Effect of Edetate Disodium and Reduced Glutathione on Absorption of Acetazolamide from GI Tract of Rats

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Abstract □ The absorption of acetazolamide suspensions from *in situ* rat gastric and intestinal loop segments was studied. In 1 hr, 66.2 and 64.3% remained unabsorbed from the rat stomach and intestine, respectively. Although 1% (w/v) reduced glutathione and 1% (w/v) (24 m*M*) edetate disodium had no effect on gastric absorption, drug absorption from the rat intestine (1 hr) was increased 1.5 and 2 times, respectively. It was hypothesized that the relatively poor intestinal absorption was due primarily to the formation of a pH-dependent (pH 4.5–10), nonabsorbable complex between acetazolamide and carbonic anhydrase present in the gut and that reduced glutathione acted as an inhibitor to promote intestinal absorption. Equilibrium dialysis studies showed that reduced glutathione could reduce the fraction of drug bound to human carbonic anhydrase B by one-half when present in a molar ratio 10 times that of acetazolamide; edetate disodium had no effect on the *in vitro* binding. It was, therefore, assumed that edetate disodium promoted an increase in intestinal absorption by altering the permeability of intestinal epithelium. Based upon present experimentation, however, the alteration of intestinal epithelium by reduced glutathione cannot be ruled out.

Keyphrases □ Acetazolamide—GI absorption, effect of edetate disodium and reduced glutathione, rats □ Edetate disodium—effect on GI absorption of acetazolamide, rats □ Glutathione, reduced—effect on GI absorption of acetazolamide, rats □ Absorption, GI—acetazolamide, effect of edetate disodium and reduced glutathione, rats

There is little information in the literature regarding the bioavailability of acetazolamide preparations. However, a plasma level comparison was presented recently (1) in which 500-mg acetazolamide compressed tablets and 500-mg sustained-release granules in capsule form were crossed over in human subjects. By comparing areas under the plasma level–time curves, it was found that the bioavailability of the sustained-release preparation was approximately one-half that of an equal dose of compressed tablets. Another study (2) reported nearly identical results.

One explanation for the reduced bioavailability of the sustained-release dosage form is that the intestinal transit time of the slow-release granules occurs at a rate faster than the drug can be released and subsequently absorbed, assuming the drug to be absorbed over a large portion of the small intestine. Conceivably, a preferential absorption site exists for acetazolamide. If acetazolamide is absorbed from a relatively small segment of the GI tract, then releasing drug after the slow release granules have passed that site would give a decreased extent of absorption compared to a rapidly dissolving tablet that releases drug before the site is reached.

The latter theory becomes more attractive by considering the results of a study by Coleman (3). In this study, the binding of acetazolamide to human carbonic anhydrase B was characterized at 4° at pH 2–11. Maximum binding occurred at pH 7, producing a 1:1 complex with an association constant of 1.25×10^7 L/M. At pH 4.5 and 10, only about 0.25 mole of acetazolamide was bound/mole of enzyme when free acetazolamide was equimolar to the enzyme at 2×10^{-5} M.

Since the GI pH is 1–3 in the stomach but occasionally as high as 5 following a meal and is 5–8 in the intestine (4), it is possible, based upon pH, that acetazolamide absorption in the intestine could be hindered by binding to carbonic anhydrase, if a sufficient concentration of enzyme existed. The concentration of carbonic anhydrase in the rat GI tract was reported (5) to be (μmoles/g wet tissue): stomach, 122; duodenum, 1.3; jejunum, 0.44; ileum, 0.32; and colon, 5.9. Although the enzyme is located intracellularly, normal extrusion of cells from human villi accounts for 250 g released into the intestine daily (6). Acetazolamide, bound to as large a molecule as carbonic anhydrase (mol. wt. = 30,000), would not be expected to transverse biological membranes passively.

Additional work by Coleman (3) showed that the binding was not only reversible and pH dependent but could be inhibited by cyanide and sulfide anions and required zinc ion. With this information, it was proposed that reduced glutathione, which contains a reactive sulfhydryl group, or edetate disodium, a chelating agent for zinc ion, could sufficiently inhibit the binding of acetazolamide and carbonic anhydrase and thus promote an increase in the rate and extent of absorption of acetazolamide in the intestine.

