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Determination of acetazolamide in human urine samples by reversed-phase high-performance liquid chromatography in the presence of xanthines

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ABSTRACT

A simple, rapid and selective high-performance liquid chromatographic assay for the determination of acetazolamide in urine samples is described. After extraction with ethyl acetate, the drug is chromatographed on an HP-Hypersil ODS-C_{18} column with a mobile phase of acetonitrile–phosphate buffer (pH 3) and ultraviolet detection at 275 nm. The efficiency of the extraction, the linearity and the reproducibility of the method permit the evaluation of acetazolamide urinary excretion a long time after its administration.

INTRODUCTION

Acetazolamide is a carbonic anhydrase inhibitor primarily employed to reduce intraocular pressure in the treatment of glaucoma, although because of its anticonvulsant properties this drug has also been used for the treatment of epilepsy.

Colorimetric [1] and enzymic methods [2–4] were initially proposed for the determination of acetazolamide from biological fluids. An electron-capture gas chromatographic procedure has also been reported [5]. However, high-performance liquid chromatographic (HPLC) methods have generally replaced these procedures. The liquid chromatographic assays are usually based on the extraction of acetazolamide from biological fluids with ethyl acetate and separation on an octadecyl silica (ODS) column under reversed-phase conditions [6–8]. Hwang *et al.* [9] reported a methodology for acetazolamide determination in plasma which permits direct injection after sample deproteinization with acetonitrile. However, elution under reversed-phase conditions is limited by the presence of caffeine and other dimethylxanthines derived from caffeine, which tend to coelute with acetazolamide, probably owing to the inappropriate selection of the mobile phase or the gradient elution program [6,10]; this indicates the possibility of interference from either dietary sources or medicaments. Then, the interference of theophylline or caffeine in the determination of acetazolamide can be significant because these drugs are commonly used in the treatment of respiratory diseases, the therapeutic concentration being in the 5–20 μg/ml range. Acetazolamide also interferes in the determination of caffeine metabolites [11]. Although the pK_a values of these compounds are very similar (acetazolamide, pK_{a1} = 7.2, pK_{a2} = 9.0; theophylline, pK_a = 8.7; paraxanthine, pK_a = 8.8), Clark [12] indicated that a proper pH adjustment

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could effect almost complete separation between acetazolamide and theophylline.

As an alternative, chromatography using silica columns and a water-saturated eluent has been proposed to avoid this interference [10], although owing to its lesser versatility, normal-phase chromatography is used less than reversed-phase chromatography in clinical laboratories.

This paper describes a simple and rapid procedure to determine acetazolamide in urine samples under reversed-phase conditions. Gradient elution with acetonitrile–phosphate buffer provides a complete resolution between this drug and caffeine and its main metabolites.

EXPERIMENTAL

Reagents

All the reagents used were of analytical grade. Methanol, acetonitrile and ethyl acetate were of HPLC grade from Scharlau (Barcelona, Spain). Water was distilled, deionized and filtered through 0.45-μm Nylon membranes from Teknokroma (Barcelona, Spain). Acetazolamide standard solution were prepared by dissolving in pure methanol (Cyanamid Ibérica, Madrid, Spain). Theobromine, 1,7-dimethylxanthine and the internal standard, β-hydroxymethyltheophylline, were supplied by Sigma (St. Louis, MO, USA). Theophylline, lead acetate and propylamine hydrochloride were supplied by Fluka (Buchs, Switzerland). Caffeine was obtained from Aldrich (Steinheim, Germany). Sodium dihydrogenphosphate monohydrate was obtained from Merck (Darmstadt, Germany). Disodium hydrogenphosphate ($Na_2HPO_4 \cdot 12H_2O$), phosphoric acid and sodium chloride were obtained from Probus (Barcelona, Spain).

Standard solutions

Standard solutions of acetazolamide, caffeine, theobromine, 1,7-dimethylxanthine and theophylline were prepared by dissolving 50 mg of the pure compound in 25 ml of methanol (2000 μg/ml). Working solutions were prepared daily by dilution of these stock solutions with the appropriate volumes of methanol. The internal standard was prepared by dissolving 25 mg of the pure compound in 500 ml of methanol (50 μg/ml). All the solutions were stored in the dark at 2°C.

Apparatus

A Hewlett-Packard 1040A liquid chromatograph, equipped with a diode-array detector linked to a Hewlett-Packard HPLC Chem Station data system (Palo Alto, CA, USA) was used for data acquisition and storage. The system was coupled to a quaternary pump Hewlett-Packard, 1050 Series (Palo Alto, CA, USA), with a 25-μl sample loop injector.

