Assay for Acetazolamide in Plasma

W. F. BAYNE ˣ, G. ROGERS, and N. CRISOLOGO

Abstract □ A method for the analysis of acetazolamide, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide, sensitive to 25 ng/ml in plasma, was developed. After extraction of acetazolamide and its propionyl analog, 5-propionamido-1,3,4-thiadiazole-2-sulfonamide, the internal standard, from plasma with ethyl acetate and removal of lipids from the residue of the ethyl acetate extract with methylene chloride, the sulfonamides were chromatographed on an octadecyl trichlorosilane bonded phase using high-pressure liquid chromatography. The method was developed to study plasma level profiles of different dosage forms of acetazolamide.

Keyphrases □ Acetazolamide—high-pressure liquid chromatographic analysis in plasma □ High-pressure liquid chromatography—analysis, acetazolamide in plasma

Acetazolamide, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide, is a carbonic anhydrase inhibitor used to lower intraocular pressure through a reduced production of aqueous humor. Its inhibitory effect on carbonic anhydrase was used to develop an assay for acetazolamide in biological fluids (1). The drug was reported to be 90% excreted, on the average, in humans after oral administration (2). The enzymatic method appears to be fairly specific; only metabolites that inhibit carbonic anhydrase should interfere with the specificity of the assay.

A number of unsuccessful attempts to develop chemical methods for acetazolamide in biological fluids were described (1). However, a modification of the Bratton and Marshall procedure (3), a colorimetric method sensitive to 5 μg/ml, was reported for acetazolamide (4). Attempts to prepare acetazolamide derivatives suitable for GLC analysis by the authors were generally unsuccessful. Secondary sulfonamides were methylated with diazomethane (5), but a number of products resulted when acetazolamide, a primary sulfonamide, was treated with diazomethane in this laboratory.

Blessington (6) successfully methylated primary and secondary sulfonamides using a method employed by Pettitt and Stouffer (7) for alkylation of amino acids. Application of this method, utilizing the sulfinyl carbanion of dimethyl sulfoxide and methyl iodide, to acetazolamide yielded one product which was not successfully characterized by GC–mass spectrometry.

Table I—Absolute Recoveries of Acetazolamide from Plasma

Acetazolamide, μg	Recovery, % (Number of Determinations)
0.1	68.0 (3)
0.25	74.9 (2)
0.5	77.3 (3)
0.75	79.3 (2)
1.0	87.0 (3)
2.5	84.0 (2)
5.0	94.2 (3)
10.0	87.0 (1)

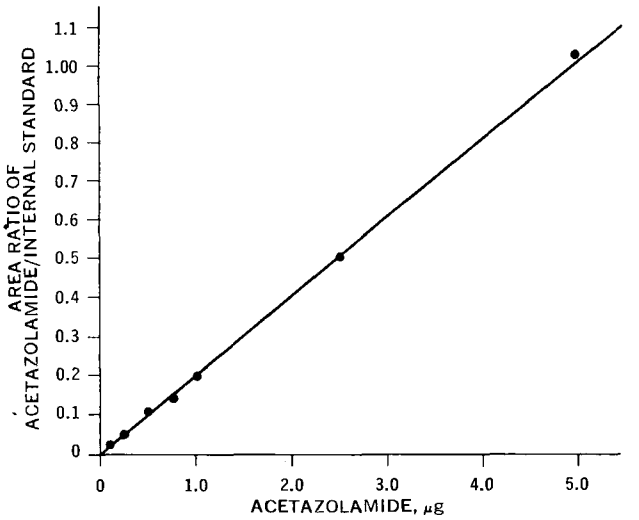

Figure 1—*Standard curve for acetazolamide (5.32 μg of internal standard).*

An extractive alkylation technique recently used to methylate chlorthalidone (8) was investigated by the authors for its potential use in preparing a volatile methylated acetazolamide derivative. Although a single peak resulted under the chromatographic conditions, the peak actually consisted of two compounds, the trimethylated derivative of acetazolamide and the tetramethylated derivative of 5-amino-1,3,4-thiadiazole-2-sulfonamide (I). If the propionyl analog of acetazolamide was methylated, the trimethylated derivative of the analog and I resulted. In this case, the two compounds were resolved, the former having the longer retention time.

Kram (9) separated a number of sulfonamides using high-pressure liquid chromatography with pellicular anion-exchange resins. A recent publication (10) indicated the feasibility of separating sulfonamides by reverse phase chromatography. Therefore, a method for acetazolamide, sensitive to 25 ng/ml plasma, was developed using high-pressure liquid chromatography that requires no prior derivatization.