The present study was undertaken to examine the effects of reduced glutathione and edetate disodium on the GI absorption of acetazolamide from ligated loops of the rat GI tract.

EXPERIMENTAL

Test Animals—Male, Charles River strain rats, 240–340 g, were used for the absorption studies. The rats were fasted 14–20 hr prior to a given experiment, but water was allowed *ad libitum*.

Apparatus and Reagents—Acetazolamide USP[1], reduced glutathione[2], and human carbonic anhydrase B[3] were used as received. All other chemicals were reagent grade. A pH meter[4] and a ratio recording spectrophotometer[5] were used.

Solubility Determinations—Saturated solutions of acetazolamide in pH 4.0 McIlvaine's citrate buffer and pH 7.0 Sorensen's phosphate buffer were prepared at 25 and 37° and equilibrated for 24 hr in a shaker bath in the presence of excess acetazolamide. Aliquots were appropriately diluted with 0.1 N sodium hydroxide, and the absorbances were determined at 288 nm.

Drug Preparation—Drug suspensions were prepared by plac-

[1] Lederle Laboratories, Pearl River, N.Y.
[2] K and K Laboratories, Plainview, N.Y.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Beckman Zeromatic II.
[5] Beckman DK-2A.

ing 125 mg of acetazolamide in a 10-ml syringe and diluting to 5 ml with either McIlvaine's citrate buffer (pH 4.0) or Sorensen's phosphate buffer (pH 7.0) to give a final drug concentration of 25 mg/ml. Suspensions were also prepared containing 1% (w/v) reduced glutathione or 1% (w/v) edetate disodium, both of which dissolved in the buffer systems. Sodium chloride was added to maintain isotonicity. The suspensions were prepared 2–3 hr prior to use and shaken for maximum dissolution to take place.

***In Situ* Absorption Procedure**—The method of Levine *et al.* (7) was employed to study the absorption of acetazolamide from the GI tract of rats. In this procedure, the rats were anesthetized with urethan (1 g/kg ip). For the studies involving the stomach, ligatures were made proximal to the cardiac and pyloric sphincters. The pyloric ligature was tied; but prior to tying off the cardiac ligature, the drug suspension was injected into the stomach by means of a syringe and blunt needle.

The intestinal preparation was made by ligating the small intestine 1 cm proximal to the cecum and midway in the jejunum between the arterioles of the first intestinal and jejunal arteries. These positions were chosen because they could be easily reproduced, did not require removal of the intestine from the abdominal cavity, and, therefore, represented a minimal amount of handling.

Drug suspension was introduced *via* the jejunal ligature just prior to tying. After 60 min, either the tied stomach or intestinal segments were excised, cut open to expose the residual acetazolamide, and washed repeatedly with about 20 ml of a pH 9–10 sodium hydroxide solution. The solution was brought to a final volume of 25.0 ml and subsequently assayed for acetazolamide content.

Equilibrium Dialysis Studies—The binding of acetazolamide to carbonic anhydrase was determined using a dialyzing system consisting of a cylindrical plexiglass cell separated into two compartments (5-ml capacity on each side) by a dialyzing membrane[6]. The membranes were stored in isotonic buffer overnight and rinsed with fresh buffer before use. Acetazolamide was dissolved in isotonic pH 4.0 or 7.0 buffer and pipetted into one compartment of the cell, designated the external compartment.

The internal compartment contained human carbonic anhydrase and in some cases reduced glutathione or edetate disodium. Equilibrium dialysis was carried out at 37° for approximately 36 hr in a water bath equipped with a shaker[7]. The extent of binding was calculated from the concentration of free acetazolamide on the external side.

Analytical Methods—A 0.25-ml lumen sample was placed into a 5-ml volumetric flask and brought to volume with 0.1 *N* HCl. Then 2 ml was transferred to a 25-ml screw-capped glass culture tube, and 5 ml of isobutyl alcohol was added to extract the acetazolamide[8]. The two phases were shaken together for 5 min and centrifuged to facilitate separation of the layers. Then 4 ml of the isobutyl alcohol layer was removed and added to a small beaker containing about 0.5 g of anhydrous sodium sulfate to remove any water solubilized in the alcohol.