The column was an HP-Hypersil ODS-C_{18} (particle size 5 μm, 250 mm × 4 mm I.D.; Merck, Darmstadt, Germany). The detector was set to collect a spectrum every 640 ms (over the range 200–400 nm) and all the assays were carried out at ambient temperature.

Mobile phase

A gradient of phosphate buffer–acetonitrile, with an increasing acetonitrile content from 12% at zero time to 15% after 3 min and to 40% after 5 min was used. After 5 min the acetonitrile content was kept constant. The phosphate buffer was prepared by dissolving 3.45 g of sodium dihydrogenphosphate monohydrate in 500 ml of distilled and deionized water, after the addition of 0.7 ml of propylamine hydrochloride. The pH was adjusted to 3 by adding the minimum amount of concentrated phosphoric acid. The solution was prepared daily, filtered through a 0.45-μm Nylon membrane (Teknokroma) and degassed with helium before use. The flow-rate was 1 ml/min. The chromatographic signal was monitored at 275 nm.

Sample treatment

To 2.0 ml of urine were added 0.5 g of sodium dihydrogenphosphate–disodium hydrogenphosphate (99:1, w/w) and 0.5 g of sodium chloride. The final pH of the mixture ranged from 5 to 5.5. It was extracted with 4.0 ml of ethyl acetate for 10 min in a mechanical agitator (Selecta, Barcelona, Spain). The aqueous phase was discarded,

and 2.0 ml of 5% lead acetate were added to the organic solution to remove urinary endogenous compounds. The mixture was then centrifuged (1000 *g* for 5 min) and the organic extract was separated and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 300 μl of the internal standard solution. The resulting solution was finally filtered through 15-mm and 0.45-μm Nylon filters (Teknokroma), and 5 μl were injected into the column.

Recovery studies

Urine samples (2.0 ml) were spiked with acetazolamide standard solutions to give different concentrations in the range 0.75–18.75 μg/ml. These samples were subjected to the previously described extraction procedure. The percentage of drug recovered for a particular extraction was calculated by comparing the peak heights obtained for acetazolamide in the spiked samples (referred to the internal standard) with the peak heights obtained for a direct injection of 5 μl of methanolic solutions containing an equivalent amount of drug. Each concentration was assayed in duplicate.

Preparation of standards for calibration

Standards for calibration were prepared by spiking 2.0-ml urine samples with the appropriate volumes of acetazolamide methanolic solution, reproducing six concentrations in the same range used in recovery studies. These samples were extracted and chromatographed as described above. Peak-height ratios of acetazolamide to β-hydroxyethyltheophylline, added to the extracts, were plotted *versus* acetazolamide concentration. A calibration curve obtained by direct injection of a methanolic solution of acetazolamide in the same concentration range was also constructed. Each concentrations was assayed in duplicate. These calibration curves were used to calculate the acetazolamide concentrations in spiked samples from different subjects. A new calibration graph was constructed to evaluate the accuracy and precision, by adding the internal standard directly to the spiked urine samples. These samples were treated as has been described, using methanol in the regeneration step.

Human studies

Urinary excretion studies were performed with

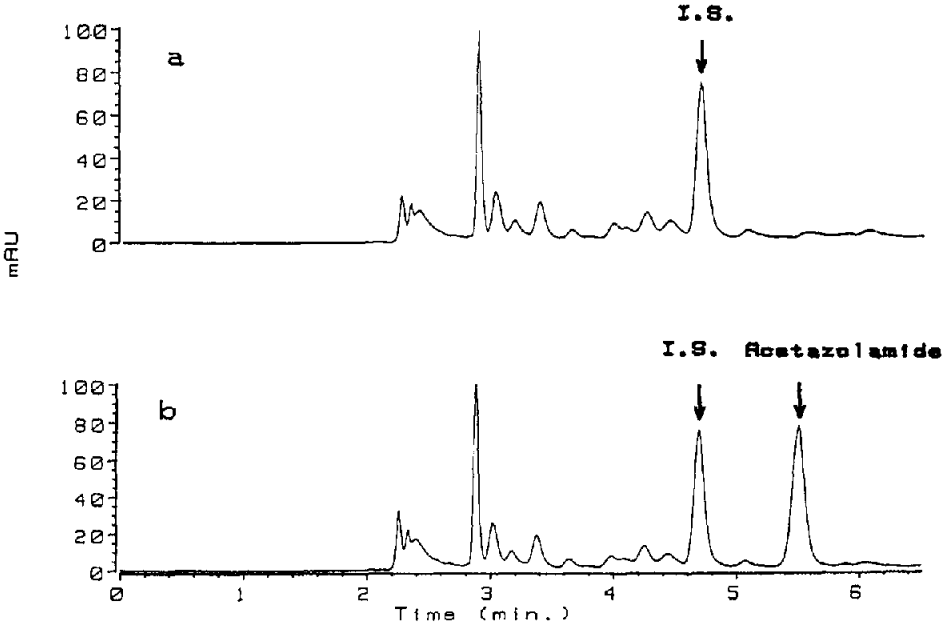

Fig. 1. Chromatograms at 275 nm of (a) a blank urine sample and (b) a urine sample spiked with 15 μg/ml acetazolamide. The peak at 4.6 min corresponds to the internal standard (I.S.).

