Uptake of Acetazolamide by Human Erythrocytes *In Vitro*

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Abstract □ The binding of acetazolamide to human erythrocytes was studied *in vitro*. Blood and plasma samples were analyzed by electron-capture GLC. At 37°, drug equilibrated between plasma and erythrocytes in approximately 40 min. The effect of plasma concentration on the steady-state level of drug within the erythrocytes was examined. Erythrocyte accumulation of acetazolamide appeared to be a composite of two processes: a nonlinear, saturable process and a linear, diffusion-controlled process. By appropriate linear transformation of the data, estimates of the erythrocyte binding capacity and the dissociation constant for the drug were obtained.

Keyphrases □ Acetazolamide—*in vitro* binding to human erythrocytes, effect of plasma drug concentration on steady-state level within erythrocytes □ Binding—acetazolamide to human erythrocytes *in vitro*, effect of plasma drug concentration on steady-state level within erythrocytes □ Erythrocytes, human—binding of acetazolamide *in vitro*, effect of plasma drug concentration on steady-state level within erythrocytes □ Plasma drug concentration—effect on steady-state level of acetazolamide within human erythrocytes □ Carbonic anhydrase inhibitors—*in vitro* binding to human erythrocytes, effect of plasma drug concentration on steady-state level within erythrocytes

The erythrocyte compartment of the blood is often dismissed as an insignificant consideration in pharmacokinetics. Blood and plasma levels are often merely *assumed* to be equivalent terms. However, many drugs accumulate significantly in erythrocytes. Concentrations of salicylate (1), phenobarbital (1), phenytoin (2), chlorpromazine (3), and chlorthalidone (4) within the erythrocyte are all in excess of the free drug concentration in the plasma. The nature of the interaction and accumulation is unknown and probably varies, and the significance of these interactions in the overall pharmacokinetics of the drugs has not been determined.

After a single oral dose of acetazolamide to humans, concentration–time profiles of the drug in erythrocytes and plasma were significantly different[1]. Twenty-four hours after the dose, erythrocyte levels were four times greater than plasma levels. This accumulation was previously attributed to binding to erythrocyte carbonic anhydrase (5). Although Maren *et al.* (6) reported that erythrocyte uptake appears to have two components—one diffusible, *i.e.*, lost by washing, and the other bound—the extent and strength of binding have not been determined.

In vivo, in a dynamic situation, a single event such as erythrocyte uptake is difficult to isolate from the multiplicity of kinetic events occurring. Therefore, to determine the strength and capacity of acetazolamide binding, erythrocyte uptake of the drug was studied *in vitro* in a closed system.

EXPERIMENTAL

Time Course to Equilibration—Acetazolamide was added to whole human blood[2] and warmed to 37°, and the mixture was swirled gently on a water bath[3]. Aliquots were withdrawn at 10, 20, 40, 60, and 120 min and centrifuged[4] for 1 min to separate the plasma.

Equilibrium Studies—Whole blood containing varying concentrations of acetazolamide was incubated for 1 hr at 37° with gentle swirling. Aliquots (50 μl) of blood were withdrawn for analysis, and the remainder was centrifuged[5] for 15 min at 1500 rpm. Plasma and blood were analyzed, and the erythrocyte concentration was calculated from the difference using the hematocrit. The free drug concentration in the plasma was determined in plasma water samples obtained by microultrafiltration (7). Results of individual representative experiments are illustrated in Figs. 1–4.

Similar experiments were conducted using a synthetic blood medium consisting of erythrocytes washed three times with isotonic Sorenson's phosphate buffer (pH 7.4) and reconstituted to a hematocrit of approximately 0.5 with the buffer. To determine if washing erythrocytes altered acetazolamide uptake, erythrocytes washed three times with buffer were reconstituted with plasma and the results were compared to those obtained with whole blood.

GLC Analysis—Acetazolamide was determined in plasma and blood by GLC using a ^{63}Ni-electron-capture detector[6]. Samples were processed and chromatographed on a glass column packed with 3% OV-17 coated on Gas Chrom Q[7] (100–120 mesh).

RESULTS AND DISCUSSION

After addition of acetazolamide to whole blood *in vitro*, equilibration of acetazolamide between plasma and erythrocytes did not occur instantaneously. Steady state was established 40 min later (Fig. 1). *In vivo*, in subjects administered a single oral dose of acetazolamide, erythrocyte levels reached peak concentrations 1–2 hr after plasma levels. Presumably, this lag time *in vivo* reflects the equilibration time noted *in vitro*.