The isobutyl alcohol was decanted off, and a quantitative volume (about 3.5 ml) was added to another screw-capped glass culture tube containing 10.0 ml of 0.1 *N* sodium hydroxide. The two phases were shaken for 5 min and centrifuged, and the aqueous layer was measured for acetazolamide content against an appropriate blank at a fixed wavelength of 288 nm.

RESULTS AND DISCUSSION

Assay—To establish the reliability of the double-extraction assay procedure, isotonic buffer solutions were first added to ligated stomach and intestinal loops of the rat. After 60 min, the buffer solutions were collected from the respective loops, spiked with drug in concentrations of 0.2, 0.4, 0.6, and 0.75 mg/ml, and then extracted and assayed for acetazolamide content.

A constant percentage of drug was extracted from the lumen perfused buffers at each concentration, yielding averaged values of 72.5% ± 3.0 (n = 4) and 78.8% ± 4.2 (n = 4) for stomach and intestinal loop preparations, respectively. A nearly identical Beer's law relationship was followed with each set of data. Neither reduced glutathione nor edetate disodium interfered with the absorbance of acetazolamide at 288 nm.

Table I—Solubility of Acetazolamide at pH 4 and 7 and 25 and 37°

Buffer	pH	Solubility[a], mg/ml 25°	37°
McIlvaine's citric acid–phosphate	4.0	0.75 (0.0064)	1.38 (0.010)
Sorensen's phosphate	7.0	0.98 (0.0051)	2.09 (0.0058)

[a] Mean (±*SD*) where n = 3.

Acetazolamide Solubility—Table I gives the results of the solubility measurements at each pH and temperature. As the solvent pH approached the pKa of acetazolamide, 7.2 (8), its solubility improved as might be expected. Because of the limited solubility of acetazolamide at physiological pH values, suspension dosage forms were prepared.

Equilibrium Dialysis Results—Table II lists the concentrations studied as well as the results obtained for the binding of acetazolamide to human carbonic anhydrase at pH 4.0 and 7.0 with and without reduced glutathione and edetate disodium. At pH 7 and 37°, acetazolamide bound to human carbonic anhydrase to the extent of 19.0%[9] when present in a molar ratio (acetazolamide–carbonic anhydrase) of 3.0. By studying the same pH and concentration of acetazolamide and carbonic anhydrase but including reduced glutathione in a molar ratio of 10.0 to acetazolamide, the percent bound was reduced approximately one-half to 10.4%.

Edetate disodium, however, in molar ratios to acetazolamide of 29.0 and 290.0, showed no tendency toward inhibiting the binding at pH 7.0. At pH 4.0[10] and the same molar ratio of acetazolamide–carbonic anhydrase (3.0), no significant binding could be detected. The presence of either reduced glutathione or edetate disodium did not alter the pH 4.0 results.

***In Situ* Absorption Results**—Table III gives the results of the effect of edetate disodium and reduced glutathione on the absorption of acetazolamide from the stomach and intestine. Without the presence of adjuvants, 66.2 and 64.3% remained unabsorbed in the rat gastric and intestinal loop segments, respectively, in 1 hr. Although the duodenum and a section of the jejunum were not included in the intestinal loop segment, the intestinal segment as such would be expected to contain greater surface area and vascularity than the stomach preparation (8).

The relatively low absorption obtained for the intestine conflicts with the generally held belief that drug absorption occurs predominately from the intestine rather than the stomach (7). If it is assumed that the pH–partition hypothesis applies to acetazolamide and that greater vascularity and surface area exist in the intestinal loop than in the gastric loop, one would expect better intestinal absorption for the drug since its higher solubility at pH 7 (37°) permits an approximately equal concentration of unionized species (Table I: 1.38 mg/ml at pH 4 and 37° and 1.28 mg/ml at pH 7 and 37°) compared to pH 4.

