(2) "British Pharmacopoeia 1973," Her Majesty's Stationery Office, London, England, 1973, p. 385.
(3) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1975, p. 409.
(4) "British Pharmacopoeia 1973," Her Majesty's Stationery Office, London, England, 1973, p. 386.
(5) *Ibid.*, p. 387.
(6) H. S. I. Tan and D. Shelton, *J. Pharm. Sci.*, **63**, 916 (1974).
(7) R. B. Smyth and G. G. McKeown, *J. Chromatogr.*, **16**, 454 (1964).

Quantitation of Acetazolamide in Plasma by High-Performance Liquid Chromatography

R. D. HOSSIE ˣ, N. MOUSSEAU, S. SVED, and R. BRIEN

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Abstract □ A method for estimating acetazolamide concentrations in human plasma is described. Buffered plasma (pH 4.8) containing chlorothiazide as an internal standard is extracted twice with ethyl acetate. The extract is evaporated, redissolved, and chromatographed on silica gel with hexane–chloroform–methanol–acetic acid (65:25:10:0.25) as the mobile phase. The extraction efficiencies were >90%, the coefficients of variation at 1 and 30 μg/ml of plasma were 3.5 and 2.0%, respectively, and the calibration curves were linear and had an intercept of essentially zero. The suitability of the method for pharmacokinetic studies was verified in a normal volunteer dosed with 250-mg (solution) and 500-mg (sustained-release tablet) acetazolamide formulations.

Keyphrases □ Acetazolamide—analysis, high-performance liquid chromatography, plasma □ High-performance liquid chromatography—analysis, acetazolamide, plasma □ Carbonic anhydrase inhibitors—acetazolamide, high-performance liquid chromatography, plasma

Acetazolamide, a carbonic anhydrase inhibitor, is used to decrease the ocular aqueous humor secretion in certain types of glaucoma. Methods for the quantitation of this drug in biological fluids include measurement of the carbonic anhydrase inhibition (1, 2), colorimetry (3, 4), electron-capture GLC (5), and high-performance liquid chromatography (HPLC) (6). However, the GLC and HPLC procedures are time consuming (2), the colorimetric method is only partially successful (4, 6), and the modified enzymatic assay (2) lacks sufficient precision.

The present study was undertaken to develop an assay without the described deficiencies that could be used to quantitate acetazolamide in human or animal plasma following administration of single doses of 250 mg of a regular acetazolamide formulation or 500 mg of a sustained-release formulation.

EXPERIMENTAL

Materials—Hexane, chloroform, and methanol were HPLC grade[1,2]. Acetazolamide and chlorothiazide were USP reference standards. Sodium acetate, ethyl acetate, sodium bicarbonate, sodium hydroxide, and acetic acid were reagent grade.

One oral acetazolamide solution, used in the plasma profile experiments, was prepared by dissolving 250 mg of the USP standard in 20 ml of ethanol and diluting to 200 ml with fresh aqueous sodium bicarbonate (0.1 *M*). The final pH was 8. Another oral solution, used in determining the absence of interference, was prepared by dissolving a commercial parenteral preparation[3] in water.

Subjects—After an overnight fast, a healthy, 40-year-old male subject (90 kg) was administered 200 ml of the solution (250 mg) and separately one commercial 500-mg sustained-release formulation[3] with 150–250 ml of water at a 1-week interval.

Blood samples (10 ml) were collected in heparinized evacuated containers[4] at the various times (Figs. 1 and 2). The samples were centrifuged at 1000×*g*, and the separated plasma was stored at −18° until analysis.

Method—To tubes containing 1 ml of plasma were added 0.5 ml of internal standard (20 μg of chlorothiazide/ml of water), 2 ml of sodium acetate buffer (0.05 *M*, pH 4.8), and 10 ml of ethyl acetate. After mixing[5] for 10 min and centrifuging for 10 min at 1000×*g*, the organic layer was removed, and the extraction steps were repeated. The combined organic layers were evaporated at 60° to dryness using a nitrogen stream. After the residue was dissolved in 10 μl of 0.45 *N* NaOH by mixing[6], 1 ml of ethyl acetate was added, and the contents were mixed again. Aliquots (200 μl) of this solution were chromatographed.

The HPLC system consisted of a pump, an injector, and a detector[7] set at 280 nm. The silica gel column[8] (5 μm, 3.2 × 250 mm) was packed using a balanced density slurry method. The mobile phase was hexane–chloroform–methanol–acetic acid (65:25:10:0.25) at a flow rate of 3 ml/min (2900 psi). Quantitation was achieved by measuring the peak height ratio of the drug to the internal standard.

