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Short communication

# Rapid determination of acetazolamide in human plasma

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## 1. Introduction

Acetazolamide (5-acetamido-1,3,4-thiazole-2-sulfonamide) is a carbonic anhydrase inhibitor which reduces the rate of aqueous humor formation and correspondingly decreases the intra ocular pressure in patients with glaucoma. It is also used, either alone or in association with other antiepileptics, for the treatment of various forms of epilepsy [1]. Absorption is fast, reaching peak plasma concentrations approximately 1–3 h after oral administration. About 80% of the drug is excreted by tubular secretion of the anionic species, and 70–90% of the administered dose is recovered unchanged within 24 h [2]. Therefore, the determination of acetazolamide concentrations in biological fluids is of particular interest in pharmacokinetic studies. In order to study the pharmacokinetics of acetazolamide in humans a selective and sensitive assay for acetazolamide in biological fluids is necessary. Methods for the quantitation of this drug in biological fluids include measurement of carbonic anhydrase inhibition [3], polarography [4], electron-capture GLC [5] and high-performance liquid chromatography [6–10]. However, the GLC method is time-consuming and the polarographic method is only partially successful and the modified enzymatic assay lacks sufficient precision. HPLC methods differ with respect to the mode of HPLC (normal-phase or reversed-phase) and sample preparation (solvent extraction and solid-phase extraction). The problem of sample preparation, in particular, has attracted much attention in recent years, as this procedure has often been a rate limiting step in HPLC analyses of biological fluids. Most of these methods required liquid–liquid extraction with evaporation of the extract or on-line solid-phase extraction and therefore sample preparation is time-consuming, complex or both. The present paper reports a simple, rapid and sensitive HPLC method with UV detection using a single-step extraction procedure for separating and quantifying acetazolamide in plasma. The sample preparation only involves protein precipitation and no evaporation step is required.

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## 2. Experimental

### 2.1. Chemicals

Acetazolamide and hydrochlorthiazide were supplied by Irandaru Pharmaceuticals (Tehran, Iran). Acetazolamide is available as an oral tablet containing 250 mg of acetazolamide and the following inactive ingredients: Avicell, corn starch and magnesium stearate. HPLC-grade acetonitrile, methanol and all other chemicals were obtained from Merck (Darmstadt, Germany).

Water was obtained by double distillation and purified additionally with a Milli-Q system.

### 2.2. Instruments and chromatographic conditions

A Perkin-Elmer HPLC system (Norwalk, CT, USA) was used with a model LC-410 pump, a model Rheodyne 7125 injector and a model LC-95 UV detector connected to a model LC-100 integrator.

The separation was performed on an analytical $250 \times 4.6$ mm i.d. μbondapak $C_{18}$ (5 μm, particle size) column. The wavelength was set at 254 nm. The mobile phase was a mixture of 0.4 M sodium acetate buffer–acetonitrile–methanol (95:3:2) adjusted to pH 5.1 at a flow rate of 2 ml/min. The mobile phase was prepared daily and degassed by ultrasonication before use.

### 2.3. Standard solutions

Stock solutions (1 mg/ml) and appropriate dilutions of acetazolamide and hydrochlorthiazide were prepared in methanol and stored at $+4$ °C. No change in stability over a period of 1 month was observed.

### 2.4. Sample preparation

To 1 ml of plasma in a glass-stoppered 15-ml centrifuge tube were added 100 μl of internal standard (50 μg/ml) and 200 μl of 24% of perchloric acid solution. After mixing (30 s), the mixture centrifuged for 15 min at 6000 rpm. Then 30 μl of supernatant was injected into the liquid chromatograph.

### 2.5. Biological samples

Acetazolamide was administered in a single dose of 500 mg to healthy volunteers after overnight fasting. Plasma samples were collected at several intervals after dosing and then frozen immediately at $-20$ °C until assayed.

### 2.6. Stability

The stability of acetazolamide was assessed during all the storage steps and during all steps of the analytical method.

### 2.7. Plasma standard curve

Blank plasma was prepared from heparinized whole-blood samples collected from healthy volunteers and stored at $-20$ °C. After thawing, stock solution of acetazolamide was added to yield final concentrations ranging from 0 to 40 μg/ml. Internal standard solution was added to each of these samples to yield a concentration of 3.85 μg/ml. The samples were then prepared for analysis as described above.

## 3. Results and discussion

Under the chromatographic conditions described, acetazolamide and the internal standard peaks were well resolved. Endogenous plasma components did not give any interfering peaks. Fig. 1 shows typical chromatograms of blank plasma in comparison to spiked samples analyzed for a pharmacokinetic study. The average retention times of acetazolamide and hydrochlorthiazide were 6.8 and 9 min, respectively. The calibration curve for the determination of acetazolamide in plasma was linear over the range 0–40 μg/ml. The linearity of this method was statistically confirmed. For each calibration curve, the intercept was not statistically different from zero. The correlation coefficients ($r$) for calibration curves were equal to or better than 0.998. The relative standard deviation (R.S.D.) values of the slope were equal to or better than 6%. For each point of calibration standards, the concen-

