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Application of column-switching techniques to the determination of medium polarity drugs: determination of acetazolamide in urine

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Abstract

A column-switching system for the determination of the medium polarity diuretic acetazolamide in urine, has been designed. An Hypersil ODS C_{18}, 30 μm (20 × 2.1 mm I.D.) pre-column was used for the pre-concentration and separation of acetazolamide from the biological matrix. The most polar urinary compounds were removed by washing the pre-column with a phosphate buffer solution (pH 3), and the fraction of eluate containing the analyte was switched to a LiChrospher RP C_{18}, 5 μm (125 × 4 mm I.D.) analytical column, where it was chromatographed using gradient elution with acetonitrile–water, and detected at 275 nm. The most apolar urinary compounds were directly discarded by means of a second switching valve. Under these conditions the recovery of drug was 96 ± 5% in the 0.50–100.0 μg/ml concentration range. The limit of detection was 10 ng/ml, the total analysis time being less than 8 min.

1. Introduction

Acetazolamide, 5-acetamino-1,3,4-thiadiazole-2-sulphonamide, is a carbonic anhydrase inhibitor diuretic, mainly used in the treatment of glaucoma and epilepsy.

Several liquid chromatographic methods have been developed for the determination of acetazolamide in biological fluids, most of them based on liquid extraction of the drug into an organic solvent. However, successive re-extractions are often required to obtain a satisfactory selectivity [1,2], resulting in very tedious and time-consuming procedures. We have recently proposed an assay for the quantification of this diuretic in which a single extraction into ethyl acetate (under acidic conditions and in presence of sodium chloride) provided a recovery of 84 ± 6% of the drug [3].

As alternative to liquid-extraction procedures, different sample pre-treatments have been tested. For example, Hwang *et al.* reported the determination of acetazolamide in plasma after sample deproteinization with acetonitrile [4]. Hartley *et al.* applied solid-phase extraction onto disposable cartridges for the analysis of acetazolamide in plasma and urine samples [5]. We have evaluated different reversed-phase solid-phase extraction columns (C_{18}, C_8, C_2, cyclohexyl, phenyl and cyanopropyl) for several diuretics

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including acetazolamide [6]. The retention of acetazolamide in these columns is low probably due to its polarity, resulting in low recoveries of the drug (the percentages of acetazolamide recovered were in the 26–71% range); therefore they are not adequate for quantification purposes.

In an effort to reduce the time required for the sample preparation step in drug analysis, an increasing number of HPLC methods incorporating on-line sample clean-up by solid-phase extraction using column switching, have been developed [7,8]. In general, most of the procedures which use solid-phase extraction onto disposable cartridges can be adapted to on-line sample clean-up by column switching. In such methods, apolar pre-columns are generally used to trap the compounds of interest. The polar compounds of the biological matrix are eluted to waste with an aqueous solvent, and subsequently the analytes are switched to an analytical column, where they are separated and quantitated.

However, the application of column switching to medium or high-polarity drugs may require some modifications in the pre-column stationary phase or in the system design [9]. For example, using a column-switching system in which all the eluate was transferred to the analytical column (after washing a C_{18} pre-column with water for 1 min), the recovery of acetazolamide was only of $46 \pm 3\%$ [10].

In this work, we demonstrate that the medium polarity diuretic acetazolamide can be satisfactorily processed by column switching. To overcome problems derived from the polarity of this drug, a cut technique is proposed to transfer the analyte from the pre-column to the analytical column.

2. Experimental

2.1. Apparatus

The chromatographic system used consisted of two quaternary pumps (Hewlett-Packard, 1050 Series, Palo Alto, CA, USA), an automatic sample injector (Hewlett-Packard, 1050 series) and two high-pressure six-port valves (Rheodyne model 7000, Cotati, CA, USA). A diode-array detector (Hewlett-Packard, 1040 series) linked to a data system (Hewlett-Packard HPLC Chem Station, Dos Series) was used for data acquisition and storage. The detector was set to collect a spectrum every 640 ms, over the range 200–400 nm. All assays were carried out at ambient temperature.

2.2. Reagents

All reagents were of analytical grade. Methanol and acetonitrile were of HPLC grade (Scharlau, Barcelona, Spain). Water was distilled, deionized and filtered over 0.45-μm nylon membranes (Teknokroma, Barcelona, Spain). Acetazolamide (Cyanamid Ibérica Madrid, Spain) stocks solutions were prepared by dissolving the pure compound in methanol. Sodium dihydrogen phosphate and phosphoric acid monohydrate were from Merck (Darmstadt, Germany) and Probus (Barcelona, Spain), respectively.