Calibration Curve—The linearity of the calibration curve was determined by adding 0.5, 2, 4, 8, and 12 μg of acetazolamide/ml to blank plasma samples. Two aliquots of each concentration were assayed as described. The coefficients of variation were determined using replicate samples of plasma standards containing 1 and 30 μg of acetazolamide/ml.

Determination of Extraction Efficiency—The extraction efficiency for the drug was estimated from the change in the peak height ratio when the drug was added to the plasma while the internal standard was added to the final extract compared to the peak height ratio when both were added to the final extract. The extraction efficiency for the internal standard was estimated from the change in the peak height ratio when the internal standard was added to the plasma while the drug was added to the final extract compared to the peak height ratio when both were added to the final extract.

Absence of Interferences—To verify the absence of metabolites and naturally occurring plasma constituents interfering with the internal standard or drug, plasma samples obtained from another volunteer 2 and 2.5 hr after ingestion of 500 mg of acetazolamide solution were pooled and extracted without the internal standard and chromatographed.

The effluents containing acetazolamide were combined and evaporated. One aliquot was rechromatographed on the same column using hexane–chloroform–methanol–acetic acid (70:25:5:0.25) as the mobile phase at a flow rate of 3 ml/min. Another aliquot was injected onto a bonded amino-silica gel column[9] (10 μm, 4.0 × 300 mm) using a mobile

[1] Burdick & Jackson Laboratories, Muskegon, Mich.
[2] Fisher Scientific, Ottawa, Canada.
[3] Diamox, Lederle, Canadian Cyanamide Ltd., Montreal, Canada.
[4] Vacutainers, Becton Dickinson, purchased through Canlab, Ottawa, Canada.
[5] Roto-rack, Fisher Scientific.
[6] Vortex, Fisher Scientific.
[7] Models 6000A, U6K, and 440, Waters Associates, Mississauga, Ontario, Canada.
[8] Li-Chrosorb Si-60, 5 μm, Brinkmann Instruments, Rexdale, Ontario, Canada.
[9] μBondapak-NH_2, Waters Associates.

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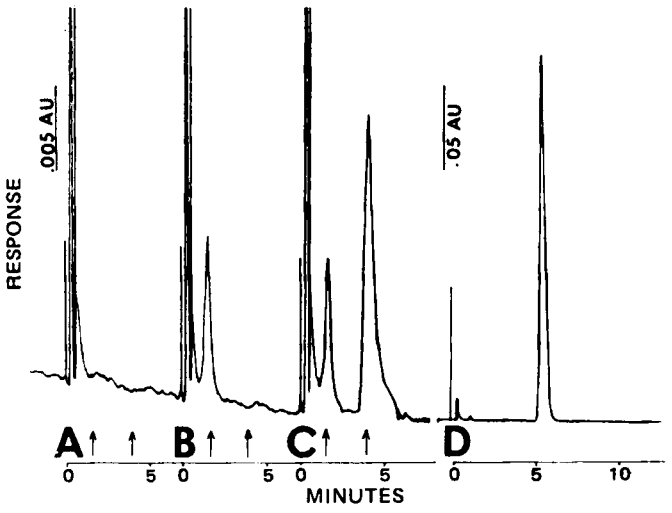

Figure 1—*Chromatograms of plasma extracts. Key: A, blank plasma; B and C, plasma of a human volunteer 4.5 hr after administration of 250 mg of an oral acetazolamide solution without (B) and with (C) the internal standard (estimated concentration, 6.03 μg/ml); and D, rechromatography of pooled acetazolamide peaks. Samples A, B, and C were chromatographed on a silica gel column with a mobile phase of hexane–chloroform–methanol–acetic acid (65:25:10:0.25). Sample D was chromatographed on a bonded amino-silica gel column with a mobile phase of 20% methanol in 0.01* M *ammonium formate. The expected locations of the drug and the internal standard are marked by arrows.*

phase of 20% methanol in 0.01 *M* aqueous ammonium formate at a flow rate of 2 ml/min.

RESULTS AND DISCUSSION

Preliminary experiments demonstrated the need for acetic acid in the mobile phase. In its absence, the peak height ratio of the drug to the internal standard diminished on successive injections of plasma extract, which may have been due to an accumulation of interfering substances. However, inclusion of acetic acid maintained the constancy of the peak height ratio in new columns, and old columns were restored to their original conditions by thorough flushing with the acidic mobile phase. Bayne *et al.* (6) had a similar problem with their acetazolamide assay. They attempted to overcome the problem by injecting alcohol between injections of the plasma extract. Unfortunately, in the present investigation this alcohol injection increased the analysis time and was not always successful.

