydroflumethiazide from aqueous solution, ether provided the highest recovery for a single extraction. As would be anticipated from the pKa's, the maximum extraction was obtained when the pH was less than 5.

The UV spectrum of 2,4-disulfamyl-5-(trifluoromethyl)aniline, the hydrolytic degradation product of hydroflumethiazide, showed absorbance maxima at 260 and 325 nm (pH 1.0). This compound fluoresces at the same wavelength as hydroflumethiazide and thus may potentially interfere with the spectrofluorometric determination of hydroflumethiazide. However, hydrolytic degradation is not known to occur *in vivo*, and interference due to the degradation product is not anticipated. To prevent hydrolysis during the analytical procedure, the drug should not be allowed to stand in acidic or basic solu-

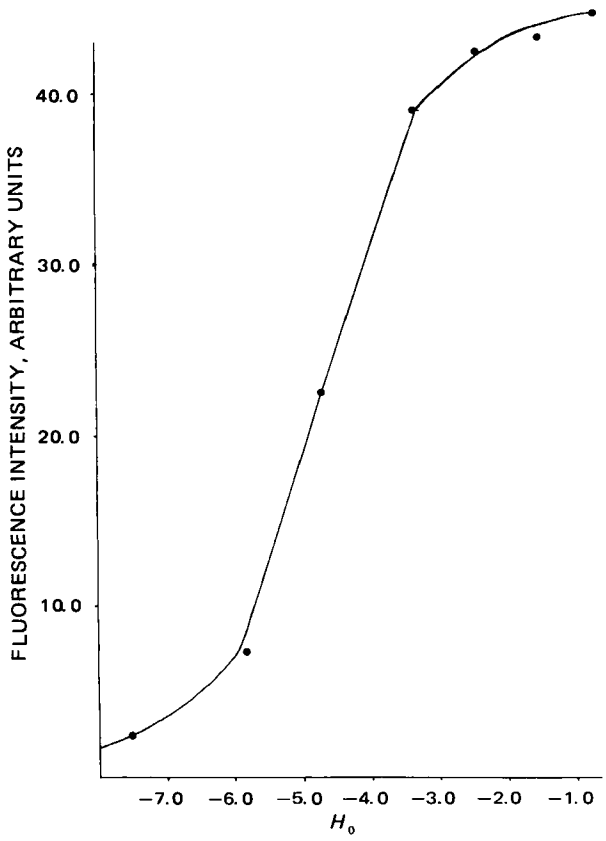

Figure 3—*Fluorescence intensity of hydroflumethiazide (2.53 × 10^{-6} M) as a function of Hammett acidity.*

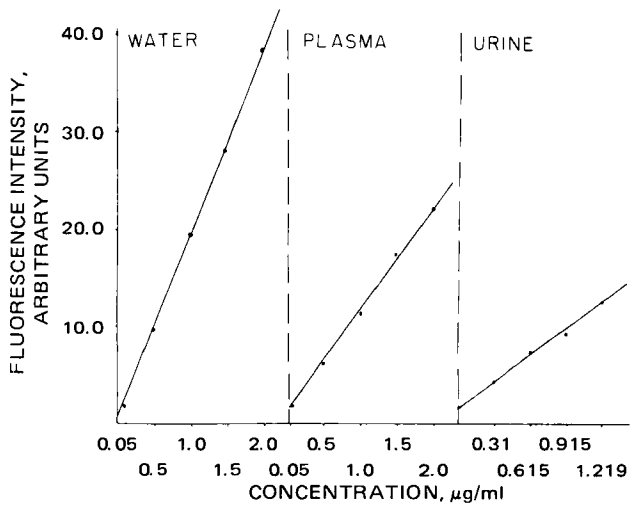

Figure 4—*Calibration curves for hydroflumethiazide added to aqueous extraction blanks and added to and extracted from plasma and urine. The lines are the least-squares linear regression fits of the data.*

Table II—Two-Way Analysis[a] of Variance for Data in Table I

Source	*df*	Sum of Squares	Mean Squares
		Extraction Blanks	
Concentration	5	4656.2422	931.24
Replication	3	2.8644	0.9548
Residual	15	7.1248	0.4749
Total	23	4666.2316	
		Plasma	
Concentration	5	1455.0633	291.0126
Replication	3	2.8283	0.9428
Residual	15	2.9066	0.1938
Total	23	1460.7983	
		Urine	
Concentration	4	308.691	77.172
Replication	3	2.241	0.747
Residual	12	2.582	0.215
Total	19	313.514	

[a] Not significant at the $p < 0.01$ level.

tions for lengthy periods and the analysis should be carried out without interruption.