Uptake of acetazolamide by the erythrocytes is concentration dependent. Although a distinct plateau is not reached if the erythrocyte concentration of acetazolamide at equilibrium is plotted against the plasma concentration, the graph is curvilinear (Fig. 2). The ratio of the erythrocyte–plasma concentration decreases as the plasma concentration increases.

For example, at a hematocrit of 0.5 and plasma concentration of 1.6 μg/ml (blood concentration of 5 μg/ml), the erythrocyte–plasma ratio was 5.2:1. However, at a plasma concentration of 23.2 μg/ml (blood concentration of 30 μg/ml), the ratio was 1.6:1. Thus, over a wide range of plasma concentrations, the erythrocyte–plasma ratio cannot be characterized by a single value. As in the case of plasma protein binding of drugs, for a given percentage bound to have any significance, the total plasma concentration must be known.

Since erythrocyte uptake of acetazolamide is concentration dependent, it is assumed to be partially composed of a saturable process. The total erythrocyte concentration can be expressed as:

$$RBC_{total} = RBC_b + RBC_f \quad \text{(Eq. 1)}$$

At equilibrium, the diffusible free drug concentration, RBC_f, within the erythrocytes is assumed to be equal to the concentration of free drug in the plasma, C_f; therefore:

$$RBC_b = RBC_{total} - C_f \quad \text{(Eq. 2)}$$

The total uptake curve is a sum of two processes: a linear, presumably diffusion-controlled process such that RBC_f is equal to C_f and a nonlinear, saturable binding process such that the relationship of RBC_b to

[1] Presented at the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, San Francisco meeting, April 1975.
[2] Irwin Memorial Blood Bank, San Francisco, Calif.
[3] Metabolyte water bath shaker, New Brunswick Scientific Co., New Brunswick, N.J.
[4] Beckman/Spinco 152 microfuge.
[5] IEC model UV centrifuge, International Equipment Co.
[6] Varian series 1200 aerograph, Walnut Creek, Calif.
[7] Applied Science Laboratories, State College, Pa.

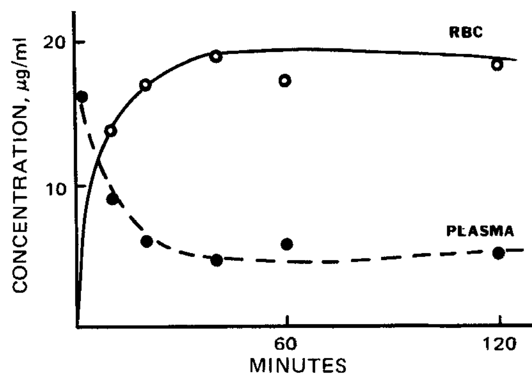

Figure 1—*Time course of erythrocyte (RBC) uptake of acetazolamide at a total blood concentration of 10 µg/ml. (All drug was assumed to be present initially in plasma.)*

C_f changes with the free concentration (Fig. 3). The curve for RBC_b is obtained by subtracting the values for RBC_f from the experimental curve, RBC_{total}.

The bound concentration, RBC_b, as calculated from Eq. 2, can also be expressed in terms of the law of mass action for the reversible macromolecule–ligand interaction:

$$RBC_b = \frac{nMk_aC_f}{1 + k_aC_f} \quad \text{(Eq. 3)}$$

Since the macromolecule concentration is unknown, only the product of the number of binding sites, n, and the macromolecule concentration, M, can be calculated. However, the product nM is a useful parameter, because it represents the maximum binding capacity of the protein.

Linear transformation (8) of Eq. 3 yields:

$$\frac{RBC_b}{C_f} = nMk_a - k_aRBC_b \quad \text{(Eq. 4)}$$

Thus, for a plot of RBC_b/C_f *versus* RBC_b, the absolute value of the slope is the association constant, k_a, and the intercept on the abscissa, the maximum binding capacity, nM (Fig. 4). The dissociation constant, k_d, is the reciprocal of the association constant.

