(6) M. K. Jain and K. S. Narag, *J. Indian Chem. Soc.*, **30,** 711 (1953).
(7) L. Novacek, J. Belusa, V. Hruskova, and D. Vavrionova, *Cesk. Farm.*, **27,** 173 (1978); through *Chem. Abstr.*, **90,** 8145c (1979).
(8) S. K. V. Seshavataram and N. V. S. Rao, *Proc. Indian Acad. Sci. Sect. A.*, **85,** 81 (1977).
(9) R. H. Mizzoni and P. C. Eisman, *J. Am. Chem. Soc.*, **80,** 3471 (1958).
(10) I. Ishii, M. Katagiri, M. Sakazume, and T. Misata, *Nippon Nogei Kagaku Kaishi*, **40,** 437 (1966); through *Chem. Abstr.*, **66,** 92704w (1967).
(11) S. M. El-Khawass, M. A. Khalil, and G. G. Tawil, *Sci. Pharm.*, **48,** 219 (1980).
(12) A. Mohsen, M. E. Omar, S. A. Shams El-Dine, A. A. Ghobashy, and M. A. Khalil, *Eur. J. Med. Chem.*, **16,** 77 (1981).
(13) S. L. Mertsalov, N. N. Vereshchagina, and I. Ya. Postovskii, *Khim. Geterotsikl. Soedin., Akad. Nauk Latv. SSR*, **2,** 215-6 (1965) Russ; through *Chem. Abstr.*, **63,** 5643b (1965).
(14) L. N. Volovel'Skii and G. V. Knorozova, *Zh. Obshch Khim.*, **34**(1), 343 (1964); through *Chem Abstr.*, *60*, 12074h (1964).
(15) J. Klosa (Privatforschungslab, Berlin), *J. Prakt. Chem.*, **31,** 140 (1966).
(16) S. R. Jain and A. Kar, *Planta Med.*, **20,** 118 (1971).
(17) N. S. Habib and A. A. B. Hazzaa, *Sci. Pharm.*, **49,** 246 (1981).
(18) D. Libermann, N. Rist, F. Grumbach, M. Moyeux, B. Gauthier, A. Rouaix, J. Maillard, J. Himbert, and S. Cals (Inst. Pasteur, Paris). *Bull. Soc. Chim. France*, 1430 (1954); through *Chem. Abstr.*, **50,** 351h (1956).
(19) El-Sebai A. Ibrahim, A.-Mohsen M. E. Omar, and M. A. Khalil, *J. Pharm. Sci.*, **69,** 1346 (1980).

ACKNOWLEDGMENTS

The authors thank Dr. G. G. Tawil, Pharmaceutical Microbiology Department, Faculty of Pharmacy, University of Alexandria, for the microbiological testing.

Determination of Acetazolamide in Biological Fluids by Reverse-Phase High-Performance Liquid Chromatography

DENNIS J. CHAPRON *‡x **and LORRAINE B. WHITE** *

Received February 24, 1983, from the *School of Pharmacy, University of Connecticut, Storrs, CT 06268 and the ‡Clinical Pharmacokinetics Laboratory, University of Connecticut Health Center, Farmington, CT 06032. Accepted for publication May 23, 1983.

Abstract □ A high-performance liquid chromatographic method for the determination of acetazolamide in whole blood, plasma, and urine was developed. Samples of biological fluids containing various concentrations of acetazolamide were spiked with the internal standard, sulfadiazine. Samples were then mixed with a 50% ammonium sulfamate solution. Whole blood samples were heated for 25 s in boiling water. All samples were extracted with ethyl acetate; a phosphate buffer (pH 8.0) was used to wash the extracts. Acetazolamide was back-extracted into a glycine buffer (pH 10.0), which was then washed with ether. Separation of acetazolamide and internal standard from other biological constituents was achieved on a 10-μm C_{18} reverse-phase column using an acetonitrile-methanol-acetate buffer (pH 4.0). The eluant was monitored at 254 nm. All calibration curves were linear, and the results from reproducibility studies were excellent. Application of the method to human pharmacokinetic studies was demonstrated.

Keyphrases □ Acetazolamide—biological fluids, reverse-phase HPLC, pharmacokinetics □ HPLC-acetazolamide, biological fluids, pharmacokinetics

Acetazolamide, a carbonic anhydrase inhibitor widely used in the medical management of glaucoma, lowers intraocular pressure by reducing the rate of aqueous humor formation. Acetazolamide is also used as an antiepileptic drug, where its effect is thought to result from inhibition of brain carbonic anhydrase. There is evidence that effective intraocular hypotensive and anticonvulsant actions are seen at plasma concentrations of 5-20 and 10 μg/mL, respectively (1-4). The drug is concentrated in erythrocytes, a site of action which has received little attention in the clinical literature. In humans, high erythrocyte levels have recently been associated with significant toxicity (4, 5). This is attributed to carbon dioxide retention resulting from inhibition of erythrocyte carbonic anhydrase, an important enzyme that greatly facilitates carbon dioxide exchange and transport in the capillary beds (6). Acetazolamide is completely eliminated unchanged *via* the kidney; determination of its clearance by this organ may prove useful for adjusting acetazolamide plasma concentrations.