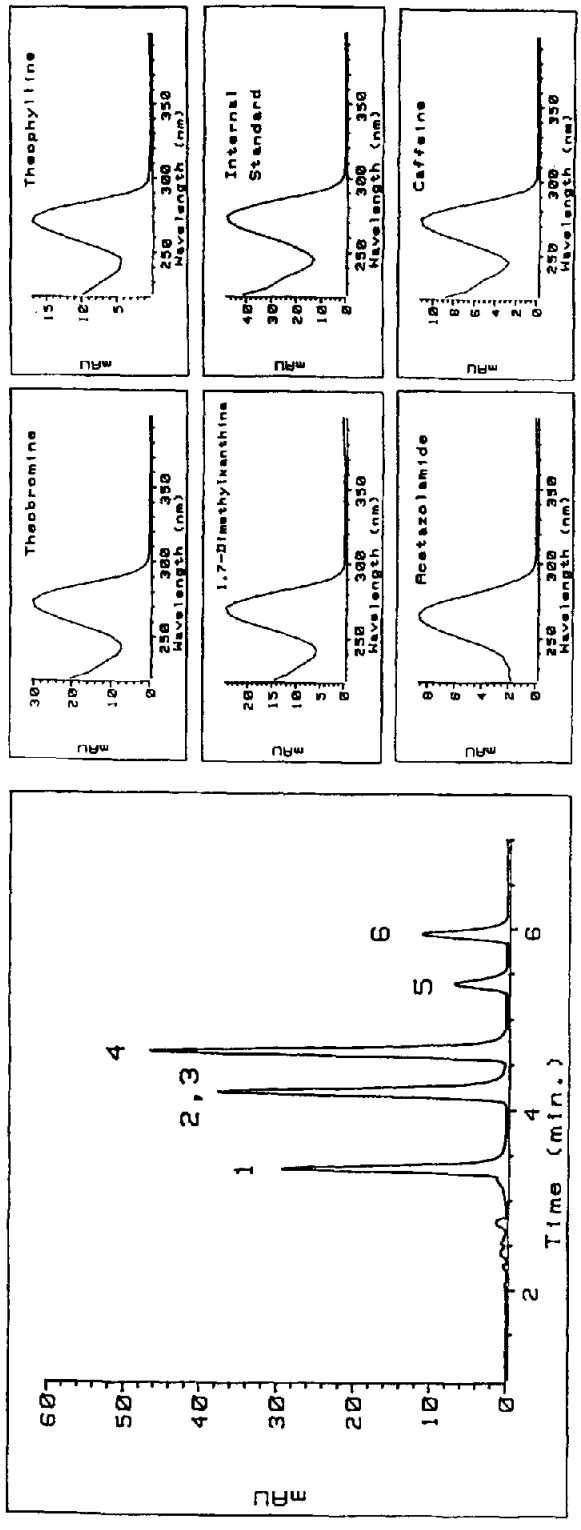

Fig. 2. Chromatogram at 275 nm of a mixture of theobromine (1), theophylline (2), 1,7-dimethylxanthine (3), acetazolamide (5) and caffeine (6) in methanol. The amount of each compound injected was 0.1 μg. The UV spectra of these compounds are also shown. Peak 4 corresponds to the internal standard.

a human healthy volunteer after a single dose of acetazolamide (250 mg). Urine samples were collected at appropriate time intervals post-dose and analysed in triplicate. After the addition of 300 μl of the internal standard solution, these samples were treated as described above, using methanol in the regeneration step.

RESULTS AND DISCUSSION

Fig. 1 shows chromatograms obtained from (a) blank and (b) spiked urine samples. Under the chromatographic conditions used, acetazolamide and the internal standard are eluted at 5.4 and 4.6 min, respectively. The components of the mobile phase used in this study are the same proposed by Cooper *et al.* [13] for the screening of diuretics. However, with the elution programme used in that procedure, caffeine and acetazolamide are eluted close together. The elution profile that we have proposed provides a suitable resolution between acetazolamide and the main xanthines compatible with a short time of analysis, as can be seen in Fig. 2, which shows a chromatogram of a mixture of acetazolamide, theobromine, theophylline, 1,7-dimethylxanthine and caffeine in methanol. Comparison of Fig. 1a and b shows that blank urine samples are free from other endogenous compounds that may interfere with the quantitation of acetazolamide.