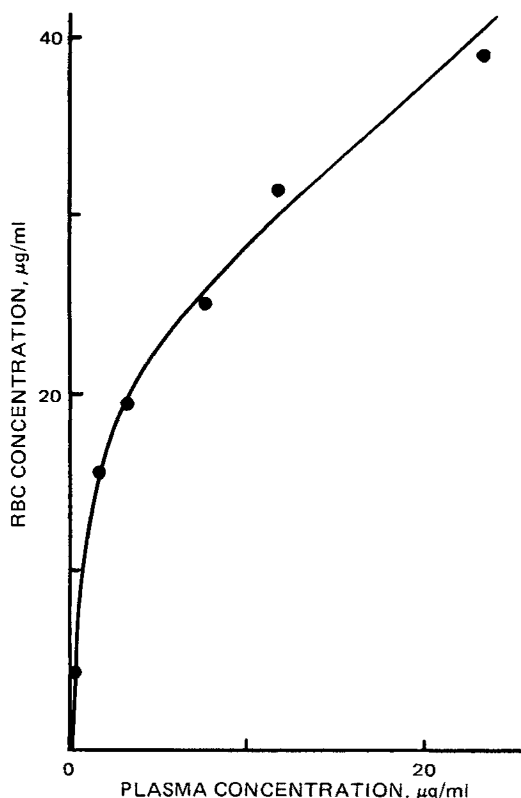

Figure 2—*Erythrocyte concentration of acetazolamide at equilibrium as a function of the total plasma concentration.*

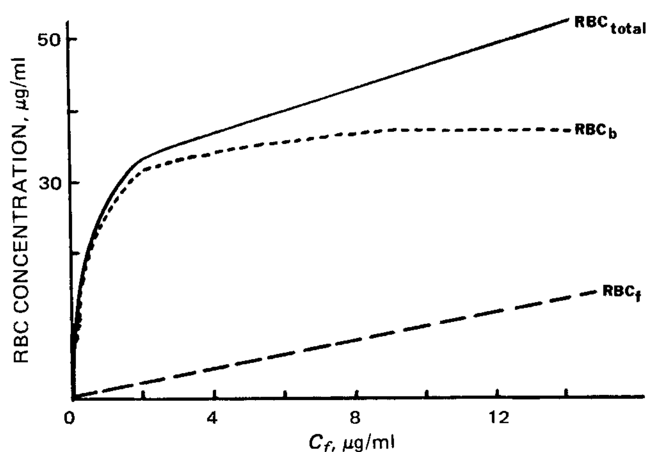

Figure 3—*Total (RBC_{total}, obtained experimentally), bound (RBC_b), and free (RBC_f) erythrocyte concentrations of acetazolamide as a function of the free drug concentration in plasma (C_f).*

Thus, to determine binding parameters for acetazolamide and erythrocytes, blood, total plasma, and free plasma concentrations were determined at equilibrium. Since greater than 90% of acetazolamide in plasma is bound to plasma protein (7), plasma ultrafiltrates were obtained to measure the freely diffusible drug in whole blood. By using a synthetic blood medium of erythrocytes suspended in isotonic buffer, the free drug concentration can be measured directly in the protein-free supernate. Washing the erythrocytes with isotonic buffer in the process of preparing the suspension does not alter the uptake of the drug. Uptake of acetazolamide from the plasma by untreated erythrocytes and by those washed with buffer is essentially the same (Fig. 4).

The concentration bound by erythrocytes was calculated by subtracting the unbound plasma concentration from the total erythrocyte concentration. Data were linearized according to Eq. 4, and the binding parameters were calculated by least-squares linear regression analysis (Fig. 4).

Blood samples varied in their maximum binding capacity and dissociation constant for acetazolamide (Table I). However, the values were in the same range as those reported (9) for human erythrocyte carbonic anhydrase B, *i.e.*, a molar concentration of 136 μM (30 µg/ml) and an inhibitory constant (K_i for acetazolamide) of 1 μM (0.22 µg/ml). These data strengthen the hypothesis (5) that the accumulation of the drug within the erythrocytes is due to binding to intracellular carbonic anhydrase.

Because blood samples varied not only in their erythrocyte binding but also in their plasma protein binding of acetazolamide, the plasma concentration at which erythrocyte binding sites become saturated cannot be closely defined. Lehmann *et al.* (9) suggested that inhibition of erythrocyte carbonic anhydrase may induce some of the side effects of acetazolamide therapy. However, attempts to delineate a maximum tolerable plasma level of drug sufficient to saturate erythrocyte enzyme may be thwarted by the large individual variation in binding parameters. Determination of the erythrocyte binding capacity for acetazolamide may