EXPERIMENTAL

Materials—A piston-driven, constant-flow, pulse-free pump[1], with a 2000-psi limit, in conjunction with a septumless injector and 254-nm UV detector[2] (8-μl flow cell), was used for analyzing samples. The output was recorded on a variable span, potentiometric recorder. Most separations were performed on a 1-m, 6.35-mm o.d., 2-mm i.d., precision-bored stainless steel column packed with an octadecyl trichlorosilane bonded phase[3] with a particle size of 30–44 μm. Sodium acetate buffer (0.05 *M*, pH 4.5) with 2% (v/v)

[1] Instrumentation Specialties Co., Lincoln, Neb.
[2] Varian Instrument Division, Palo Alto, Calif.
[3] Vydac Reverse Phase, Applied Science Laboratories, State College, Pa.

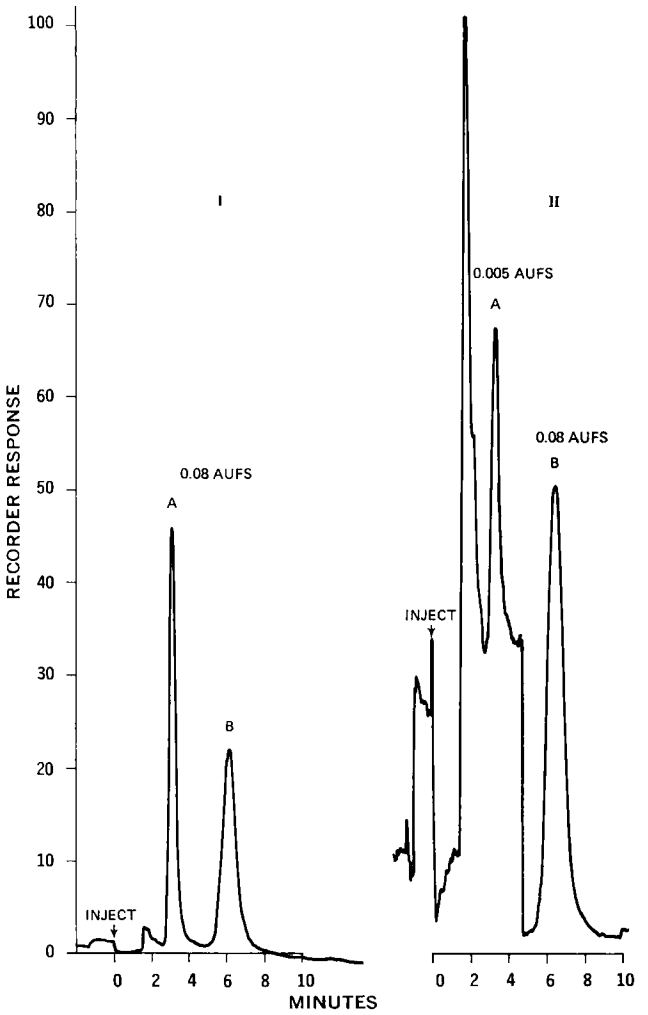

Figure 2—*Chromatograms of acetazolamide (A) and internal standard (B) in 1 ml of plasma. Key: I, 0.1 μg of acetazolamide and 5.32 μg of internal standard; and II, 5.0 μg of acetazolamide and 5.32 μg of internal standard.*

methanol was employed as the mobile phase. The flow rate was 48 ml/hr with pressures in the neighborhood of 1050 psi.

Some separations were performed on a 30-cm, 4-mm i.d. column packed with octadecyl trichlorosilane bonded to less than 10 μm diameter packing material which recently became available[4]. In this case, the same buffer was used but contained 15% (v/v) methanol. The flow rate was 120 ml/hr with pressures of about 1500 psi.

Reagents—Glycine buffer was prepared by addition of sulfuric acid to an aqueous glycine solution to obtain a pH 2 buffer. Sodium acetate and ammonium acetate buffers were prepared by titrating acetic acid with sodium hydroxide or ammonium hydroxide, respectively, to obtain pH 4.5 buffers.

Extraction Procedure and Standard Curve—Solutions of acetazolamide[5] and internal standard, 5-propionamido-1,3,4-thiadiazole-2-sulfonamide[6], were prepared daily in 0.01 *N* NaOH at concentrations of 1 mg/ml. The acetazolamide solution was diluted 1:100 and 1:20 with 0.01 *N* NaOH prior to addition to plasma. To 1-ml quantities of plasma, contained in 40-ml Teflon-stoppered[7] centrifuge tubes, 0.1, 0.25, 0.5, 0.75, 1.0, 2.5, and 5.0 μg of acetazolamide were added. The 1:100 dilution was used for the first four samples, while the 1:20 dilution was used for the last three samples.