2.3. Standard solutions

Standard solutions of acetazolamide were prepared by dissolving 50 mg of the pure compound in 25 ml of methanol (2 mg/ml). These solutions were stored in the dark at 2°C. Working solutions were prepared daily by dilution of these stock solutions with the appropriate volumes of water.

2.4. Columns and mobile phases

The pre-column (20×2.1 mm I.D.) was dry-packed with an Hypersil ODS C_{18}, 30 μm (Hewlett-Packard, Darmstadt, Germany) stationary phase. The analytical column was an HP-LiChrospher 100 RP 18, 125×4 mm I.D., 5 μm, column (Merck).

A 0.05 M phosphate buffer (pH 3) was used for washing the pre-column (solvent 1). This solution was prepared by dissolving 3.45 g of sodium dihydrogen phosphate monohydrate in 500 ml of distilled and deionized water. The pH was adjusted to 3 by adding the minimum amount of concentrated phosphoric acid. The

analytical separation was performed in the gradient elution mode with an acetonitrile–water mixture (solvent 2). The mobile phases were prepared daily, filtered with over a 0.45-μm nylon membrane (Teknokroma) and degassed with helium before use.

2.5. Column-switching operation

At the beginning of each assay, 5 μl of sample were injected from the injector to the pre-column. By pumping solvent 1, the most polar components of the matrix were directly eluted to waste, whereas the analyte was retained on the pre-column. At $t = 0.3$ min, valve 1 was rotated to position 2 (see Fig. 1), so the fraction of eluate containing the analyte was transferred from the pre-column to the analytical column. At $t = 1.5$ min, valve 2 was rotated to position 2; thus, the late-eluting matrix components were discarded, and the analyte is separated from possible endogenous compounds by pumping solvent 2. During 0–2 min water was used as solvent 2; at $t = 2$ min the acetonitrile content in solvent 2 was increased from 0% to 50% at $t = 4$ min. After 4 min the acetonitrile concentration was kept constant. In order to prevent contamination of the pre-column, the acetonitrile content in solvent 1 was also increased from 0% at $t = 3$ min to 50% at $t = 5$ min.

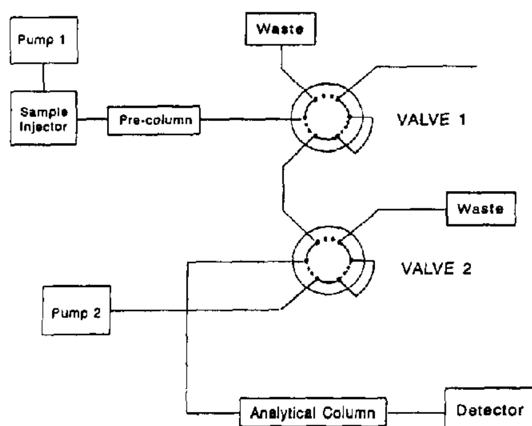

Fig. 1. Schematic representation of the chromatographic system used for the determination of acetazolamide. Switching valves: position 1 (—), position 2 (· · · · ·).

At $t = 7$ min the switching valves were turned back to their original position.

2.6. Urine samples

Volumes of 1 ml of untreated urine samples were placed into glass injection vials, and 5-μl aliquots were directly injected onto the chromatographic system.

2.7. Recovery studies

Free urine samples of 5.0 ml were spiked with acetazolamide standard solutions giving different concentrations in the 0.50–100.0 μg/ml range. The percentage of drug recovered for a particular injection was calculated by comparing the peak areas obtained for the spiked samples with the values obtained for direct injections onto the analytical column of 5-μl aliquots of aqueous solutions containing the same concentrations of drug. Each concentration was assayed in triplicate.

2.8. Preparation of standards for calibration

Standards for calibration were prepared by spiking 5.0-ml volumes of urine samples with the appropriate volumes of acetazolamide aqueous solutions resulting in different concentrations in the 0.50–100.0 μg/ml range. These samples were processed in triplicate as described above. Peak areas were plotted *versus* acetazolamide concentration, and the resulting calibration curve was used to calculate the acetazolamide concentrations in the unknown samples.

2.9. Human studies

Urinary excretion studies were performed with a healthy volunteer receiving a single dose of acetazolamide (250 mg). Urine samples were collected at appropriate time intervals post-dose, and processed as described above.