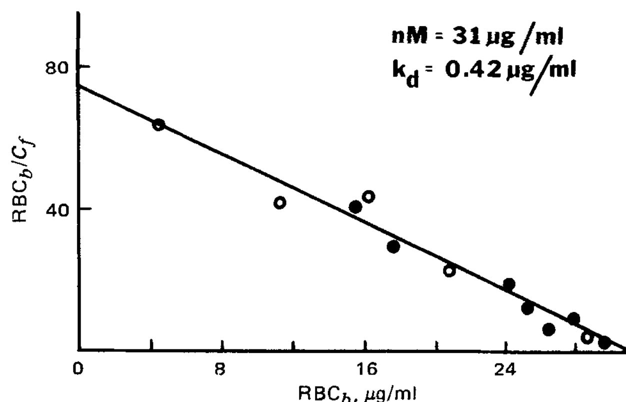

Figure 4—*Rosenthal plot of the binding of acetazolamide to human erythrocytes* in vitro, *Experiment 4. Key:* O, *washed blood cells reconstituted with pH 7.4 phosphate buffer; and* ●, *whole blood.*

Table I—Binding of Acetazolamide to Human Erythrocytes *In Vitro*

		k_d, µg/ml	nM, µg/ml
1[a]	Erythrocytes in buffer, pH 7.4	0.50	21
2	Whole blood	0.23	27
	Erythrocytes in buffer, pH 7.4	0.23	28
3	Whole blood	0.41	29
	Erythrocytes in buffer, pH 7.4	0.24	27
4	Whole blood	0.43	32
	Erythrocytes in buffer, pH 7.4	0.40	31
	Mean	0.35	28
	SD	0.11	3.6

[a] Numbers indicate experiments performed with different units of whole blood.

still provide an estimate of the safe therapeutic limit for the plasma concentration of the drug.

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ACKNOWLEDGMENTS AND ADDRESSES

Received February 26, 1976, from the *Department of Pharmacy, School of Pharmacy, University of California, San Francisco, CA 94143.*

Accepted for publication June 21, 1976.

Presented at the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Atlanta meeting, November 1975.

Supported in part by U.S. Public Health Service Grant NIGMS 16496 and Lederle Laboratories.

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Direct Analysis of Salicylic Acid in Keratolytic Plaster by Gas–Solid Chromatography

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Abstract ☐ A rapid gas-solid chromatographic method is reported for the direct analysis of salicylic acid in keratolytic plaster. The method requires no preseparation or derivatization and takes about 14 min. Since it requires only 2–7 mg of sample, it can determine the salicylic acid concentration in a particular area of plaster. The analyzing column is packed with porous polymer beads of 2,6-diphenyl-1,4-phenylene oxide. An automatic solid sampling system is used in connection with the gas chromatograph employing dual flame-ionization detectors. The average recovery of salicylic acid in synthetic samples containing 400–2700 µg was 100.6 ± 1.6%. The average correlation coefficient of the peak area against the standard was +0.9995 ± 0.0003 for samples in the 300–3000-µg range.

Keyphrases ☐ Salicylic acid—gas-solid chromatographic analysis, commercial plaster pads ☐ Gas–solid chromatography—analysis, salicylic acid in commercial plaster pads ☐ Keratolytics—salicylic acid, gas-solid chromatographic analysis in commercial plaster pads

The 40% salicylic acid (I) keratolytic plaster is a uniform mixture of I in a suitable base spread on a backing material such as paper or cloth. The USP (1) requires the plaster mass to contain not less than 90.0% and not more than 110.0% of the labeled amount of I.

To check for uniform distribution of I and for specification compliance, a specific method that does not require a fairly large sample is needed. Since the USP method (1) requires more than 1 g of the plaster base, excluding the backing material, it lacks the sensitivity desired for the type of product[1] with considerably less than 10 mg of the plaster base in a small disk. Furthermore, the USP method is very lengthy.

BACKGROUND

Literature methods for the determination of I that are based on measurement of UV absorption (2–4), color intensity of its ferric chelate (5, 6), fluorescence (7, 8), and alkalimetric titration (9) either required extensive separation manipulations or gave unsatisfactory results due to interference by the excipients in the plaster base.

Several investigators reported the use of GLC for the determination of I in pharmaceuticals and biological media (10–14). However, the methyl ester and methyl ether derivatives (11, 12) or the trimethylsilyl derivative (13, 14) of I had to be prepared before GLC analysis for sufficient volatility; otherwise, severe tailing occurred (10).

Because of the low solubility of the plaster base in most common solvents, methods requiring additional steps to extract I prior to GLC determination or other functional group analyses are less desirable than a direct method by which I in a plaster base can be analyzed without preseparation.

A direct method by gas-solid chromatography (GSC) for determining underivatized I with three other ingredients in a mixture was reported previously (15). This paper reports a rapid and specific GSC method using the same column (15) for the direct determination of I in the plaster base of callus pads without preliminary separation or derivatization.

[1] Zino Corn Pads, Scholl, Inc., Chicago, IL 60610.