GLC Analysis of Acetazolamide in Blood, Plasma, and Saliva following Oral Administration to Normal Subjects

SYLVIA M. WALLACE *, VINOD P. SHAH ‡, and SIDNEY RIEGELMAN ˣ

Abstract □ An electron-capture GLC assay for acetazolamide in biological fluids was developed. Extraction efficiency was 96–104%. The minimum detectable amount of acetazolamide was 10 ng/sample. Concentrations of acetazolamide were determined by GLC in blood, plasma, plasma water, and saliva after oral administration of a single 250-mg dose to five volunteers. Erythrocyte levels were calculated from whole blood and plasma data. Concentration of free drug in the plasma was measured in samples of plasma water obtained by microultrafiltration. Peak plasma levels of 10–18 μg/ml were reached 1–3 hr after the dose. At least 1 hr later, erythrocyte levels reached peak concentrations of 13–29 μg/ml. Over 31 hr, plasma levels declined more rapidly than erythrocyte levels. Saliva concentrations averaged 1% of those in plasma and decreased at a rate equal to that of plasma. Saliva levels were proportional to, but not equal to, plasma water concentration. Saliva to plasma ratios were consistent for any given individual and, therefore, offer a means of monitoring drug dosage without resorting to frequent blood sampling.

Keyphrases □ Acetazolamide—GLC analysis, human blood, plasma, and saliva □ GLC—analysis, acetazolamide, human blood, plasma, and saliva □ Carbonic anhydrase inhibitors—acetazolamide, GLC analysis, human blood, plasma, and saliva

Acetazolamide is used primarily to control intraocular pressure in the treatment of glaucoma. Side effects of the drug, although not life threatening, are often severe enough to warrant discontinuing therapy. At present, dosage regimens are adjusted empirically to balance therapeutic and toxic effects. However, by using as a guideline the minimum effective plasma concentration for lowering intraocular pressure (10 μg/ml) established by Lehmann *et al.* (1), a more efficient, individualized control of therapy should be possible.

The present study was designed to:

1. Develop a rapid, sensitive method for quantitating acetazolamide in biological fluids. Until recently (2), techniques for analyzing acetazolamide have been limited to the carbonic anhydrase inhibition method (3). However, the lower limit of the enzyme assay is 0.2 μg/ml of plasma.

2. Determine the feasibility of monitoring acetazolamide dosage in glaucoma patients by measurement of saliva concentrations. Saliva has been suggested (4–6) as a satisfactory alternative to plasma for routine monitoring of pharmacokinetic parameters. Use of saliva as the sampling fluid precludes the discomfort and inconvenience of multiple venipunctures but still yields the requisite information for dosage regimen adjustment.

3. Establish the relationship between plasma, saliva, and erythrocyte (red blood cell) concentrations of acetazolamide after oral administration of a single 250-mg dose to humans. Acetazolamide has a high affinity for binding sites, purportedly carbonic anhydrase enzymes, within the red blood cells (7). To define the kinetics of multiple dosing, the pool of drug within the red blood cells must be examined.

EXPERIMENTAL

Materials—Acetazolamide[1], fluoranthene[2], analytical grade solvents[3] (ether, dichloromethane, and 2-propanol), and trimethylphenylammonium hydroxide[4] (0.1 *M* in methanol) were used as obtained from standard sources without further purification. Tetramethylammonium hydroxide[5] (20% in methanol) diluted with methanol to 0.2% was used as an alternative derivatizing reagent, since many lots of trimethylphenylammonium hydroxide produce interfering peaks on electron-capture GLC.

Sample Preparation—Aliquots of blood, plasma (50–100 μl), and saliva (1–2 ml), adjusted to approximately pH 5 by the addition of 0.5 ml of 0.1 *M* acetate buffer, were extracted with 10 ml of organic solvent (six volumes of ether, four volumes of dichloromethane, and two volumes of 2-propanol). Tubes were centrifuged[6] for 15 min at 1500 rpm. The lower aqueous layer was frozen in a dry ice–acetone bath, and the upper organic layer was decanted quantitatively. Alternatively, if the concentration of acetazolamide in plasma or saliva exceeded the linear range of the standard curve, an aliquot of the organic layer was removed. The organic extract was evaporated to dryness under a slow stream of nitrogen.