3. Results and discussion

3.1. Switching parameters

As previously indicated, the retention of acetazolamide onto reversed-phase packings is usually very low. For example, when water is used as solvent 1, acetazolamide is completely eluted from the Hypersil ODS C_{18}, 30 μ (20 × 2.1 mm I.D.) pre-column between 0.25 and 1.6 min, the dead volume being 0.15 min. This is illustrated in Fig. 2a, which shows the chromatogram obtained for a standard solution of acetazolamide with the pre-column directly connected to the detector. Under the same conditions, most of the matrix components eluted between 0.15 min and 1.0 min (Fig. 2b). Therefore, if a high recovery of acetazolamide is to be obtained, a large amount of matrix components will reach the analytical column when a simple (straight or back-flush) switching technique is used. As a result, the selectivity is poor and the operative life of the analytical column is short.

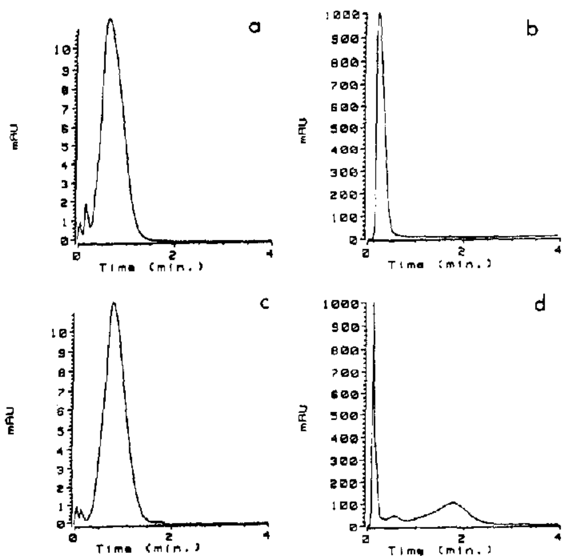

Fig. 2. Elution of acetazolamide and a urine sample from the pre-column: chromatograms obtained using water as solvent 1 for acetazolamide (a) and a urine sample (b); chromatograms obtained using 0.05 M phosphate buffer (pH 3) as solvent 1 for acetazolamide (c) and a urine sample (d). Concentration of drug, 5.0 μg/ml; $\lambda = 275$ nm.

To achieve an adequate pre-separation of acetazolamide from urinary endogenous compounds, a more polar bonded-phase pre-column should be used [9]. Instead, we have modified the switching-system design and the washing solvent. Since an acidic medium can increase the non-ionic form of acetazolamide [3,6], we tested different phosphate buffers over the range of pH 3–5 for flushing the pre-column, but no significant differences in the elution of this diuretic were observed. This can be seen in Fig. 2c, which shows the elution of acetazolamide from the pre-column, when a 0.05 M phosphate buffer of pH 3 is used as flushing solvent. However, with this latter solvent, several urinary compounds are retarded and eluted at retention times higher than 1.0 min (see Fig. 2d). According to these results, we have incorporated a second switching valve (see Fig. 1) to discard late eluting matrix components (heart-cut technique). The optimum selectivity compatible with a suitable recovery of acetazolamide was obtained by rotating the second switching valve at $t = 1.5$ min. A switching system consisting of only one valve may be used with the same objective (by connecting pump 2 to the free connection at valve 1), but in such a configuration we observed greater baseline distortions.

An acetonitrile–water mixture was used for the analytical separation. Thus, the matrix components that reach the analytical column can be separated from acetazolamide, which was the late eluting component. Fig. 3 shows the chromatograms obtained for a blank urine sample (a) and a urine sample spiked with acetazolamide (b) under the proposed conditions. The retention time of acetazolamide was 5.27 min. The selectivity is adequate and time of analysis is minimum. The xanthines caffeine, theophylline, theobromine and 1,7-dimethylxanthine (the main metabolite of caffeine), which tend to coelute with acetazolamide under reversed-phase conditions [5,11], are eluted at retention times lower than 5 min, and therefore do not interfere with the assay.

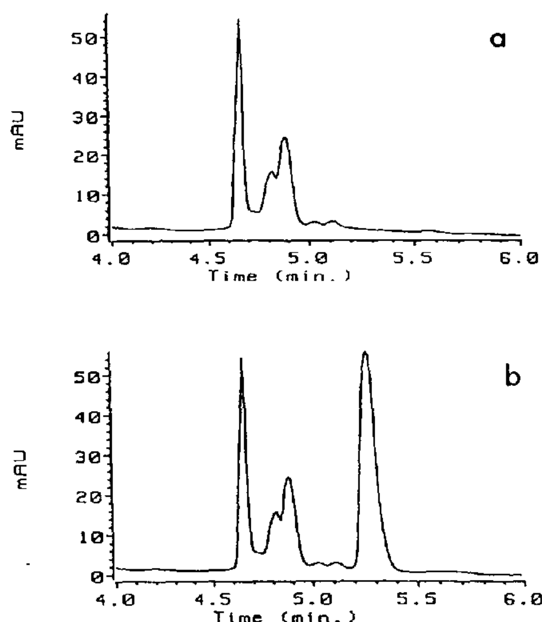

Fig. 3. Chromatograms of blank urine sample (a) and urine sample spiked with 50.0 μg/ml of acetazolamide (b). Retention time of acetazolamide, $t_R = 5.27$ min; $\lambda = 275$ nm.