Several high-performance liquid chromatographic (HPLC) assays have been published for determining acetazolamide levels in plasma or serum (7-9), and they have been an improvement on the older, less sensitive enzymatic assays (10, 11). A sensitive GC method is available, but it requires an electron-capture detector (12). Some of the recent HPLC procedures require repeated and/or successive extractions and dry-down procedures. None of the HPLC methods can quantitate acetazolamide in whole blood [erythrocytes indirectly (12)] or urine, fluids from which clinically important information may be ascertained.

The present report describes an HPLC method that (*a*) obviates time-consuming solvent evaporation steps, (*b*) displays excellent sensitivity and reproducibility, and (*c*) can be applied to several different biological fluids.

EXPERIMENTAL SECTION

Reagents and Materials—Acetazolamide[1] and sulfadiazine[2] were used as supplied. The solvents used for extraction and chromatography were all HPLC grade[3]. All chemicals were analytical reagent grade[4].

Standards—Separate stock solutions containing 1.0 mg/mL of acetazolamide and sulfadiazine (internal standard) were prepared weekly by dissolving these agents in 0.005 and 0.01 M NaOH, respectively. Aqueous solutions of lower concentrations (*i.e.*, 0.01-0.5 mg/mL) were prepared extemporaneously as needed from the stock solutions. Buffer solutions of 0.032 M glycine (pH 10.0) and 0.1 M phosphate buffer (pH 8.0) were prepared monthly. All solutions were stored at 4°C when not in use.

Extraction—Urine and heparinized whole blood were obtained from drug-free, normal, human volunteers. Plasma was obtained from an aliquot of whole blood using heparin as anticoagulant. Two hundred-microliter aliquots of plasma, whole blood, or urine (diluted 1:10) were added to 13 × 100-mm borosilicate glass tubes.

[1] Sigma Chemical Co., St. Louis, Mo.
[2] ICN Pharmaceuticals Inc., Cleveland, Ohio.
[3] Burdick and Jackson, Muskegon, Mich.
[4] Fisher Scientific, Fair Lawn, N.J.

0022-3549/84/0700-0985$01.00/0
© 1984, American Pharmaceutical Association

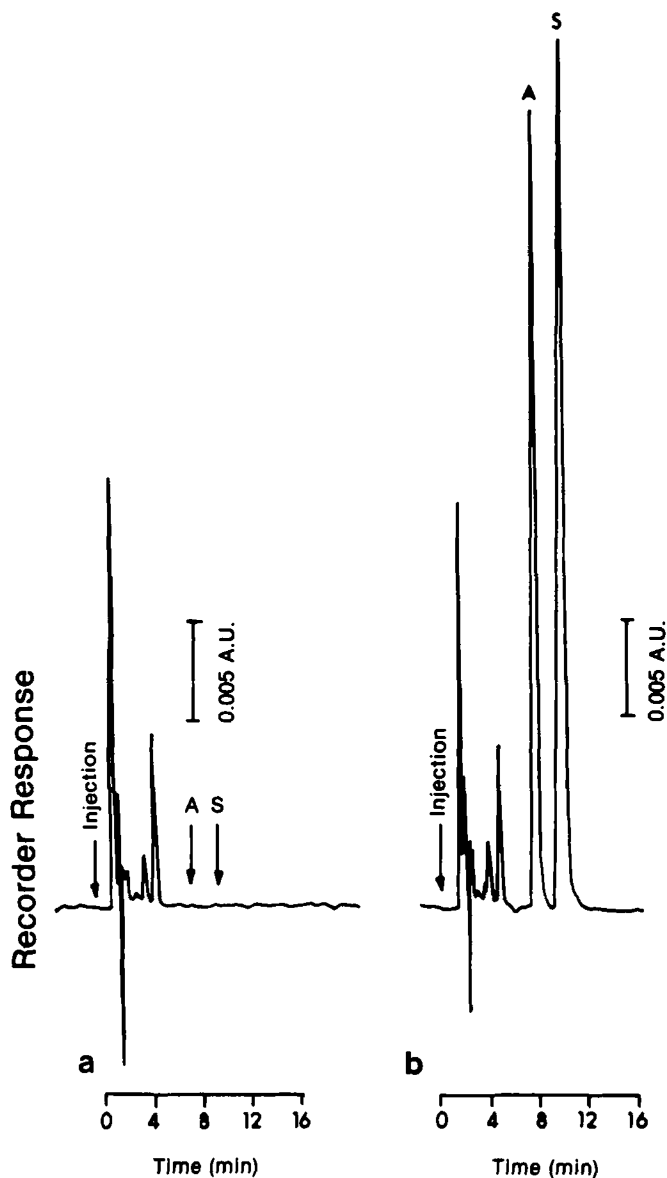

Figure 1—*Chromatograms of an extract of (a) blank whole blood and (b) an extract of whole blood containing 10.0 and 12.5 μg/mL of acetazolamide (A) and sulfadiazine (S), respectively.*