Chromatographic Analysis—The internal standard, fluoranthene (2.5 μg in an ether solution), was added to the dried organic extract of plasma and saliva samples. The solvent was evaporated to dryness under nitrogen. After addition of 50 μl of methylating reagent (0.1 *M* trimethylphenylammonium hydroxide or 0.2% tetramethylammonium hydroxide in methanol), 2-μl aliquots were chromatographed. Chromatograms obtained with the two methylating reagents were identical.

The gas chromatograph[7] equipped with a ^{63}Ni-electron-capture detector was operated at 280, 215, and 340° for the injector, column, and detector, respectively. Solutions were chromatographed on a 1.8-m, 0.3-cm (6-ft, 0.124-in.) o.d. glass column packed with 3% OV-17 on Gas Chrom Q[8] (100–120 mesh). Carrier gas (95% argon, 5% methane) flow was 30 ml/min.

Sample Collection—Five healthy volunteers (two females and three males) between the ages of 24 and 31 years were each given a 250-mg tablet of acetazolamide[9] on an empty stomach. Blood samples were collected in heparinized tubes[10] at 0, 0.25, 0.5, 1, 2, 3, 5, 7, 9, 24, 27, and 31 hr after drug administration. At the same time, saliva (stimulated by chewing an elastic band) was collected in wide mouth vials. Saliva and plasma (separated from whole blood by centrifugation) were stored at 0° prior to analysis. Aliquots (100 μl) of plasma water were obtained from the 1-, 2-, 3-, 5-, 7-, and 9-hr plasma samples using a microultrafiltration procedure (8).

Calculation of Red Blood Cell Levels—Concentrations of acetazolamide in plasma, plasma water, blood, and saliva were measured directly by electron-capture GLC. Concentrations of the drug in the red blood cell fraction were calculated using the hematocrit:

$$C_B = C_{RBC}(H) + C_P(1 - H) \quad \text{(Eq. 1)}$$

$$C_{RBC} = \frac{C_B - C_P(1 - H)}{H} \quad \text{(Eq. 2)}$$

where C_B is the concentration in the blood, C_P is the concentration in the plasma, C_{RBC} is the concentration in the red blood cells, and H is the hematocrit.

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Chem Service, Inc., West Chester, Pa.
[3] Mallinckrodt Chemical Works, St. Louis, Mo.
[4] Eastman Organic Chemicals, Rochester, N.Y.
[5] Aldrich Chemical Co., San Leandro, Calif.
[6] Centrifuge model UV, International Equipment Co., Needham, Mass.
[7] Model 1200, Varian Aerograph, Walnut Creek, Calif.
[8] Applied Science Laboratories, State College, Pa.
[9] Diamox, Lederle Laboratories, Pearl River, N.Y.
[10] Becton-Dickinson Co., Rutherford, N.J.

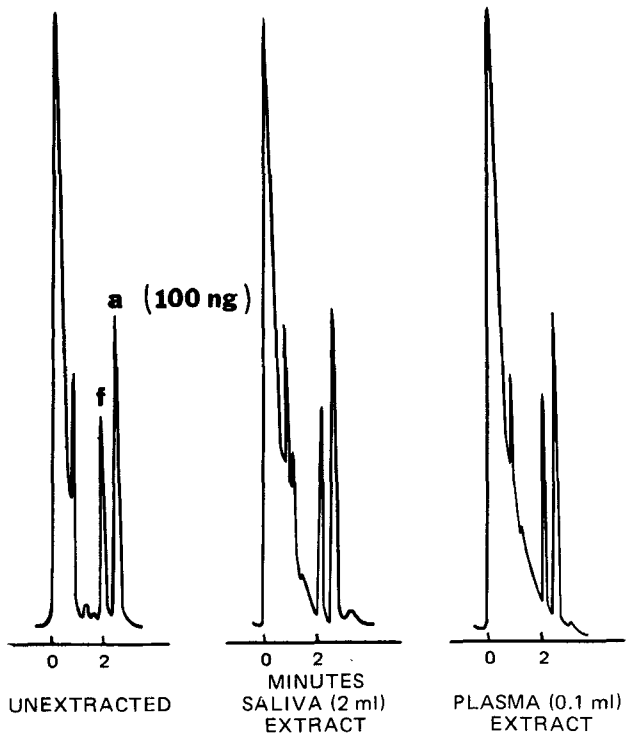

Figure 1—*Gas chromatogram of 2.5 μg of fluoranthene (f) internal standard and 100 ng of acetazolamide (a) unextracted, extracted from 2 ml of saliva, and extracted from 100 μl of plasma.*