Extraction recoveries for the different acetazolamide concentrations tested are listed in Table I.

TABLE I

RECOVERIES OF ACETAZOLAMIDE AT THE DIFFERENT CONCENTRATIONS TESTED

Acetazolamide concentration (μg/ml)	Recovery (%)
3.75	90
7.50	82
11.25	83
15.00	89
18.75	77
Mean	84 ± 6

TABLE II

ACETAZOLAMIDE CONCENTRATIONS OBTAINED FOR DIFFERENT URINE SAMPLES FROM CALIBRATIONS WITH URINE SAMPLES AND WITH STANDARD SOLUTIONS

Subject	Acetazolamide added (μg/ml)	Acetazolamide found (μg/ml)	
		Urine samples	Standard solutions
1	1.50	0.8	1.3
2		1.9	2.4
3		1.9	2.4
Mean		1.3 ± 0.6	2.0 ± 0.6
1	3.75	3.7	3.6
2		3.8	3.9
3		3.3	3.8
4		3.5	3.7
Mean		3.6 ± 0.2	3.7 ± 0.2
1	7.50	6.3	6.1
2		8.1	8.0
3		7.6	7.5
4		8.5	8.3
5		8.6	8.8
6		8.7	8.9
Mean		7.9 ± 0.9	8 ± 1

The extraction efficiency, 84 ± 6%, is comparable with that obtained by sample treatment involving back-extraction or large volumes of ethyl acetate [6,7]. The percentage of acetazolamide recovered is also similar to that obtained by Hwang *et al.* [9] for plasma samples after deproteinization with acetonitrile and by Hartley *et al.* [10] for plasma and urine samples using C_{18} solid-phase extraction columns. We tested different solid-phase extraction columns for acetazolamide in urine samples, and the recoveries obtained ranged from 71 ± 4% for C_8 columns to 26 ± 6% for C_2 columns [14]. The liquid–liquid extraction procedure is therefore preferred over solid-phase extraction techniques for quantification purposes.

The calibration graphs for both standards and urine samples are linear over the working range 0.75–18.75 μg/ml, and their intercepts are essen-

tially zero. The slopes of these calibration graphs were 0.074 for standards in methanol and 0.066 for urine samples, with mean correlation coefficients of 0.998 and 0.997, respectively. The slopes for both calibration graphs are statistically equivalent if the mean percentage of recovery is considered. Therefore, the matrix of the sample does not modify the slope of the calibration graph obtained with standards.

Control urine samples from different volunteers were spiked with acetazolamide and tested to determine acetazolamide levels (Table II). The concentrations were calculated from both the calibration graph corresponding to a random urine sample spiked with acetazolamide and the calibration graph obtained from standard solutions of acetazolamide and the mean percentage of recovery. The precision between samples is generally good, and therefore it seems that this determination does not depend on the matrix of the urine samples for all cases tested.

In order to evaluate the precision and accuracy of the method, different samples form subject 1 were spiked with acetazolamide in the same concentrations assayed in Table II. The internal standard solution was directly added to these samples, and 300 μl of methanol were used in the regeneration step (the recovery of internal standard was 79 ± 4%, for n = 3). The concentrations found were 1.46 ± 0.04, 3.9 ± 0.2 and 7.6 ± 0.4 μg/ml of urine (n = 3). These results show that the accuracy of the method is improved when the internal standard is directly added to the samples, the precision being good.

The limit of detection (for a signal-to-noise ratio of 3) corresponds to an injected amount of 0.25 ng, which is equivalent to an acetazolamide concentration in urine of 8 ng/ml.

Pharmacokinetic studies of urine samples [15] indicate that this diuretic is primarily excreted as unchanged drug. The acetazolamide concentration is usually higher than 10 μg/ml in the 0–36 h period after administration of a normal single dose. This indicates that the proposed procedure can be applied to the determination of acetazolamide with sufficient accuracy and precision a long time after its administration. The assay has been applied to the measurement of the urine levels of acetazolamide afer a single dose of 250 mg to a human volunteer (Table III).

TABLE III

ACETAZOLAMIDE LEVELS IN URINE AFTER A SINGLE DOSE OF 250 mg

Time (h)	Acetazolamide concentration (μg/ml)
16	15.1 ± 0.5
32	6.9 ± 0.2
44	4.3 ± 0.2

CONCLUSION

An HPLC assay for the determination of acetazolamide in urine had been validated for the therapeutic concentration range. Sample preparation is rapid and provides clean chromatograms. The time required for the elution of acetazolamide is less than 6 min, and the interference of caffeine and its main metabolites, noted in the literature for reversed-phase separations of acetazolamide, is eliminated.

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