In the presence of 1% (w/v) reduced glutathione and 1% (w/v) edetate disodium (24 m*M*), acetazolamide absorption in the stomach was not significantly improved (Table III). However, the intestinal results showed 2 and 1.5 times the extent of absorption at 1 hr when drug suspensions contained edetate disodium and reduced glutathione, respectively; both values were statistically significant. These results are qualitatively in agreement with the equilibrium dialysis results insofar as they support the hypothesis that the binding of carbonic anhydrase and acetazolamide could be inhibited in the presence of reduced glutathione at pH 7, potentially to promote acetazolamide absorption in the intestine but having no consequence on drug absorption from the stomach at pH 4.0.

Even though the stomach (parietal and epithelial cells) has the highest concentration of enzyme, its pH is unfavorable for binding.

[6] Dialyzing cellophane, Union Carbide Corp., New York, N.Y.
[7] American Optical model A002156, Dallas, Tex.
[8] Robert Goldman, Garden Laboratories, Springfield Gardens, N.Y., personal communication.

[9] Acetazolamide was found to bind to the dialysis cell and/or membrane on the order of 5.09 ± 2.1% (n = 5); the fraction bound was corrected accordingly.
[10] Because of the limited solubility of human carbonic anhydrase in pH 4.0 buffer, its concentration was reduced.

Table II—Binding of Acetazolamide to Human Carbonic Anhydrase B in the Presence and Absence of Edetate Disodium and Reduced Glutathione at 37° Using Equilibrium Dialysis

Determination	Concentration[a] Acetazolamide (×10⁵)	Human Carbonic Anhydrase B (×10⁵)	Reduced Glutathione (×10³)	Edetate Disodium (×10²)	pH	Percent Bound[b]
1	8.28	2.76	—	—	4.0[c]	0.930[d] (2.23)
2	8.28	2.76	0.828	—	4.0	0.160[d] (1.63)
3	8.28	2.76	—	1.2	4.0	−0.160[d] (3.30)
4	41.4	13.8	—	—	7.0[e]	19.0 (5.82)
5	41.4	13.8	4.14	—	7.0	10.4 (2.66)
6	41.4	13.8	—	1.2	7.0	20.4 (1.46)
7	41.4	13.8	—	12.0	7.0	19.1 (4.60)

[a] Expressed as molarity. [b] Mean (± *SD*) where $n = 3$ for Determinations 1–3, $n = 4$ for Determinations 4–6, and $n = 2$ for Determination 7. [c] McIlvaine's citric acid–phosphate buffer. [d] Taken as no perceptible binding. [e] Sorensen's phosphate buffer.

Table III—Effect of Edetate Disodium and Reduced Glutathione on Absorbance of Acetazolamide after 1 hr from the Rat Stomach (pH 4.0) and Intestinal (pH 7.0) Loop Segments

Experimental Conditions	Number of Gut Segments	Percent Unabsorbed[a]	*p*
Stomach			
Acetazolamide, pH 4.0[b]	5	66.2 (6.4)	—
Acetazolamide, 1% (w/v) reduced glutathione, pH 4.0	5	55.3 (12.2)	N.S.[c]
Acetazolamide, 1% (w/v) edetate disodium, pH 4.0	5	60.4 (9.52)	N.S.[c]
Intestine			
Acetazolamide, pH 7.0[d]	5	64.3 (3.52)	—
Acetazolamide, 1% (w/v) reduced glutathione, pH 7.0	5	45.4 (14.4)	0.02[e]
Acetazolamide, 1% (w/v) edetate disodium, pH 7.0	5	28.2 (10.4)	0.001[e]

[a] Mean (±*SD*). [b] McIlvaine's citric acid–phosphate buffer. [c] Paired *t*-test, N.S. = nonsignificant or $p > 0.05$ compared to drug alone in the stomach preparation. [d] Sorensen's phosphate buffer. [e] Paired *t*-test, compared to drug alone in intestinal loop segment.

Throughout the small intestine, however, the pH is favorable for maximum binding. The enhancement effect could not be attributed to an increase of acetazolamide solubility in the presence of reduced glutathione or edetate disodium. This was determined in an additional study in which excess acetazolamide was shaken in pH 7 buffer alone and with 1% (w/v) reduced glutathione and 1% (w/v) edetate disodium for 4 hr at 37°, the approximate total length of time the *in vivo* suspension dose was allowed to equilibrate. The solubility determinations, performed in triplicate, were all within a range of 2.0 mg/ml ± 5%.