Calibration curves for hydroflumethiazide extracted from plasma and urine and for hydroflumethiazide added to extraction blanks are shown in Fig. 4. Each point represents the mean of four replications for plasma, urine, and water. The data on which the curves are based are shown in Table I. The curves were fit by the method of least squares.

The slopes of the curves were 18.91 (spectrofluorometric response units per micrograms per milliliter at 333 and 396 nm at range setting of minimum sensitivity) for direct analysis of drug added to extraction blanks, 10.86 for drug extracted from plasma, and 9.13 for drug extracted from urine (after 1:31 dilution). The average fraction of the drug extracted could be determined from the ratio of the slopes of the urine and plasma curves to the slope of drug in extraction blanks. The mean percent of drug extracted was 57.4% from plasma or 48.3% from urine.

A blind analysis of hydroflumethiazide in spiked human plasma samples was performed. Recoveries ranged from 87.0 to 114.5%, and the mean was 98.8 ± 7.4% (*SD*) for 10 samples over the 0.2–2.0-μg/ml range.

The analysis of variance for the data in Table I is shown in Table II. These data demonstrate that there were no significant variations between extractions performed on the same day for urine, plasma, or drug added to extraction blanks. These results indicate the high reproducibility of the procedure.

An *in vivo* study was conducted similar to that of Garceau *et al.* (1) in which hydroflumethiazide was administered to a beagle dog by intravenous injection. A plot of plasma levels of hydroflumethiazide following a single intravenous injection of 15 mg is shown in Fig. 5. The determined plasma levels parallel those reported previously (1) and demonstrate the validity of the assay method.

The developed methodology appears to have sufficient sensitivity to determine hydroflumethiazide levels following the administration of therapeutic doses. The method is more convenient for routine clinical determinations of hydroflumethiazide than any previously reported methods with equivalent sensitivity.

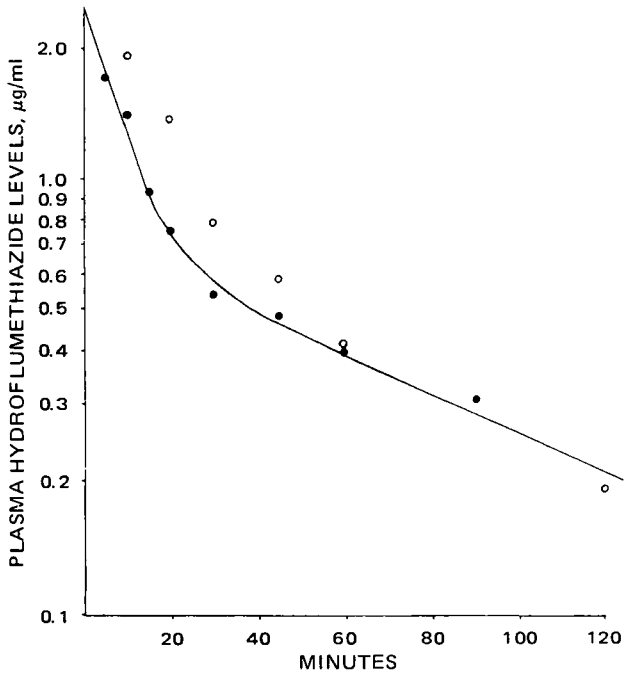

Figure 5—*Plasma levels of hydroflumethiazide following a single intravenous injection of 15 mg to a beagle dog. Key: ●, data from the present study; and ○, data from Ref. 1.*

REFERENCES

(1) Y. Garceau, I. Davis, and J. Hasegawa, *J. Pharm. Sci.*, **63**, 1793(1974).

(2) H. Sheppard, T. F. Mowles, and A. J. Plummer, *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 722(1960).

(3) V. B. Pilsbury and J. V. Jackson, *J. Pharm. Pharmacol.*, **18**, 713(1966).

(4) "Scientific Tables," 7th ed., K. Diem and C. Lentner, Eds., Geigy Pharmaceuticals, Ardsley, N.Y., 1974, pp. 280–282.

(5) "Handbook of Analytical Toxicology," I. Sunshine, Ed., Chemical Rubber Co., Cleveland, Ohio, 1969, p. 58.

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