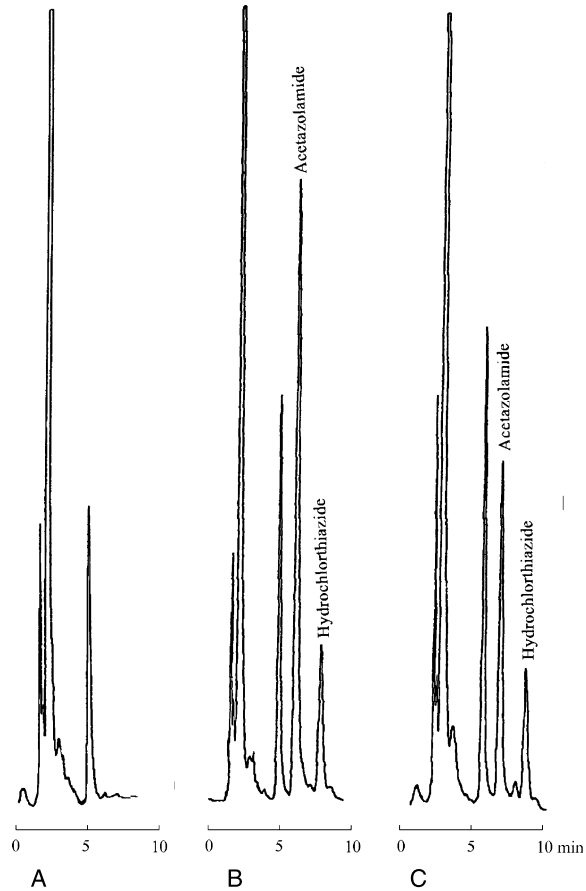


Fig. 1. Chromatograms of (A) blank plasma; (B) blank plasma spiked with 10 ng/ml acetazolamide and 3.85 ng/ml hydrochlorthiazide (internal standard); (C) plasma sample taken 3 h after oral administration 500 mg acetazolamide with 3.85 ng/ml hydrochlorthiazide.

trations were recalculated from the equation of the linear regression curves (Table 1). The relative analytical recovery for plasma at three different concentrations of acetazolamide was determined. Known amounts of acetazolamide were added to drug-free plasma in concentrations ranging from 0.25 to 5 μg/ml. The internal standard was added and the relative recovery of acetazolamide was calculated by comparing the peak areas for extracted acetazolamide from spiked plasma and a standard solution of acetazolamide in methanol containing internal standard with the same initial concentration. The average recovery was 91.9 ± 3.5% ($n = 6$). The limit of detection was defined as the acetazolamide concentration that produced a signal-to-noise ratio greater than three. The limit of detection in plasma was 5 ng/ml based upon this criterion. At this level, the R.S.D. was lower than 12%. This is sensitive enough for drug monitoring and other purposes such as pharmacokinetic studies. We assessed the precision of the method by repeated analysis of plasma specimens containing known concentrations of acetazolamide. As shown in Table 2, coefficients of variation were less than 8% which is acceptable for the routine measurement of acetazolamide. The aim of our study was to develop a simple and fast method for the routine analysis of biological samples in pharmacokinetic acetazolamide research.

Table 2
Reproducibility of the analysis of acetazolamide in human plasma ($n = 5$)

| Concentration added (μg/ml) | Concentration measured (mean ± S.E.) | |
|---|---|---|
| | Intra-day | Inter-day |
| 1 | 0.92 ± 0.04 (4.3) | 0.99 ± 0.05 (5.1) |
| 5 | 4.63 ± 0.34 (7.3) | 5.30 ± 0.22 (4.2) |
| 20 | 19.91 ± 0.19 (0.9) | 19.92 ± 0.25 (1.3) |

Values in parentheses are coefficients of variation (%).

Table 1
Assay linearity

| | Coefficient of the linear regression analysis ($r \pm$ S.D.) | Slope ± S.D. | Intercept ± S.D. |
|---|---|---|---|
| Intra-assay | $0.999 \pm 6.81 \times 10^{-4}$ | 0.267 ± 0.0133 | 0.0371 ± 0.0121 |
| $n = 6$ | R.S.D. = 0.0682% | R.S.D. = 4.98% | |
| Inter-assay | $0.9991 \pm 7.85 \times 10^{-4}$ | 0.259 ± 0.0141 | 0.0442 ± 0.0161 |
| $n = 9$ | R.S.D. = 0.0786% | R.S.D. = 5.44% | |

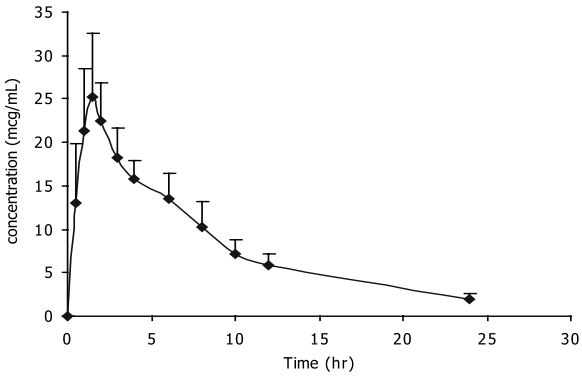


Fig. 2. Mean plasma concentration–time profile of acetazolamide in healthy volunteers ($n = 11$) after a single 500-mg oral dose of acetazolamide.

This method is well suited for routine application in the clinical laboratory because of the simple extraction procedure and high sensitivity. Over 300 plasma samples were analysed by this method without problems thus proving its suitability. In this study, plasma concentrations were determined in 11 healthy volunteers, who received 500 mg of acetazolamide each. Fig. 2 shows the mean plasma concentration–time curve of acetazolamide: plasma concentration reached a maximum $1.54 \pm 0.35$ h after dosing with a level of $27.48 \pm 7.75$ μg/ml.

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