It is conceivable that glutathione enhanced the *in situ* intestinal absorption of acetazolamide by altering the permeation of intestinal epithelium. Figure 1 depicts the physicochemical events proposed at the intestinal site of absorption. It is assumed that the rate of absorption is proportional to the concentration of free drug in solution, A_f. If it also can be assumed that a rapid, reversible

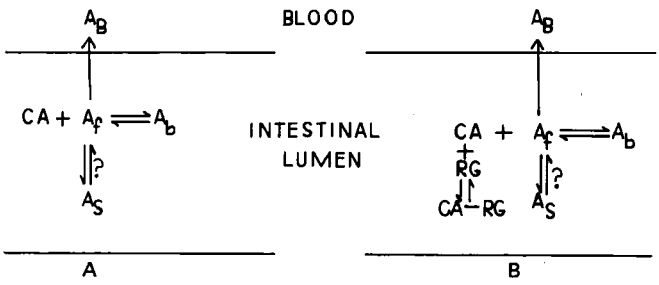

Figure 1—*Schematic representation of the hypothesized competitive mechanisms representing acetazolamide absorption in the rat intestinal tract at pH 7 in the presence (B) and absence (A) of the proposed inhibitor, reduced glutathione (RG). All symbols represent intestinal concentrations, where CA = carbonic anhydrase; A_f = free acetazolamide; A_b = hypothesized, bound, nonabsorbable complex; A_s = excess solid acetazolamide; and CA–RG = bound complex. The symbol ? questions the rapidity with which A_s can dissolve to maintain a constant A_f as free drug is absorbed from the intestinal lumen of the rat (A_B).*

equilibrium exists between the A_f and the postulated nonabsorbable complex of drug and carbonic anhydrase (Fig. 1A) and between A_f and excess solid drug, A_f would be expected to remain constant in spite of the fact that the fraction bound could be altered by the presence of reduced glutathione, RG (Fig. 1B). Excess solid could maintain the A_f regardless of the degree of binding that might occur; hence, the absorption rate and the amount of drug remaining in the intestinal lumen could only increase 1.5 times if the permeability of the absorbing membrane to acetazolamide increased due to the presence of reduced glutathione.

However, it was questioned whether the dissolution process near saturation levels would be rapid enough to maintain the A_f as drug was absorbed. An *in vitro* experiment was conducted to test the assumption that a rapid readjustment of the equilibrium exists between free drug in solution and excess solid under near saturation conditions (2 mg/ml) at 37°. Drug suspensions were prepared by placing 625 mg of acetazolamide into 25 ml of pH 7 buffer in a shaking water bath at 37°. After 3 hr, 1.0 ml of solution was filter pipetted and assayed for drug content; this value represented a zero-time value. Over the following hour, 3.0 ml was removed at 10-min intervals. An appropriate volume of pH 7 buffer was added at each 10-min interval to maintain the original volume of 25 ml.

The drug concentration was determined for each sample that was removed; the experiment was performed in triplicate. This sampling procedure represented an attempt to approximate the net rate that the absorbing membrane was removing drug from the intestinal lumen in the presence of reduced glutathione, *i.e.*, 45% remaining after 1 hr. After 3 hr of shaking at 37°, a concentration of 2.2 ± 0.075 mg/ml was measured. With each succeeding sample, the concentration of acetazolamide was lower. At the end of 1 hr, the concentration measured 1.91 ± 0.0095 mg/ml, which was statistically different from the zero-time value ($p < 0.03$) but greater than one would expect if no dissolution occurred under the experimental conditions or at 1.02 mg/ml.

Consequently, it is feasible that the absorption process occurs at a rate faster than the dissolution process, such that the increased amount of drug absorbed from the intestinal loop in the presence of reduced glutathione could be a consequence of competitive inhibition. However, the alteration of the intestinal epithelium by reduced glutathione cannot be ruled out without further experimen-

tation since the results are also compatible by this mechanism.