The tubes containing whole blood underwent two freeze-thaw cycles. Internal standard was then added (1–20 μL), the amount dependent on the range of concentrations expected in the unknown samples. One milliliter of a 50% solution of ammonium sulfamate was added, and the tubes were vortexed for a few seconds. The tubes containing whole blood were then placed in boiling water for 25 s, removed, and then quickly placed in cool water. One-half milliliter of water was added to the whole blood samples, and the tubes were vortexed for a few seconds.

Three milliliters of ethyl acetate was added to all tubes. The tubes were sealed with an extraction-tube plug[5] and shaken (200 oscillations/min) for 15 min. The phases were separated by centrifugation at 444×g for 5 min. The ethyl acetate layer was transferred to an identical tube containing 1.5 mL of pH 8.0 phosphate buffer (3.0 mL was used for urine workup). The tubes were then plugged and shaken, and the phases were separated by centrifugation as previously described. The organic phase was then transferred to tapered tubes[6] containing 200 μL of glycine buffer (pH 10.0), capped, and vortexed for 3 min. The phases were separated by centrifugation (444×g, 5 min), and the ethyl acetate layer was removed and discarded. To the remaining glycine layer, was added 1.0 mL of ether. The tubes were capped, vortexed for 2 min, the ether layer was removed and discarded, and the tubes were allowed to vent

[5] Oxford Labs., Foster City, Calif.
[6] Corning centrifugation tube, Cat #8122.

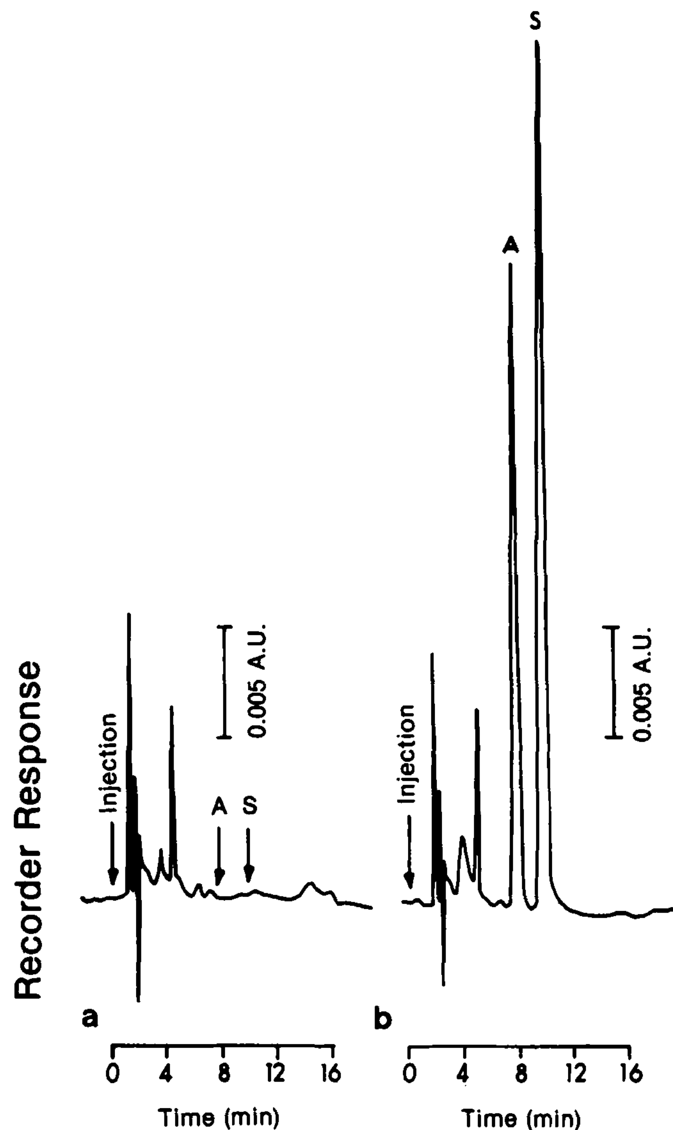

Figure 2—*Chromatograms of an extract of (a) blank plasma and (b) an extract of plasma containing 6.0 and 10.5 μg/mL of acetazolamide (A) and sulfadiazine (S), respectively.*

the ether vapors for ~15 min. An aliquot of the glycine phase (25–50 μL) was sampled from the tube and injected into the chromatograph.

