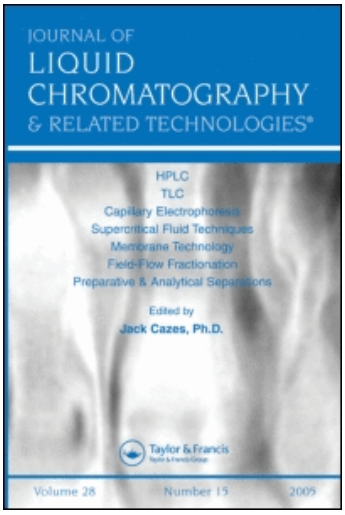

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ACETAZOLAMIDE IN HUMAN PLASMA

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ABSTRACT

A simple, rapid and specific HPLC method has been developed to determine acetazolamide concentrations in human plasma. The assay procedure requires only 250 µl of sample with direct injection of the organic supernatant after protein precipitation with acetonitrile. Chlorothiazide was used as an internal standard. A reversed-phase C_{18} µBondapak column was employed for the chromatographic separation. The eluent was monitored at 265 nm using a UV variable wavelength detector. The retention times for acetazolamide (ACZ) and chlorothiazide (CTZ) were 6 and 8 min respectively. A linear relationship ($r \geq 0.995$) was obtained over the 1-20 µg/ml concentration range. The limit of sensitivity for ACZ was 0.5 µg/ml, with greater than 85% recovery of ACZ and internal standard. The method was applied to human plasma samples obtained after administration of a 250 mg acetazolamide tablet.

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INTRODUCTION

Acetazolamide, 5-acetoamino-1,3,4-thiadiazole-2-sulfonamide, (ACZ) is a carbonic anhydrase inhibiting drug which is used for the treatment of certain types of glaucoma. Since our laboratories were engaged in a project to assess the pharmacokinetics of 250 mg oral doses of ACZ in humans, a reliable, simple assay was needed. The anticipated human plasma ACZ concentrations ranged from 0 to 20 μg/ml (1,2). While electron-capture GLC (3), and carbonic anhydrase inhibition (1,4,5)assays have been used, HPLC methods have generally replaced these procedures. However, the reported HPLC methods involve an ethyl acetate extraction step before injection (6-9). The purpose of this report is to describe a simple and rapid procedure which eliminates the solvent extraction process. The technique uses an acetonitrile precipitation with direct injection after centrifugation.

EXPERIMENTAL

Reagents and Materials

Acetazolamide was obtained from Sigma Chemical Co. (St. Louis, MO). Chlorothiazide, analytical reagent grade was a gift from Merck, Sharp and Dohme, Analytical Research Division (Rahway, NJ). Acetonitrile, HPLC Grade, distilled-in-glass was purchased from Burdick and Jackson (Muskegeon, MI). Glacial acetic acid, analytical reagent grade, and sodium acetate anhydrous were obtained from Mallinkrodt Chemical Inc.(St. Louis, MO).

Instrumentation and Conditions

The HPLC system consisted of a Hewlett-Packard Model 1081B pump and autosampler. A Lambda-Max Model 480 UV detector operated at 265 nm, with a sensitivity setting of 0.005 AUFS; a guard column containing μBondapak/Corasil 37-50 micron particles; and a pre-packed 30 cm x 3.9 mm i.d. stainless steel μBondapak C_{18} column, were all from Waters Associates (Milford,MA). The chromatograms were recorded using a 10 mV strip chart recorder (Fisher Recordall, Series 500, Fairlawn, NJ) at a chart speed of 0.1 in/min. A Hewlett-Packard Model 3353 Laboratory Data System was used to analyze chromatographic peak areas.

The mobile phase consisted of an 0.05 M acetate buffer/acetonitrile (94:6 v/v) solution adjusted to pH 4.5 with glacial acetic acid. The mobile phase was filtered through a 47 mm, 0.45 μ, Nylon-66 filter (Rainin, Woburn, MA) under vacuum prior to use. The chromatographic system was operated at ambient temperature with a flow rate of 2 ml/min.

Standard Solutions

An aqueous stock solution of 100 μg/ml ACZ was prepared daily. Aliquots of the solution were diluted with deionized water to provide standard solutions ranging from 5 μg/ml to 100 μg/ml. The aqueous internal standard solution contained 250 μg/ml of chlorothiazide, and was stored at $5^{o}C$.

