Quantitative Fluorometric TLC Procedure for Determination of Hydroflumethiazide in Biological Fluids

Y. GARCEAU, I. DAVIS, and J. HASEGAWA ˣ

Abstract □ A rapid, sensitive, and specific fluorometric TLC procedure for the estimation of hydroflumethiazide in human biological fluids is described. The drug was removed from plasma in a single extraction step and applied to a TLC plate of silica gel. After development, the plate was scanned in a spectrodensitometer equipped to measure fluorescence below 400 nm. Quantitation was achieved by comparing the areas under the peaks obtained from the unknowns to those obtained from standards applied to the same plate. The limit of detection was 10 ng of drug/ml of plasma. A mean recovery value of 99.7% was obtained when the drug was extracted from spiked human plasma samples, while 97.5% of drug was recovered from spiked human urine samples. To test the validity of the method, a beagle dog was given an intravenous dose of hydroflumethiazide, and postadministration blood and urine samples were collected and analyzed.

Keyphrases □ Hydroflumethiazide—fluorometric TLC analysis in plasma and urine □ TLC, spectrofluorodensitometry—analysis, hydroflumethiazide in plasma and urine □ Spectrofluorodensitometry—analysis, hydroflumethiazide

Although the benzothiadiazine diuretics have been in use for more than 10 years, the scientific literature contains little information on specific assay methods for the determination of these drugs in biological fluids. Procedures have been described for the determination of chlorothiazide (1), hydrochlorothiazide (2), and bendroflumethiazide (3), but they relied on nonspecific colorimetric reactions, involving diazotization and coupling to form an azo dye, and they lacked sensitivity. Qualitative procedures using UV spectrophotometry, paper chromatography, and TLC have also been reported (4, 5).

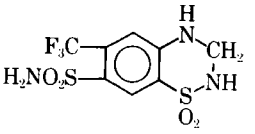
Hydroflumethiazide (I) is a potent diuretic, about 10 times as effective as chlorothiazide on a weight basis. A literature survey failed to reveal any published report on a specific assay method for hydroflumethiazide in biological fluids. This paper describes a quantitative fluorometric TLC procedure for the estimation of this drug in human plasma and urine.

EXPERIMENTAL

Materials and Chemicals—TLC plates (20 × 20 cm) of silica gel[1] were used. No activation was required. The plates were divided into 20 channels of 1-cm width with a scoring device[2]. Samples were applied to the plates with 10-, 25-, or 50-μl syringes[3].

Stock solutions of hydroflumethiazide[4] were prepared by dissolving accurately weighed amounts of drug in methanol. Working standards were made by appropriate dilutions of the stock standards. All solvents and chemicals were analytical reagent grade.

Procedure for Assay of Plasma and Urine Samples—One-milliliter samples of human plasma were spiked with aliquots of 50



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μl of standard solutions of hydroflumethiazide. The amounts of drug added were chosen to cover the concentration range of 0.05–1.00 μg/ml of plasma. To each plasma sample, 0.5 g of sodium chloride was added and the mixture was extracted for 15 min on a mechanical shaker[5] with 15 ml of a solution of ether–ethylene dichloride (1:1).

After centrifugation, the organic layer was transferred and dried for 5 min with 1.5 g of sodium sulfate. A 10-ml aliquot from the organic layer was transferred to a clean conical tube and evaporated to dryness[6] under nitrogen at 40°. The inside of the tube was washed with 1 ml of the extracting solvent mixture, and the solution was again evaporated to dryness.

The dry residue was dissolved in 100 μl of chloroform–acetone (7:3 v/v). Aliquots from this solution, 25–50 μl, were applied to prescored TLC plates, generally in duplicate. Standards of hydroflumethiazide of three different concentrations were also spotted so that a calibration line was included on each plate.