Chromatography—The HPLC system consisted of a constant-flow pump[7], a variable-volume sampling valve[8], and a fixed-wavelength detector[9]. A 3.9 mm × 30-cm reverse-phase column[10] was used with a 2.3 × 3.9 mm guard column[11], which was packed with a commercially available bulk packing material[12]. The output from the detector was monitored on a 10-mV recorder[13].

Analyses were performed using a mobile phase of acetonitrile–methanol–0.05 M sodium acetate (3:2:95), the pH adjusted to 4.0 with glacial acetic acid. The mobile phase was deaerated before use. The system was operated at ambient temperature at a flow rate of 2.0 mL/min. The UV detector was fixed at a wavelength of 254 nm.

Calibration and Reproducibility—Known quantities of acetazolamide were added to blank plasma and whole blood. Samples (0.2 mL) were then assayed for acetazolamide. Calibration curves were constructed by plotting peak height ratio (acetazolamide–internal standard) *versus* the concentration of acetazolamide. Acetazolamide is rapidly eliminated from plasma; in order to accommodate a broad range of concentrations, two different standard curves

[7] Model M6000A; Waters Associates, Milford, Mass.
[8] Model U6K; Waters Associates, Milford, Mass.
[9] Model 440; Waters Associates, Milford, Mass.
[10] μ-Bondapak C_{18}; Waters Associates, Milford, Mass.
[11] Waters Associates, Milford, Mass.
[12] Bondapak C_{18}/Corasil; Waters Associates, Milford, Mass.
[13] Omniscribe, model B5117-5; Houston Instruments, Austin, Tex.

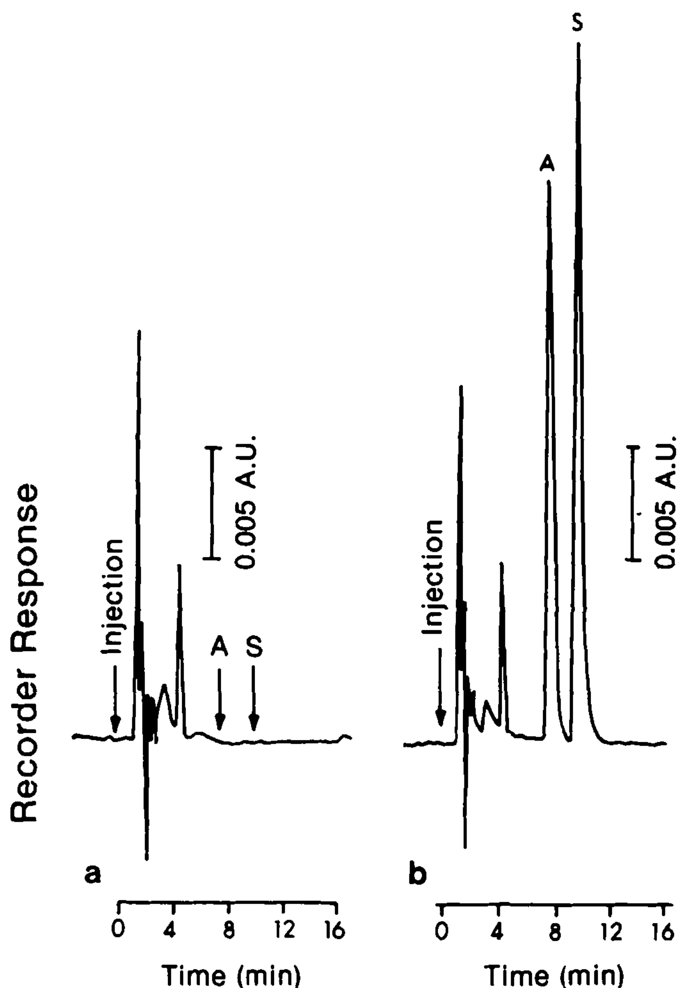

Figure 3—*Chromatograms of extracts of (a) blank urine and (b) urine containing acetazolamide (A) and sulfadiazine (S). Prior to extraction, each urine sample was diluted 1:10 with distilled water to yield final concentrations of 4.0 and 6.4 µg/mL for acetazolamide and sulfadiazine, respectively.*

were prepared. For plasma, standard calibration curve ranges were 0.05–1.5 µg/mL and 2.0–50.0 µg/mL. Because of significant accumulation and persistence of acetazolamide in erythrocytes, only a single calibration curve was needed for whole blood (*i.e.*, 1–50 µg/mL). Standard curves for urine were prepared in diluted urine (1:10) over the concentration range of 1–12 µg/mL. Day-to-day reproducibility and within-run precision of the analytical procedures were examined by processing spiked samples of plasma, whole blood, and urine.