Edetate disodium has promoted an increase in intestinal (rat) absorption of salicylate anion (9), phenolsulfonphthalein (10), heparin (11), mannitol (11), inulin (11), decamethonium (11), and sulfanilic acid (11). It has been postulated that edetate disodium alters the aqueous permeability of the intestinal epithelium by depleting magnesium and calcium ions, resulting in separation of the epithelial cells of the rat intestine (12, 13). This mechanism is likewise suspected with acetazolamide, since the lack of effect of edetate disodium in inhibiting the binding of acetazolaminde to human carbonic anhydrase discounts the possibility of enzyme inactivation by removal of zinc ion. The possibility of edetate disodium acting as an *in vivo* inhibitor to the binding of acetazolamide to carbonic anhydrase is made more difficult by the fact that an endogenous source of free calcium exists to bind preferentially to the divalent edetate and to prevent calcium removal from the enzyme.

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GLC Assay of Belladonna Extracts

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Abstract □ Samples of belladonna pilular and powdered extract and tincture from two suppliers were analyzed by GLC as a cooperative effort between two laboratories to develop methodology with substantial improvements in sensitivity, specificity, precision, and working time over the present official method. This goal was achieved, but marked differences in response of the individual extracts to different isolation schemes were noted.

Keyphrases □ Belladonna extract—GLC analysis in pharmaceutical formulations, suitability of anhydrous sodium sulfate in isolation, compared to official method □ Alkaloids, belladonna—GLC analysis in pharmaceutical formulations, suitability of anhydrous sodium sulfate in isolation, compared to official method □ Atropine—GLC analysis, pharmaceutical formulations of belladonna extract □ Scopolamine—GLC analysis, pharmaceutical formulations of belladonna extract □ Hyoscyamine—GLC analysis, pharmaceutical formulations of belladonna extract □ GLC—analysis, belladonna alkaloids, pharmaceutical formulations

Reliable GLC assays (1, 2) of atropine and scopolamine in dosage forms have come into general use in recent years and have been the basis of extensions (3) to other drug mixtures. Official titrimetric assays (4, 5) for the crude belladonna extracts (powdered and pilular) and the directly derived tincture are tedious, unspecific, and unreliable because of emulsion problems. Application of the GLC method to these mixtures of plant extracts would be desirable, and this paper reports the results of a cooperative effort between two laboratories to accomplish this goal. Some earlier assay work on plant extracts from belladonna by a GLC method was reported (6), and inaccuracies of the titrimetric approach also were noted then.

EXPERIMENTAL

All reagents were USP, NF, or ACS grade. Samples of pilular and powdered extracts were received from manufacturers, and the tincture was obtained locally. The optical isomers in atropine are not separated, so the combined atropine–hyoscyamine peak is expressed solely as atropine herein for convenience.

Chromatographic System[1]—A 1.2-m × 4-mm i.d. glass column packed with 3% methyl phenyl polysiloxane oil on 100–120-mesh acid-washed, flux-calcined, diatomaceous earth[2] was cured and conditioned as specified elsewhere (1, 2, 5). Minimal pledgets of silanized glass wool were used to minimize the catalytical conversion of atropine to apoatropine (6, 7). The column was maintained at 215°, and the injection port and flame-detector block temperatures were 240 and 245°, respectively; dry helium was used as a carrier gas at a flow rate of 65 ml/min. Electronic peak measurement was employed[3].

System Suitability—Chromatograph six to 10 injections of the assay preparation, and record peak areas as directed under *Procedure*. The analytical system is suitable for conducting this assay if the relative standard deviation for the ratio, R_A, calculated by the formula 100 × (standard deviation/mean ratio), does not exceed 2.0%; the resolution factor between A_H and A_A is not less than 5; and the tailing factor (the sum of the distances from peak center to the leading edge and to the tailing edge divided by twice the distance from the peak center to the leading edge), measured at 5% of the peak height of A_A, does not exceed 2.0.

[1] HP 5750B gas chromatograph with flame-ionization detector fitted for on-column injection.

[2] OV-17 on Gas Chrom Q, Applied Science Laboratories.

[3] Infotronics CRS 204 digital integrator.