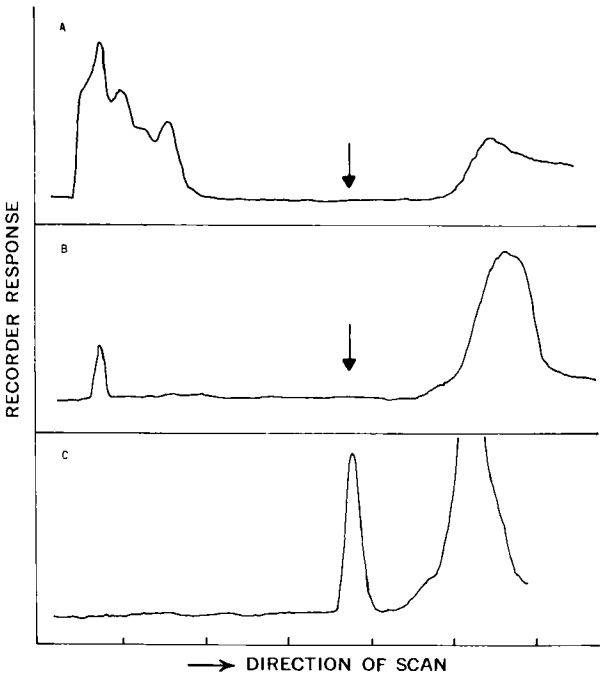

Figure 1—*Typical chromatograms. Key: A, blank human urine; B, extract of blank human plasma (R_f value of hydroflumethiazide is shown by arrow); and C, extract of a human plasma sample spiked with hydroflumethiazide at a concentration of 0.2 μg/ml. Additional peaks represent urine or plasma components.*

[1] Sil G-25-22, Brinkmann Instruments Inc.
[2] SDA 320, Schoeffel Instrument Corp.
[3] Hamilton Co.
[4] Linson Ltd., Dublin, Ireland.
[5] Eberbach, Ann Arbor, Mich.
[6] N-Evap, Organomation Assoc.

Table I—Summary of Recovery Experiments

Amounts Spiked, μg/ml	Amounts Recovered, μg/ml		
	Run 1	Run 2	Run 3
	Plasma		
0.05	0.048	0.048	0.048
0.10	0.093	0.096	0.096
0.20	0.204	0.197	0.204
0.40	0.401	0.428	0.390
0.60	0.612	0.636	0.606
0.80	0.762	0.756	0.780
1.00	1.044	1.056	1.068
Mean recovery: 99.7% *SD*: 4.4%			
	Urine		
5.0	4.7	5.0	5.0
10.0	9.5	9.5	9.5
20.0	20.0	20.5	20.0
30.0	28.0	29.0	29.5
40.0	38.5	39.0	39.5
Mean recovery: 97.5% *SD*: 2.7%			

Urine samples were analyzed directly without any extraction step. Spiked human urine samples were prepared in the concentration range of 5–40 μg/ml. Unspiked samples served as blanks. Duplicate aliquots of 10 μl were spotted directly on a TLC plate. Standards of at least three different concentrations were also spotted.

TLC—The plates were developed in a saturated tank containing 100 ml of ethyl acetate–methanol–0.1 *N* ammonium hydroxide (96:2:2). After developing 15 cm, the plates were air dried. They were then scanned in a spectrodensitometer[7] equipped with a density computer[8]. The light source was a xenon mercury 200-w lamp[9].

The instrument was operated in the fluorescence mode. The excitation wavelength was 265 nm. Emission was filtered with a UV-transmitting, visible-absorbing filter[10] having a transmission band