Stability and Recovery—The stability of acetazolamide as a refrigerated aqueous solution (1 mg/mL), a frozen glycine extract, and frozen plasma and whole blood samples was assessed periodically by HPLC assay. Recovery of acetazolamide was calculated by comparing the peak height ratio of drug added to the biological fluid against the peak height ratio of drug added to the final glycine phase. In both treatments, the internal standard was added to the final glycine phase.

RESULTS AND DISCUSSION

Acetazolamide is a relatively polar drug [chloroform-water partition coefficient at pH 7.0 ≅ 0.001 (13)] which coextracts with endogenous blood and urinary constituents that create detection and quantification difficulties. The present results showed that a phosphate buffer washing step, which removes a moderate amount of acetazolamide, dramatically eliminates interfering endogenous substances. Blank urine, whole blood, and plasma (Figs. 1–3) do not show any endogenous interfering peaks. The retention times for acetazolamide and sulfadiazine were 7.6 and 9.8 min, respectively. Extraction of acetazolamide from whole blood was markedly improved by heating in boiling water for 25 s prior to processing. Perhaps heating irreversibly inactivates carbonic anhydrase and liberates the bound acetazolamide. Degradation of either acetazolamide or sulfadiazine does not occur during this heating step.

Table I—Calibration Curves for Acetazolamide in Various Biological Fluids

Fluid[a]	Concentration, µg/mL	r^2 [b]
Plasma	0.05–1.5	0.9984 ± 0.0025
	2.0–50	0.9988 ± 0.0022
Whole blood	1.0–50	0.9997 ± 0.0004
Urine	1.0–12	0.9996 ± 0.0004

[a] Based on five calibration curves. [b] Mean ± *SD*.

Calibration curves for all the biological fluids were linear over the concentrations described, and the intercepts were essentially zero. The mean coefficient of determination (r^2) for five successive calibration curves for urine, whole blood, and plasma are shown in Table I. Table II shows the coefficient of variation (*CV*) for the reproducibility studies. These data indicate the overall variability of the assay to be excellent, with all *CV* values <5.0%. With a 200-µL sample, the lowest quantifiable concentration providing reasonable reproducibility (*CV* ≤10%) was 0.05 µg/mL.

Absolute recoveries from plasma, whole blood, and urine were found to be 36, 33, and 20%, respectively. The significant loss of acetazolamide during processing was expected (acetazolamide has a pK_a of 7.4 and would be significantly ion-trapped in the phosphate buffer during the washing step) and considered very acceptable, because endogenous interfering substances were completely eliminated, sensitivity was adequate for human pharmacokinetic studies, and reproducibility of the assay was excellent. Absolute recovery can be increased by increasing the volume of ethyl acetate used for the initial extraction (*e.g.*, 4.0- and 5.0-mL ethyl acetate extraction of plasma provided absolute recoveries of 45 and 50%, respectively). We found it more convenient, however, to work with smaller extraction volumes. During sample processing the net loss of sulfadiazine was found to exceed that of acetazolamide. Across the biological matrices examined, sulfadiazine loss was found to parallel that of acetazolamide. A processed sample will produce a peak height ratio ~1 when initially spiked with a concentration of sulfadiazine 30% greater than that of acetazolamide.

Several stability studies were conducted. The aqueous refrigerated solution of acetazolamide (1 mg/mL) was found to be stable for ≤30 d. Frozen (−20°C) glycine extracts of acetazolamide and sulfadiazine were stable for ≤7 d. Acetazolamide in frozen plasma and whole blood was stable ≤180 d.

The HPLC procedures described have been successfully applied to pharmacokinetic studies of acetazolamide disposition in humans after intravenous administration. Figure 4 shows typical acetazolamide concentration–time curves following intravenous administration to a young female volunteer. Because urinary concentrations of the drug were expected to vary considerably, being very high initially and then rapidly declining thereafter, the following formula was used to estimate acetazolamide urinary concentrations:

$$\text{Estimated urinary acetazolamide concentration} = \frac{\text{AUC } (\mu g \cdot \text{min/mL}) \times CL \text{ (mL/min)}}{\text{Urine Volume (mL)}}$$

where AUC is the area under the acetazolamide plasma concentration–time curve and *CL* is its estimated clearance from plasma. Estimated urinary acetazolamide concentrations were then diluted to produce concentrations that would fall at the midpoint of the standard curve for urine (*i.e.*, 1–12 µg/mL). Cumulative urinary and excretion rate plots are shown in Fig. 5 for the same subject. It is noteworthy that 98.8% of the injected dose (320 mg) was recovered during a 72-h urine collection period. Studies designed to better define

Table II—Summary of Data for Reproducibility

Within-Run Precision[a]		Day-to-Day Reproducibility[b]	
Conc., µg/mL	*CV*, %	Conc., µg/mL	*CV*, %
Plasma			
0.1	2.8	0.5	4.2
1.0	3.6	10.0	1.1
10.0	1.5		
20.0	2.0		
Whole Blood			
1.0	1.3	10.0	3.7
10.0	1.6		
20.0	2.6		
Urine			
5.0	1.2	6.0	1.3
10.0	1.5		

[a] $n = 8$. [b] $n = 6$.