RESULTS AND DISCUSSION

With a single extraction into the organic solvent mixture, recovery of acetazolamide from blood, plasma, and saliva was 96–104%. Two milliliters of saliva and up to 100 μl of plasma did not produce any significant interfering peaks. With a column temperature of 215° and a carrier gas flow of 30 ml/min, retention times for fluoranthene and acetazolamide were 2 and 2.5 min, respectively (Fig. 1). Peak height ratios of acetazolamide relative to fluoranthene were calculated, and standard curves were plotted as peak height ratio *versus* acetazolamide concentration.

Standard curves, prepared daily using at least four concentrations of acetazolamide, were linear ($r^2 = 0.98$) to a maximum concentration of 100 ng of acetazolamide/50 μl of methylating reagent. Concentrations of 10 ng/50 μl were easily visualized. Thus, for a 2-ml sample of saliva and a 100-μl sample of plasma or blood, the GLC method could quantitate saliva levels of 5 ng/ml and plasma or blood levels of 100 ng/ml.

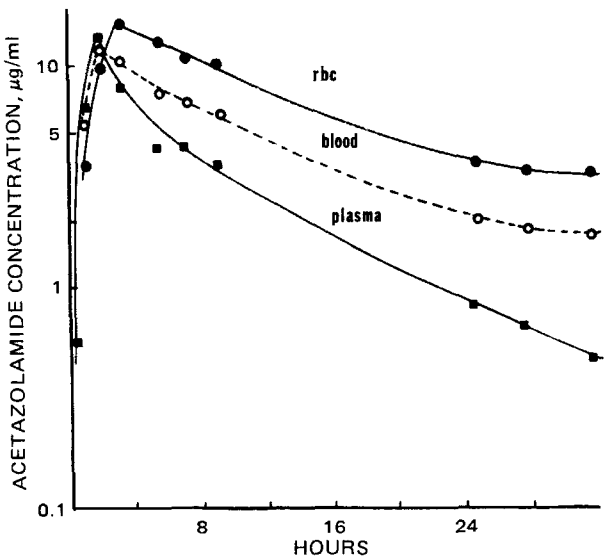

Figure 3—*Semilogarithmic plot of plasma, blood, and red blood cell levels of acetazolamide in Subject TS after a 250-mg oral dose.*

In the five subjects studied, peak plasma levels of 10–18 μg/ml were reached 1–3 hr after oral administration of the 250-mg dose (Figs. 2 and 3). These levels were approximately half those reported (1) after oral administration of a 500-mg dose. At least 1 hr later, whole blood and red blood cell levels reached peak concentrations of 10–19 and 13–29 μg/ml, respectively (Table I). This lag between the time for plasma and red blood cell levels to reach peak concentrations was apparent in all subjects.

Previous studies of elimination kinetics of acetazolamide in humans rarely analyzed plasma beyond 12 hr. These levels, if determined, were usually single-point measurements at 24 hr. Subsequent plasma concentrations were frequently too low to be measured accurately by the carbonic anhydrase inhibition method. Thus, data have heretofore not permitted evaluation of more than one exponential decay phase. With the more sensitive GLC assay, elimination from the plasma no longer appears to be a simple monoexponential process.

Over the 31 hr studied, plasma levels decayed more rapidly than red blood cell or blood levels. The area under the blood level–time curve from 0 to 31 hr was 1.5 times that under the plasma curve. If sufficient data are available, *i.e.*, for times greater than 31 hr after the dose, plasma levels should eventually decline parallel to red blood cell levels. This conjecture is suggested by the data for Subject PJ (Fig. 2). The plasma decay curve appeared biexponential with a half-life of just over 2 hr for the initial phase and 13 hr for the terminal phase. The half-life for the decay curve in the red blood cells was also 13 hr.