From a 1:10 dilution of the internal standard solution, a constant amount of internal standard, 5.32 μg, was added to the solutions containing the different quantities of acetazolamide. Three milliliters of 0.5 *M* ammonium acetate buffer (pH 4.5) was added to the plasma. The aqueous phase was extracted twice with 10-ml portions of ethyl acetate, the contents being shaken for 5 min and centrifuged at 2500 rpm after each extraction. The combined organic phase was transferred to another 40-ml centrifuge tube and evaporated at 40° with a stream of nitrogen.

To the yellow residue, 4 ml of 0.25 *M* glycine buffer (pH 2) was added. The aqueous phase was extracted twice with 5-ml portions of methylene chloride and the organic phase, which retained the yellow pigments, was discarded. During this extraction, the contents were shaken gently to prevent emulsion formation. The aqueous phase was then extracted twice with 6-ml portions of ethyl acetate, and the combined organic extracts were evaporated to dryness. The residue was dissolved in 100 μl of 0.01 *N* NaOH, and the contents were sonified to ensure dissolution. Then 10–25 μl of the 0.01 *N* NaOH solution was injected onto the chromatographic column by the stop-flow technique. The peak areas of acetazolamide and the internal standard at 254 nm were determined by multiplying the peak height times the width at half-height.

The standard curve and typical chromatograms obtained in its preparation are presented in Figs. 1 and 2, respectively. Figure 3 illustrates the chromatogram obtained from a sample containing 25 ng/ml acetazolamide, together with a blank, using the highly efficient, small-diameter packing material.

Analysis of Samples—Approximately 5 μg of the internal stan-

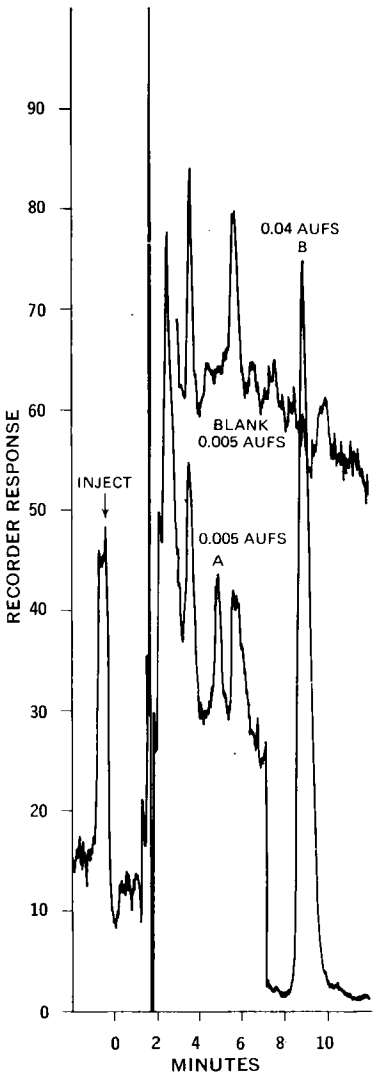

Figure 3—*Chromatograms of acetazolamide (A: 25.2 ng/ml plasma), internal standard (B: 2.58 μg/ml plasma), and blank.*

[4] μBondapak C18, Waters Associates, Milford, Mass.
[5] Sigma, St. Louis, Mo.
[6] Gift of Lederle Laboratories, Pearl River, N.Y.
[7] Kontes, Vineland, N.J.

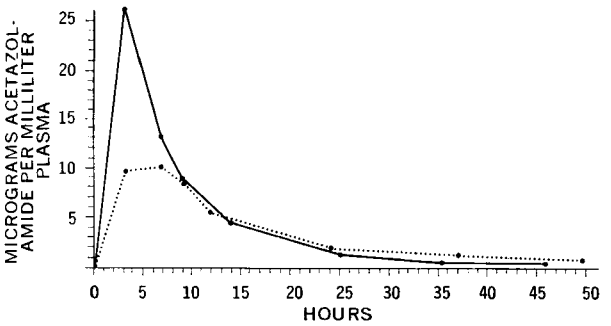

Figure 4—*Plasma level profiles for different dosage forms of acetazolamide. Key: —, tablet, 500 mg; and . . . ., timed-release formulation, 500 mg.*

dard, 5-propionamido-1,3,4-thiadiazole-2-sulfonamide, accurately known through weighing and dilution, was added to the plasma samples to be analyzed. The extraction procedure was then performed as described. The standard curve was used to determine the quantity of acetazolamide present in a known volume of sample. To reduce build-up of highly retained compounds, which reduced the efficiency of the column after repeated injections, it was convenient to inject 50 μl of methanol or dimethylformamide after each sample injection.