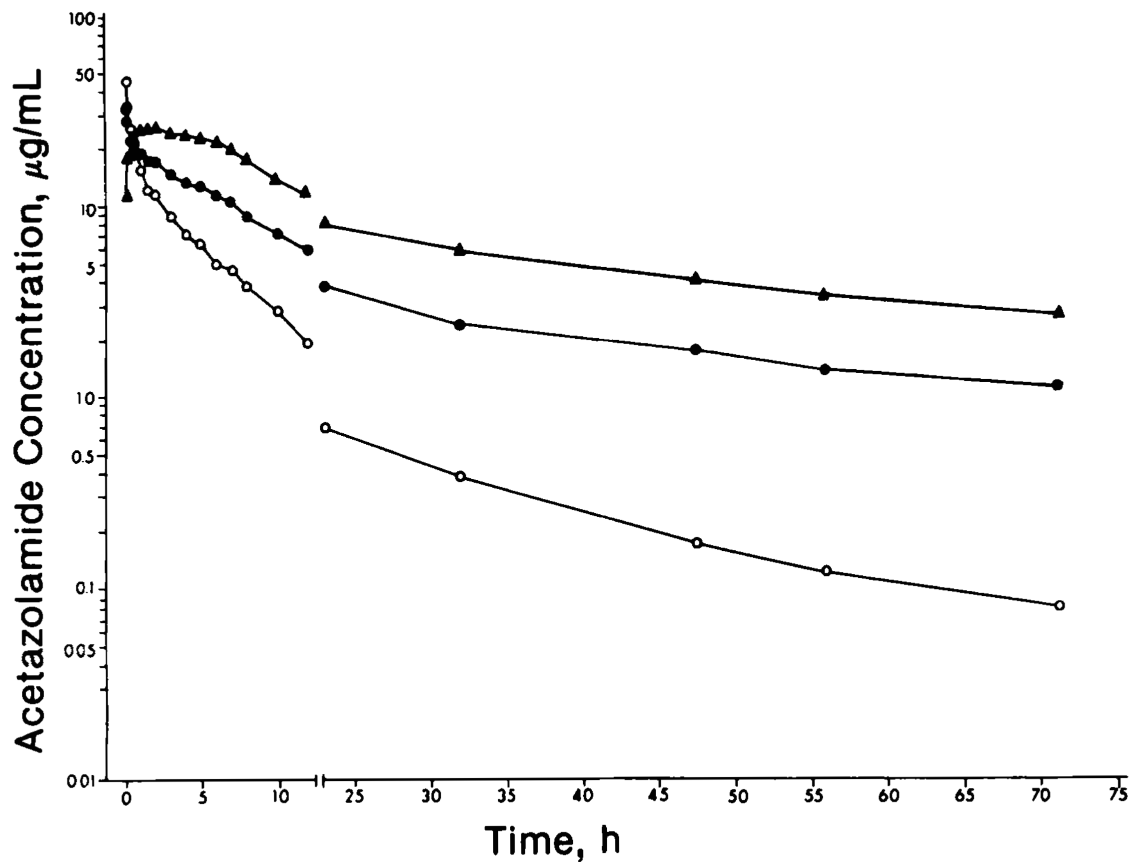

Figure 4—*Concentration-time profiles of acetazolamide in erythrocytes (▲), whole blood (●), and plasma (O) following an intravenous bolus injection of 320 mg.*