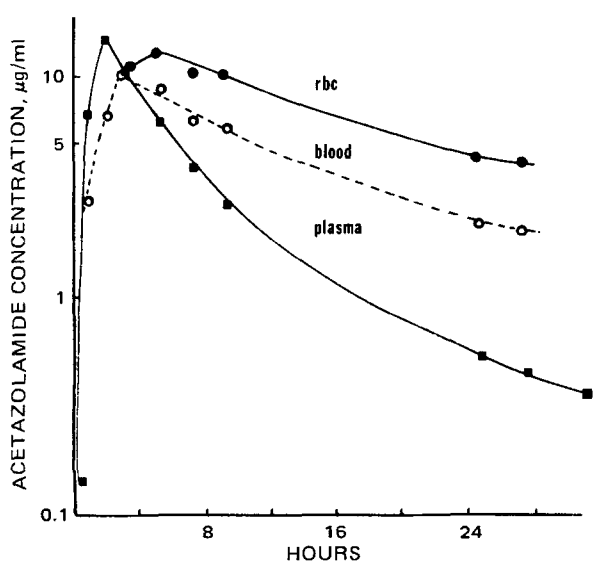

Figure 2—*Semilogarithmic plot of plasma, blood, and red blood cell levels of acetazolamide in Subject PJ after a 250-mg oral dose.*

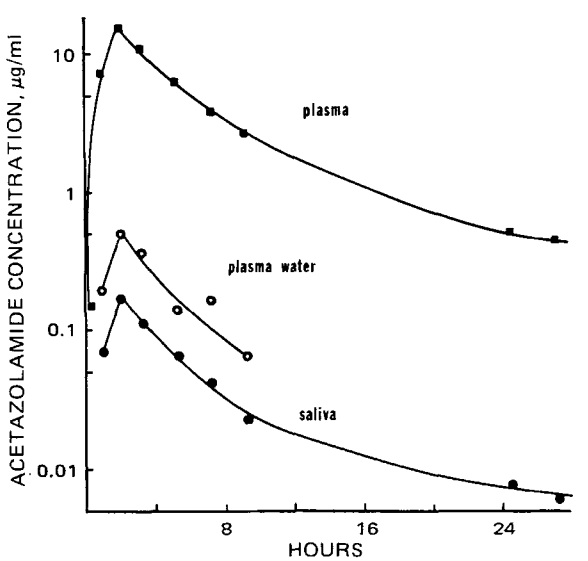

Figure 4—*Semilogarithmic plot of plasma, plasma water, and saliva levels of acetazolamide in Subject PJ.*

Table I—Blood, Plasma, and Red Blood Cell Levels (Micrograms per Milliliter) of Acetazolamide in Five Normal Subjects following Oral Administration of a 250-mg Tablet

	Hours										
	0.25	0.5	1	2	3	5	7	9	24	27	31
Subject PJ											
Blood	0	—	2.77	6.96	10.50	9.21	6.56	5.92	2.06	2.00	—
Plasma	0	0.15	7.23	14.54	10.33	6.30	3.87	2.69	0.52	0.45	0.35
Red blood cell	0	—	0	0	10.72	13.07	10.14	10.21	4.11	4.06	—
Subject CA											
Blood	0	0	0	4.79	8.42	10.54	10.65	9.61	2.91	2.68	—
Plasma	0	0	0	8.74	10.61	8.92	8.04	6.26	1.06	0.76	0.63
Red blood cell	0	0	0	0.16	5.85	12.44	13.71	13.54	5.09	4.93	—
Subject SG											
Blood	1.40	9.16	14.53	19.37	—	12.92	11.26	8.26	2.47	2.05	—
Plasma	1.20	7.91	13.77	12.95	9.12	8.97	5.97	5.24	0.84	0.78	0.35
Red blood cell	1.69	11.04	15.66	29.00	—	14.70	19.20	12.79	4.92	3.95	—
Subject AS											
Plasma	0	7.90	12.95	18.12	10.43	7.01	6.61	4.60	0.74	0.64	0.45
Subject TS											
Blood	0	0.29	5.34	11.71	11.12	7.89	7.12	6.41	1.87	1.73	1.61
Plasma	0	0.60	6.66	13.31	7.95	4.41	4.41	3.58	0.80	0.67	0.45
Red blood cell	0	0	3.51	9.49	15.47	12.71	10.86	10.12	3.40	3.19	3.21