Solutions for standard curves were prepared by combining 250 μl of drug-free plasma and 50 μl of a standard drug solution of 5,

25, 50 or 100 µg/ml ACZ in a 15 ml silanized conical centrifuge tube. A 50 µl aliquot of an internal standard solution and 1 ml of acetonitrile were added and vortexed. Blank plasma samples were also carried through the procedure, substituting deionized water for drug and internal standard, to determine endogenous interferences. The mixtures were vortexed for 20 seconds and centrifuged at 3000 rpm and 5°C for 10 min. Approximately 500 µl of the supernatant was transferred to autosampler vials and 25 µl was injected into the HPLC.

Recovery Studies

A standard curve was constructed using aqueous standard drug solutions using the procedure described above. Both the aqueous and plasma standard curves were prepared on the same days. The injection volumes used for all samples was 25 µl. A comparison was made of the peak areas from the aqueous and plasma standard curves to determine percent recovery.

RESULTS

Acetonitrile samples containing ACZ demonstrated no evidence of degradation over a 24 hour period when stored at 5°C. There was less than a 4% change in the slopes of standard curves from 1-20 µg/ml when fresh samples and samples stored in autosampler vials for 24 hours at room temperature were compared. The aqueous internal standard solution (CTZ) showed no significant degradation over a period of one month when stored at 5°C.

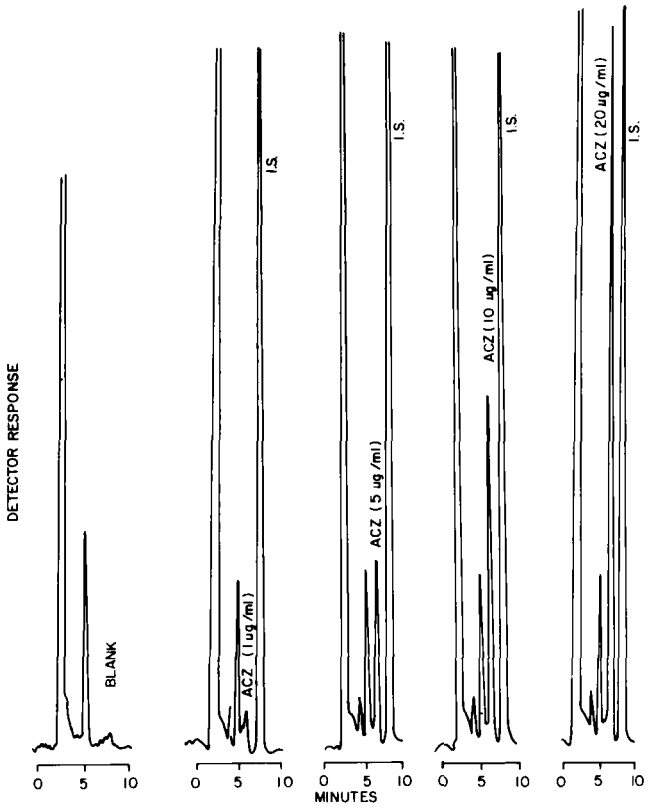

FIGURE 1 - HPLC Chromatograms of Fortified Human Plasma Containing 0, 1, 5, 10 and 20 μg/ml of Acetazolamide (ACZ) and Internal Standard (I.S.).

Standard curves were plotted as the peak area ratio (ACZ/CTZ) versus the ACZ plasma concentration. Figure 1 illustrates a representative HPLC chromatogram obtained during the assay of fortified, pooled human plasma samples. The retention times for ACZ and CTZ were 6 and 8 min, respectively. Typical chromatograms obtained from human plasma samples following a 250 mg oral dose of acetazolamide are shown in Figure 2. No metabolites of ACZ have been reported and