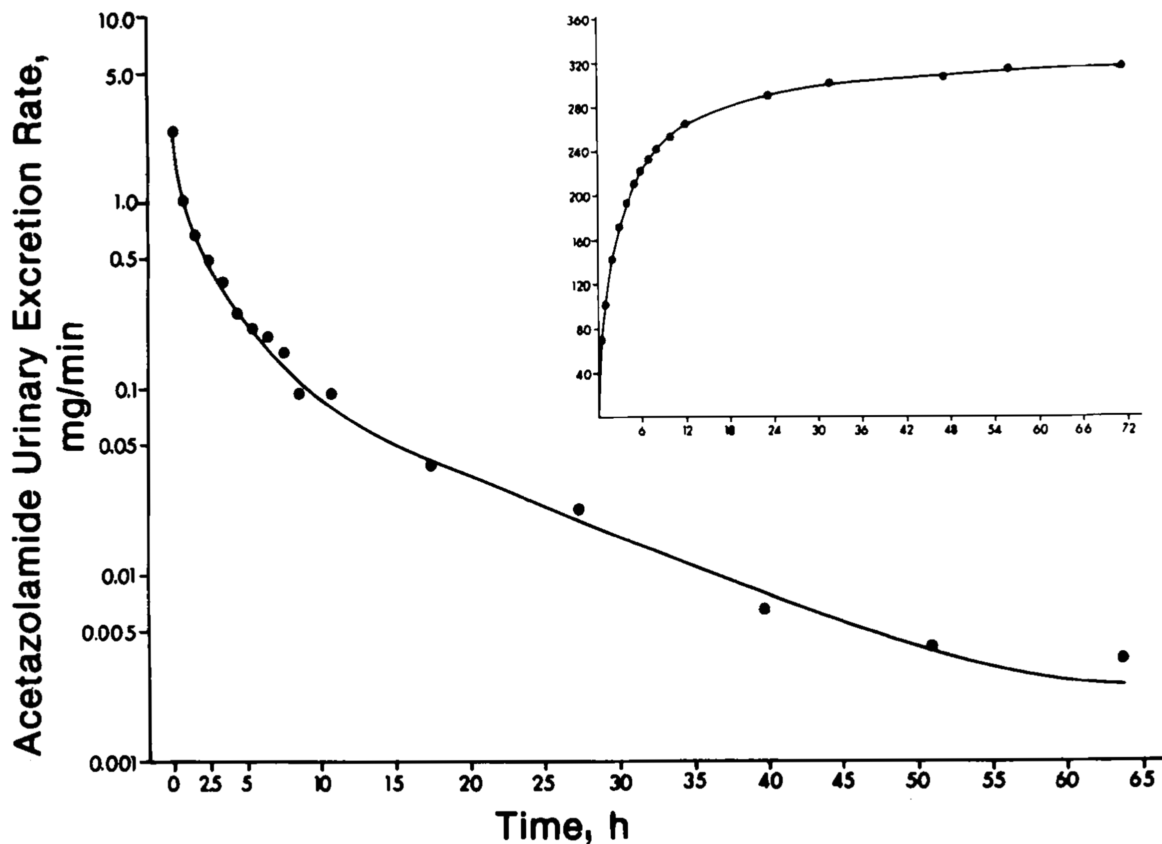

Figure 5—*Urinary excretion rate plot of acetazolamide following an intravenous bolus injection of 320 mg. The inset depicts cumulative amounts of acetazolamide excreted in urine (mg) over time (h) in the same subject.*

the factors that influence the disposition and response to acetazolamide are in progress.

REFERENCES

(1) B. Lehmann, E. Linner, and P. J. Wistrand, in "Schering Workshop in Pharmacokinetics," G. Raspe, Ed., Pergamon, Oxford, 1969, p. 197.
(2) B. R. Friedland, J. Mallonee, and D. R. Anderson, *Arch. Ophthalmol.*, **95**, 1809 (1977).
(3) F. G. Berson, D. L. Epstein, W. M. Grant, T. Hutchinson, and P. C. Dobbs, *Arch Ophthalmol.*, **98**, 1051 (1980).
(4) M. Inui, H. Azuma, T. Nishimura, and N. Hatada, in "Advances in Epileptology: XIIIth Epilepsy International Symposium," H. Akimoto, H. Kazamatsuri, M. Seino, and A. Ward, Eds., Raven, New York, N.Y., 1982, p. 307.
(5) D. M. Woodbury and J. W. Kemp, in "Antiepileptic Drugs," D. M. Woodbury, J. K Penry, and C. E. Pippenger, Eds., Raven Press, New York, N.Y., 1982, p. 771.
(6) J. B. West, "Respiratory Physiology," Williams and Wilkins Co., Baltimore, Md., 1979, p. 74.
(7) W. F. Bayne, G. Rogers, and N. Crisologo, *J. Pharm. Sci.*, **64**, 402 (1975).
(8) R. D. Hossie, N. Mouseau, S. Sved, and R. Brien, *J. Pharm. Sci.*, **69**, 348 (1980).
(9) D. M. Chambers, M. H. White, and H. B. Kostenbauder, *J. Chromatogr.*, **225**, 231 (1981).
(10) T. H. Maren, *J. Pharmacol. Exp. Ther.*, **130**, 26 (1960).
(11) G. J. Yakatan, C. A. Martin, and R. V. Smith, *Anal. Chim. Acta*, **84**, 173 (1976).
(12) S. M. Wallace, V. P. Shah, and S. Riegelman, *J. Pharm. Sci.*, **66**, 527 (1977).
(13) T. H. Maren, J. R. Haywood, S. K. Chapman, and T. J. Zimmerman, *Invest. Ophthalmol. Vis. Sci.*, **16**, 730 (1977).