Red blood cell levels obtained up to 27 hr could be fit adequately to a one-compartment pharmacokinetic model with an elimination half-life of 10–12 hr. However, when more samples were obtained beyond 24 hr, a second elimination phase was apparent. In Subject TS (Fig. 3), where data at 24, 28, and 31 hr were available, the down slope of the red blood cell *versus* time curve was biphasic with half-lives of 6 and 33 hr. For Subjects PJ (Fig. 2), CA, and SG, data were only available at 24 and 28 hr. Obviously, although a straight line with a half-life of 40–50 hr can be drawn through these last two data points, such estimates of a terminal slope are highly questionable. Maren *et al.* (7) reported similar biphasic decay curves for red blood cell binding of acetazolamide in dogs with half-lives of 12 hr for the initial phase and 2–3 days for the terminal phase.

Saliva levels were approximately 1% of plasma levels and decreased at a rate equal to that of the plasma rather than the blood (Fig. 4). The proportionality factors relating plasma to saliva concentration calculated by least-squares linear regression analysis (Figs. 5 and 6) and by an arithmetic average were similar (Table II). The saliva–plasma ratio did not change with time or plasma concentration of acetazolamide. However, saliva samples obtained within the 1st hr after oral administration frequently gave unpredictable results (Fig. 5). In the population tested, saliva levels were 0.7–1.1% of plasma levels. Maren and Robinson (9) reported that in hydrocephalic children treated with acetazolamide, cerebrospinal fluid levels were also 1% of plasma levels. Although there was no significant difference (analysis of variance, $p < 0.05$) between the saliva–plasma ratio of the five subjects, it is still advisable to obtain individual estimates of the ratio rather than rely on a population average.

The fraction of drug bound to plasma protein did not change significantly for the first 9 hr after oral administration. Thereafter, free drug concentration in the 100 μl of ultrafiltrate [using the microultrafiltration apparatus (8)] was too low to measure accurately. Plasma water, or free drug, concentrations averaged 6% of plasma concentrations (Table II). Thus, saliva levels were proportional to, but not equal to, levels of unbound drug in plasma (Fig. 4). In addition to protein binding, other parameters such as the oil-in-water partition coefficient and the degree of ionization obviously affect membrane transport and secretion into the saliva and cerebrospinal fluid.

CONCLUSIONS

In this single-dose study, although saliva–plasma ratios varied in the normal population, they were consistent for a given individual. Thus, saliva measurements offer a convenient means of monitoring plasma acetazolamide levels in glaucoma patients. The biexponential decay of saliva levels mirrors that of plasma.

Blood and plasma levels of acetazolamide were not equivalent. From

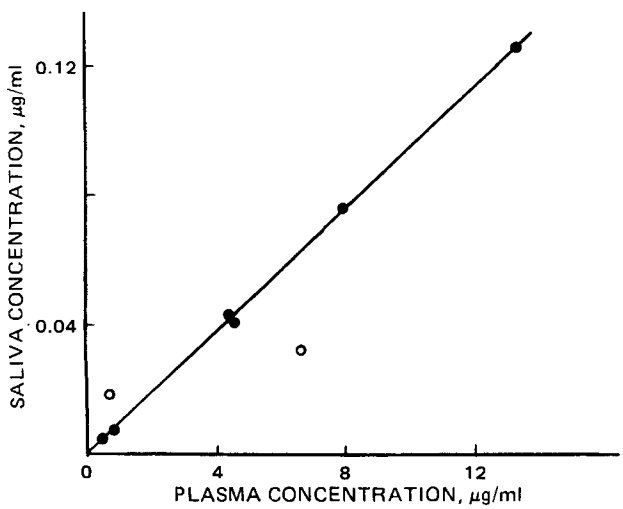

Figure 5—*Correlation of saliva and plasma concentrations of acetazolamide in Subject TS. Open circles represent samples taken within the 1st hr after oral administration. The line was calculated by least-squares linear regression analysis.*