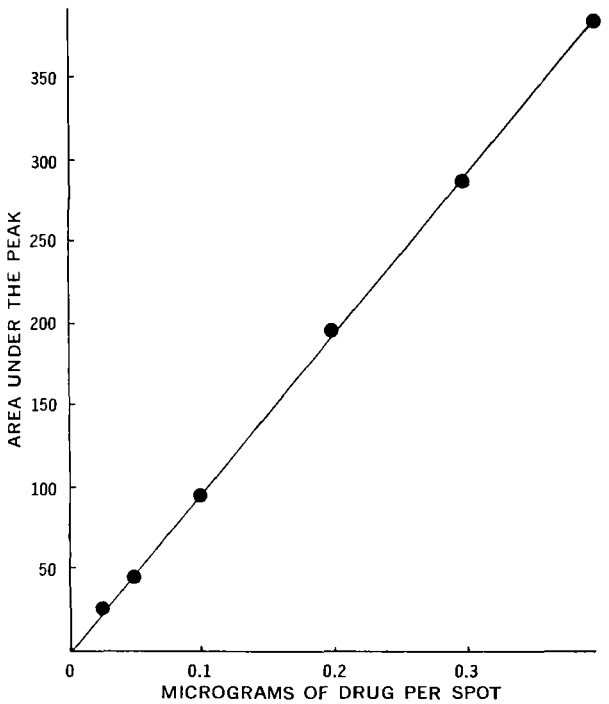

Figure 2—*Typical calibration line for hydroflumethiazide.*

[7] Model SD 3000, Schoeffel Instrument Corp.
[8] Model SDC 300, Schoeffel Instrument Corp.
[9] Hanovia Lamp Division, Camrand Precision Ind.
[10] Corning, C.S. 7.51.

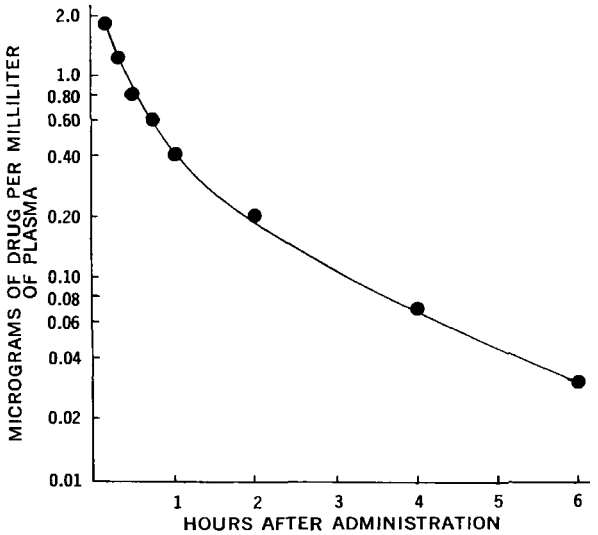

Figure 3—*Plasma levels of hydroflumethiazide following intravenous administration of a 15-mg dose to a beagle dog.*

between 290 and 420 nm, with maximum transmission at 365 nm. Gain was set at 700 on the arbitrary scale of the instrument, and the density computer was set at O.D. 0.2. Scanning and chart speeds were 10.2 cm (4 in.)/min.

***In Vivo* Experiment**—To assess the applicability of the method, a beagle dog was given 15 mg of hydroflumethiazide intravenously (3 ml of a 5-mg/ml solution in 0.02 *N* NaOH). Heparinized blood samples were collected prior to and at 10, 20, 30, and 45 min and at 1, 2, 4, and 6 hr after injection. The blood samples were centrifuged immediately, and the plasma was frozen until analyzed. Urine was collected for 24 hr after administration.

RESULTS AND DISCUSSION

Hydroflumethiazide fluoresces strongly, and its emission peak is below 400 nm on a thin layer of silica gel. Since the spectrodensitometer used was not originally equipped for fluorescence analysis below 400 nm, it was adapted to that purpose by using a UV filter transmitting between 290 and 420 nm. This filter accomplished the dual purpose of filtering off the excitation energy and passing the fluorescence emission. It also filtered off visible fluorescence, thus increasing specificity.

Under the experimental conditions described, hydroflumethiazide had an R_f value of 0.52 and was separated from a known degradation product, 2,4-disulfamyl-5-trifluoromethylaniline, R_f 0.64. Hydroflumethiazide is not known to be metabolized in humans. It was well separated from plasma or urine components (Fig. 1) and from the common diuretics chlorothiazide, R_f 0.25, and hydrochlorothiazide, R_f 0.44. The latter compounds do not fluoresce but can be detected on a TLC plate of silica gel from their UV absorption at 290 nm. The lowest detectable amount of these substances is around 50 ng.

A typical calibration line is shown in Fig. 2. The correlation coefficient was 0.999.

Recovery experiments are summarized in Table I. Recoveries from spiked human plasma samples ranged from 93 to 107%, and the mean was 99.7 ± 4.4% *SD*, n = 21. Recoveries from spiked human urine samples ranged from 94 to 103%, and the mean was 97.5 ± 2.7% *SD*, n = 15.