RESULTS

When the slope (0.205) for the regression line of the data for the standard curve was multiplied by the amount of internal standard employed (5.32 μg), a value of 1.09 was obtained. This value was very close to the molecular weight ratio (1.06) of the acetamido and propionamido analogs. The molar extinction coefficient for the two compounds was 8.52×10^3 at 254 nm.

The plasma level–time curves for a 500-mg oral tablet and a 500-mg timed-release formulation[8] are presented in Fig. 4. Significant plasma concentrations exist for both dosage forms 45 hr after administration. This finding can be explained by the strong affinity of carbonic anhydrase in the red blood cells and other tissues for acetazolamide. This affinity results in extremely small concentrations of free drug within these tissues and hence small gradients for the transport of drug from these tissues (11). A full analysis of the pharmacokinetics will be presented in the future.

DISCUSSION

Acetazolamide was initially chromatographed on pellicular anion-exchange column. However, the efficiency of the column rapidly declined with repeated injections of biological samples. Acetazolamide was reported to have two pKa's of 7.4 and 9.1 (12). The absorption maximum shifts from 265 nm for the nonionized form to 290 nm for the totally ionized form. Since a 254-nm UV detector was employed, sensitivity was progressively lost as the pH of the buffers was increased from 7 to 9 to increase the retention of acetazolamide. Therefore, reverse phase chromatography was employed. There was no evidence of column degradation with continuous use.

[8] Sequel, Lederle Laboratories, Pearl River, N.Y.

Table II—Area Ratios for Timed-Release Formulation

Hours	Area Ratio, Acetazolamide/ Internal Standard
3.3	1.96; 1.96
6.25	2.02; 2.11
9.25	1.76; 1.73
12.25	1.11; 1.12
24.25	0.409; 0.422
37.25	0.224; 0.234
49.25	0.182; 0.174

The absolute recoveries of acetazolamide from 1 ml of plasma (Table I) ranged from 68 to 94% for concentrations of 0.1–10.0 μg/ml. These recoveries were determined by adding known quantities of acetazolamide to plasma and comparing the amounts recovered, using the previously discussed extraction scheme, to known amounts of acetazolamide injected directly into the liquid chromatograph from the solution used to spike the plasma samples. The recoveries showed the generally expected trend of higher percent recoveries with higher concentrations. This variability was overcome by using an appropriate internal standard with similar partitioning properties.

To check the precision in performing the assay procedure with an internal standard, plasma concentrations from the orally administered timed-release formulation were determined in duplicate. The measured ratios, corrected to the 5.32 μg of internal standard used in the construction of the standard curve, are presented in Table II.

The described analysis, with some modification, can obviously be extended to other sulfonamides and their metabolites.

REFERENCES

(1) T. H. Maren, *J. Pharmacol. Exp. Ther.*, **130**, 26(1960).

(2) T. H. Maren, *Physiol. Rev.*, **47**, 595(1967).

(3) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537(1939).

(4) W. Harke, C. Schirren, and R. Wehrmann, *Klin. Wochenschr.*, **37** 1040(1959).

(5) R. J. Daun, *J. Ass. Offic. Anal. Chem.*, **55**, 1277(1971).

(6) B. Blessington, *Org. Mass Spectrom.*, **6**, 347(1972).

(7) B. C. Pettitt and J. E. Stouffer, *J. Chromatogr. Sci.*, **8**, 735(1970).

(8) M. Ervik and K. Gustavii, *Anal. Chem.*, **46**, 39(1974).

(9) T. C. Kram, *J. Pharm. Sci.*, **61**, 254(1972).

(10) "DS 72-015," Waters Associates, Inc., Milford, Mass., 1972.

(11) T. H. Maren, *Biochem. Pharmacol.*, **9**, 39(1962).

(12) T. H. Maren, *J. Pharmacol. Exp. Ther.*, **117**, 385(1956).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 15, 1974, from *Alza Research, Palo Alto, CA 94304*

Accepted for publication September 26, 1974.

[x] To whom inquiries should be directed.