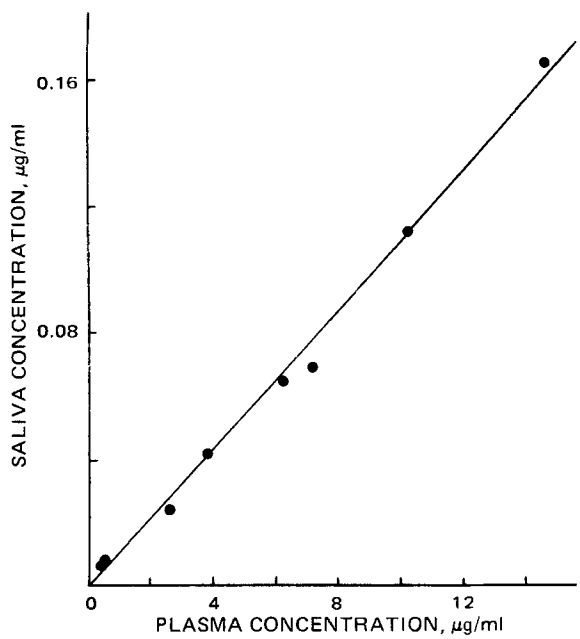

Figure 6—*Correlation of saliva and plasma concentrations of acetazolamide in Subject PJ. The line was calculated by least-squares linear regression analysis.*

Table II—Relationship of Saliva, Plasma, and Plasma Water Concentrations of Acetazolamide in Five Subjects after Oral Administration of a 250-mg Dose

Subject	Saliva Concentration / Plasma Concentration × 100			Plasma Water Concentration / Plasma Concentration × 100	
	Mean	(± *SEM*)	Linear Regression Slope	Mean	(± *SEM*)
PJ	1.11	(±0.07)	1.12	3.02	(±0.32)
CA	0.70	(±0.10)	0.51	4.22	(±0.41)
SG	0.85	(±0.09)	0.77	10.74	(±0.73)
AS	0.94	(±0.14)	0.93	5.17	(±0.52)
TS	0.84	(±0.06)	0.92	5.35	(±0.40)
Pooled data	0.90	(±0.04)	0.88	5.66	(±0.58)

24 to 31 hr after administration of a single oral dose, the ratio of red blood cells to plasma levels was greater than 4:1. Therefore, to ensure accurate determination of plasma levels, plasma should be separated from blood immediately, before hemolysis occurs. An appreciable degree of hemolysis may lead to overestimation of plasma concentrations.

The significance of the slowly declining red blood cell levels during chronic therapy with the drug remains to be determined.

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Pharmacokinetic Analysis of Drug Concentration Data Obtained during Repetitive Drug Administration

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Abstract □ A digital computer curve-fitting method, designed to estimate pharmacokinetic model constants by utilizing all drug concentration–time data collected during repetitive dosing studies, was applied to data manifesting systematic dose-to-dose variability in one or another of the pharmacokinetic parameters. The method accurately determined dose-to-dose changes in absorption or elimination rate constants or in the apparent volume of distribution, and it would be useful for detecting phenomena such as self-induction and self-inhibition that may occur during multiple-dose administration. The method can also be used to analyze multiple-dose data of drugs exhibiting capacity-limited elimination and to obtain estimates of the Michaelis–Menten parameters.

Keyphrases □ Pharmacokinetics—model constants estimated by computer curve-fitting method, drug concentration data obtained during repetitive drug administration □ Computer curve-fitting method—pharmacokinetic model constants estimated, drug concentration data obtained during repetitive drug administration □ Repetitive drug administration—drug concentration data obtained, model constants estimated by computer curve-fitting method

A previous report (1) described the development of a digital computer method which utilized all plasma or blood concentration data collected during repetitive dosing studies to estimate pharmacokinetic model constants and, simultaneously, to fit the entire time course of drug concentration in plasma or blood. The method readily accommodates changes in dose or dosing interval during a multiple-dose regimen.

Several investigations found that pharmacokinetic parameters of certain drugs change in a systematic way