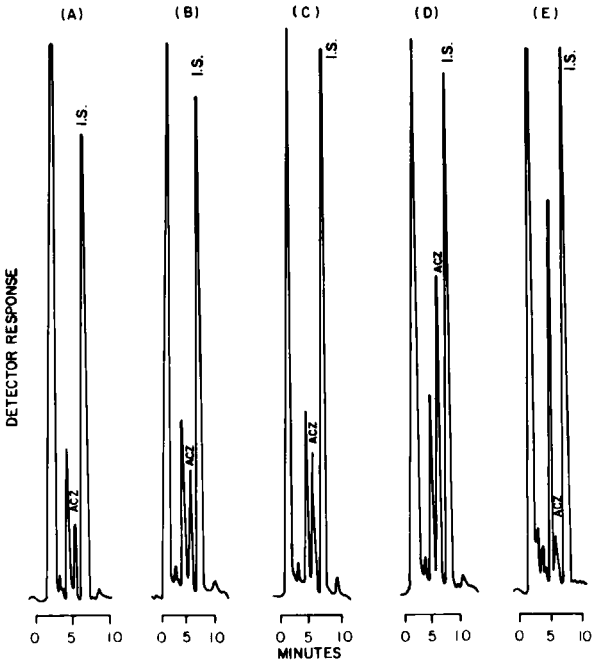

FIGURE 2 - HPLC Chromatograms for Human Plasma Samples Obtained 0.5 (A), 1 (B), 1.5 (C), 2 (D) and 25 (E) Hours After a 250 mg Oral Dose of Acetazolamide.

blank pre-dose plasma obtained from subjects participating in a single-dose ACZ bioequivalency study did not exhibit any interfering peaks in the chromatograms. Small peaks were noted in the vicinity of the internal standard for some blank plasma samples, but these were less than 2 percent of the internal standard peak. Standard curves consistently exhibited excellent linearity over the ACZ concentration range of 1-20 μg/ml, with negligible intercepts and correlation coefficients in the range of 0.995-0.999.

Fortified control plasma samples containing 1 or 20 μg/ml of ACZ (N= 8-10) were analyzed using the standard curves to determine

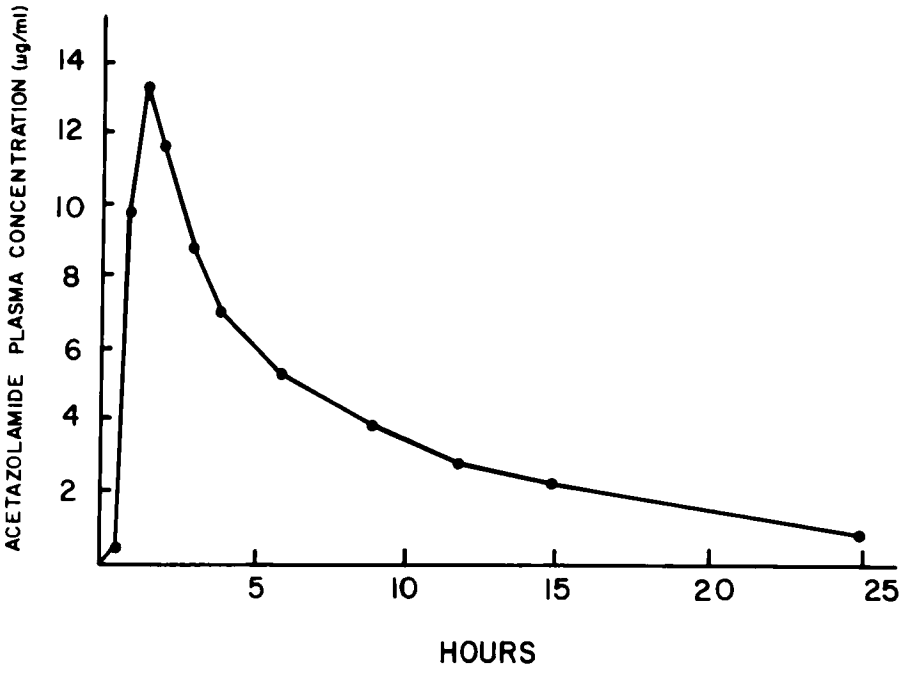

FIGURE 3 - Typical Acetazolamide Plasma Concentration-Time Profile After Administration of a 250 mg Oral Dose to a Human Subject.

drug concentrations. It was calculated that the 1 and 20 μg/ml samples contained 0.86 (S.D.=0.11) and 20.10 (S.D.=0.59) μg/ml, respectively. The lower limit of sensitivity was found to be 0.50 μg/ml with a signal-to-noise ratio of 2. The recovery of ACZ over the range 5 to 20 μg/ml was 90% (%C.V.=6.70,N=4) and 86.6% for CTZ (%C.V.=9.80,N=4). Figure 3 illustrates a representative ACZ plasma concentration-time profile, when the assay was applied to plasma samples obtained from a human who had received a 250 mg oral dose of drug.

In conclusion, a rapid method has been developed for the assay of acetazolamide in plasma. To date the method has been applied to the analysis of over 500 plasma samples obtained during a 0-25 hr period after administration of a 250 mg acetazolamide tablet to human subjects.

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