Plasma samples spiked with hydroflumethiazide were allowed to stand at room temperature for up to 24 hr before extraction to check for degradation and possible recovery loss. Recovery was constant for the period of time tested.

Under the conditions described, the instrument can reliably integrate an "area under the peak" of 10 units, which sets the limit of detection to 5 ng of hydroflumethiazide/spot. This sensitivity corresponds to 10 ng of drug/ml of plasma or 0.5 μg/ml of urine. Urine samples were applied directly to the TLC plates and much greater sensitivity could be obtained by including the extraction step used for plasma. However, this was considered unnecessary. The method is adequate to quantitate hydroflumethiazide in plas-

ma or urine following administration of a single 50-mg oral dose to humans[11].

An *in vivo* study was conducted to confirm the applicability of the method. A plot of plasma levels of hydroflumethiazide following a single intravenous injection to a beagle dog is shown in Fig. 3. The curve shows a typical biphasic pattern, with a half-life of 10 min for the α-phase and of 90 min for the β-phase. The amount of drug excreted in a 24-hr urine collection was 8.6 mg or 57% of the dose. The reason for incomplete excretion is not known at present.

The analytical method was developed using human plasma and urine while the pilot experiment was conducted in dogs. However, dog plasma and urine were free of interfering impurities, as were the human fluids.

The procedure described in this paper is accurate and reproducible and has a high degree of sensitivity. It is quick and easy to carry out. One analyst can assay 20–25 plasma samples in a normal working day.

[11] Unpublished data.

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Diphenylhydantoin Microdetermination in Serum and Plasma by UV Spectrophotometry

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Abstract □ A modified UV spectrophotometric procedure for determining diphenylhydantoin in 0.1–0.2 ml of serum is described. Improvement of previously described techniques was accomplished by modifying the extraction conditions and refluxing assembly. Continuous extraction of the oxidized diphenylhydantoin provided the maximum yield of benzophenone, resulting in optimal sensitivity as well as reproducibility of analytical values. Reliable clinical assays are achieved on 0.1 ml of serum containing 0.5 μg of diphenylhydantoin. Recovery of the drug from biological fluids is approximately 94%.

Keyphrases □ Diphenylhydantion—microdetermination in serum and plasma, UV spectrophotometry □ Microdetermination—diphenylhydantoin in serum and plasma, UV spectrophotometry □ UV spectrophotometry—microdetermination of diphenylhydantoin in plasma and serum

The requirement for plasma levels of diphenylhydantoin in the clinical management of epileptic patients has been well documented (1–4). Earlier colorimetric and spectrophotometric methods for determining this common anticonvulsant were plagued by interference from other drugs, necessitating extensive separation techniques (5–7). Wallace *et al.* (8) developed the first sensitive and specific spectrophotometric determination for diphenylhydantoin not requiring preliminary separation from other drugs, a technique based upon the oxidation of the parent drug to benzophenone. Numerous modifications of that method have been published (4, 9–14), with a primary objective of determining the drug in as little as 0.4–2.0 ml of specimen (4, 12–14).

GLC techniques are available both for the determination of unchanged diphenylhydantoin (15–18) and derivatized diphenylhydantoin (19–25). GLC techniques are often complicated and usually demand a time interval exceeding that required for a spectrophotometric scan. Additionally, the lability of the hydantoins makes them highly susceptible to degradation at the injection temperature conditions of the chromatograph.

The present report describes two modifications of the spectrophotometric analysis: one utilizing conventional condensers and requiring 1 ml of plasma and the other utilizing a modified reflux condenser[1] and requiring only 0.1–0.2 ml plasma. The methods, in addition to providing a greater sensitivity than that achieved by available methods, eliminate the time-consuming evaporation of chloroform required in the Lee (4) and Saitoh (13) methods. Additionally, a far greater product stability than exists in the "bomb oxidation techniques" is obtained.

EXPERIMENTAL

Apparatus—Reflux condensers were mounted on a support[2]. The semimicrodetermination utilizes a conventional condenser[3]; the microdetermination utilizes a previously described "external cold finger" reflux condenser[1]. Heating mantles[4], 270 w, 500-ml ca-

[1] Unpublished data.
[2] Flexaframe.
[3] Allihn.
[4] Glas-Col.