Amperometric Determination of Hydralazine Hydrochloride in a Flowing Stream at the Glassy Carbon Electrode

MUMTAZ H. SHAH and JAMES T. STEWARTˣ

Received March 11, 1983, from the *Department of Medicinal Chemistry, College of Pharmacy, The University of Georgia, Athens, GA 30602.* Accepted for publication May 25, 1983.

Abstract □ A flow-injection method for the determination of hydralazine hydrochloride based on electrochemical oxidation at the glassy carbon electrode is presented. The amperometric method is highly specific and may be used to determine hydralazine hydrochloride in the presence of other drugs commonly found in its pharmaceutical dosage forms or administered concurrently in therapeutic situations. By using an electrode potential of +650 mV *versus* an Ag/AgCl reference electrode, a calibration curve was found to be linear in the 1-50-μg/mL concentration range, with minimum detectability at 10 ng (signal-to-noise ratio, 2). When the method was applied to the analysis of hydralazine hydrochloride in selected pharmaceutical dosage forms, it showed good accuracy and precision. Although automation was not used in this study, the method could readily be incorporated in automated systems because it employs the technique of continuous analysis in a flowing stream.

Keyphrases □ Hydralazine hydrochloride—amperometric determination in a flowing stream, glassy carbon electrode □ Flow-injection method—amperometric determination, hydralazine hydrochloride, glassy carbon electrode □ Electrochemical oxidation—glassy carbon electrode, flow-injection method, determination of hydralazine hydrochloride

Hydralazine hydrochloride, a widely prescribed antihypertensive agent, has been analyzed by diverse methodologies, including titrimetry (1, 2), spectrophotometry (3, 4), fluorometry (5), GC, and HPLC (6-10). Interest in this laboratory in the development of new continuous assay methods for drugs in flowing streams led us to investigate the oxidation of hydralazine at the glassy carbon electrode. There is almost no information in the literature concerning the electrochemistry of hydralazine. One report has indicated that the drug can be reduced at the dropping mercury electrode to give two half-wave potentials of −700 and −950 mV *versus* the standard calomel electrode (11). The reduction occurs in a solution containing 1 M HCl as a supporting electrolyte and gelatin as a surfactant. The report did not comment on the sensitivity, accuracy, precision, and specificity of the method. There appears to be no data in the literature on the electrochemical oxidation of the drug.

This laboratory has reported previously on continuous analysis in flowing streams by oxidation of drugs such as ascorbic acid and methyldopa at the tubular carbon electrode (12, 13). The glassy carbon electrode has supplanted the tubular carbon electrode and has shown general usefulness as a sensitive tool for the determination of oxidizable drugs in flowing stream systems such as HPLC (14-16). These kinds of electrodes can be easily incorporated into automated or semiautomated systems such as would be used in dosage form analysis.

In this report, amperometric determination of hydralazine hydrochloride in a flowing stream utilizing oxidation at the glassy carbon electrode is reported. The flow-injection method

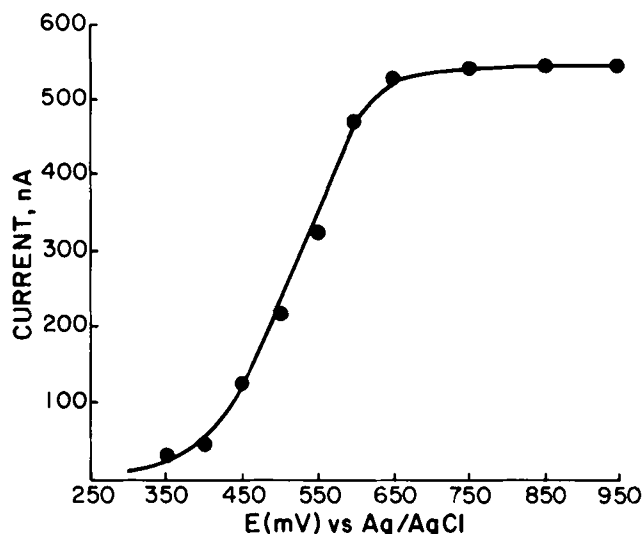

Figure 1—*Hydrodynamic voltammogram of hydralazine hydrochloride (50 μg/mL) in a 40:60 mixture of Walpole acetate buffer (pH 4.2)-absolute methanol at a flow rate of 1 mL/min.*

0022-3549/84/0700-0989$01.00/0
© 1984, American Pharmaceutical Association