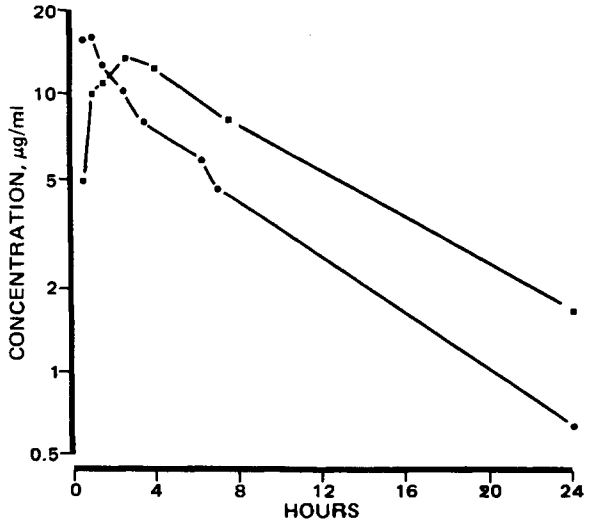

Figure 2—*Plasma concentration profiles of acetazolamide in a volunteer following the oral administration of 250 mg in solution (●) and a 500-mg sustained-release tablet (■).*

Table I—Typical Acetazolamide Calibration Curve

Drug Added, μg/ml	Peak Height Ratio[a]	Ratio[b]
0.5	0.062	0.125
2.0	0.264	0.132
4.0	0.521	0.130
8.0	1.043	0.130
12.0	1.595	0.133

[a] Ratio of the drug to the internal standard. [b] Ratio of the peak height ratio to the drug concentration.

The final method was evaluated in terms of extraction efficiency, interferences, linearity, and precision. The extraction efficiency of acetazolamide from plasma to ethyl acetate was 94% while that for chlorothiazide was 91%. Neither naturally occurring plasma constituents nor metabolites interfered with the peak height determination of either the drug or the internal standard. With blank plasma, when the internal standard was eliminated from the assay, no peaks absorbing at 280 nm and having the same retention times as acetazolamide or chlorothiazide were observed (Fig. 1A). Plasma extracts from a volunteer 4.5 hr after ingestion of 250 mg of drug (Fig. 1B) showed no peak with a retention time similar to that of the internal standard. Furthermore, the pooled effluents corresponding to the acetazolamide peak from the plasma of a volunteer 2–2.5 hr after a 500-mg drug dose showed a single peak when rechromatographed using a different column and mobile phase (Fig. 1D).

A typical calibration curve is shown in Table I. The excellent linearity of the method is illustrated by the constant values of the ratio of the peak height ratio to the drug concentration. The regression of the peak height ratio against drug concentrations gave a slope (±95% confidence interval) of 0.13 (±0.003) ml/μg and a peak height ratio intercept of nearly zero (−0.006 ± 0.02). In another series of experiments, the coefficients of variation determined at 1 and 30 μg/ml (n = 5 and 6, respectively) were 3.5 and 2.0%, respectively.

The suitability of the method for studying acetazolamide pharmacokinetics following single-dose administration was ascertained by administering separately 250 mg of the drug in solution and 500 mg in a sustained-release formulation to a normal human volunteer (Fig. 2). The solution showed rapid absorption with apparently biphasic elimination. A peak concentration of 16 μg/ml was produced at 1 hr compared to the lower peak concentration (13 μg/ml) produced at 2.5 hr with the larger dose, sustained-release product. These figures are in good agreement with values published previously (7).

This assay is relatively quick and precise and is not subject to interference either from metabolites or from naturally occurring plasma constituents.

REFERENCES

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

(2) G. J. Yakatan, C. A. Martin, and R. V. Smith, *Anal. Chim. Acta*, **84**, 173 (1976).

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

(4) T. H. Maren, V. I. Ash, and E. M. Bailey, Jr., *Bull. Johns Hopkins Med. J.*, **95**, 244 (1954).

(5) S. M. Wallace, V. P. Shah, and S. Riegelman, *J. Pharm. Sci.*, **66**, 527 (1977).

(6) W. F. Bayne, G. Rogers, and N. Crisologo, *ibid.*, **64**, 402 (1975).

(7) R. D. Schoenwald, M. E. Garabedian, and G. J. Yakatan, *Drug Dev. Ind. Pharm.*, **4**, 599 (1978).