3.2. Recovery

The efficiency and precision obtained in the sample clean-up process are excellent, and do not depend on the drug concentration in the studied interval, the mean recovery of drug being 96 ± 5% ($n = 12$). This value is comparable with the values obtained with conventional off-line extraction procedures, which are in the 84–96% range [1–6,12], and is clearly better than that obtained with octadecyl solid-phase extraction cartridges (63 ± 6%) [6]. The efficiency is also better than that obtained with a switching system in which the eluate is completely transferred to the analytical column (using the same pre-column and water as washing solvent for 1 min); in that case, we observed a recovery of acetazolamide of 46 ± 3% [10].

Although in our system the switching steps were manually effected, the reproducibility is greatly improved compared with liquid–liquid or solid-phase extraction on disposable cartridges, since no off-line operation is involved. Therefore, addition of an internal standard is not necessary, thus avoiding sample dilution. Furthermore, the time required for processing the samples with the present technique is drastically shortened.

3.3. Precision and accuracy

The calibration curves obtained are unbiased and linear over the working interval 0.50–100.0 μg/ml. The mean correlation coefficient was 0.9995. Spiked urine samples obtained from different subjects were analyzed in triplicate by the present procedure. The results obtained are summarized in Table 1. The concentrations found were close to the actual concentrations in all cases tested. From these results it can be concluded that the accuracy and precision of the method is suitable.

3.4. Sensitivity

The limit of detection at 275 nm (for a signal-to-noise ratio of 3) corresponds to a acetazolamide concentration in urine of 10 ng/ml.

3.5. Human studies

The described assay has been applied to the measurement of urinary levels of acetazolamide after administration of a single dose of 250 mg to a volunteer. Since acetazolamide is mainly excreted in unchanged form [13], no interference from metabolites was observed (Fig. 4). Table 2

Table 1
Precision and accuracy for acetazolamide in urine ($n = 3$)

Added concentration (μg/ml)	Found concentration (μg/ml)		
	Subject 1	Subject 2	Subject 3
2.5	2.4 ± 0.2	2.6 ± 0.3	2.4 ± 0.1
10.0	9.8 ± 0.6	10.7 ± 0.4	10.4 ± 0.2
25.0	26 ± 1	27 ± 1	25.7 ± 0.3
75.0	74.9 ± 0.6	77 ± 1	76 ± 1

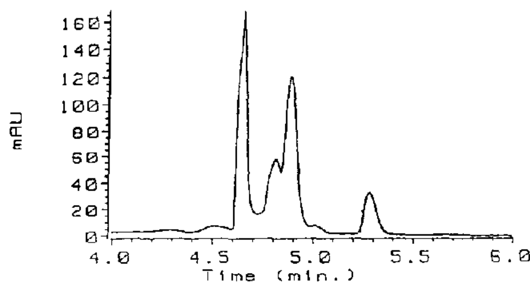

Fig. 4. Chromatograms of a urine sample obtained 24 h after administration of a single dose of 250 mg of acetazolamide. Retention time of acetazolamide, $t_R = 5.27$ min; $\lambda = 275$ nm.

gives the concentrations at different times post-dose. The values are in agreement with previously reported pharmacokinetic data [3,13].

3.6. Utility

Although untreated samples were directly injected and the duration of the flushing was very short, more than 50 samples were processed without increase of the back-pressure, either in the pre-column or in the analytical column. This indicates that the efficiency of the clean-up process is good. In principle, the described assay can be applied to any biological fluid; however, prior sample conditioning (centrifugation, dilution, filtration *etc.*) may be required to ensure a satisfactory pre-column lifetime.

Since equilibration of both the pre-column and the analytical column after an analysis can be achieved in a few minutes, the total analysis time is lower than 10 min.

With the proposed procedure, the urinary concentration of acetazolamide can be adequately determined in the 0–52 h period after administration of a minimum single dose of the drug, although it can still be detected 72 h after dosing.

Table 2
Acetazolamide levels in urine after a dose administration of 250 mg ($n = 3$)

Time (h)	Acetazolamide concentration (μg/ml)
8	78 ± 1
24	41.2 ± 0.8
48	5.6 ± 0.2

4. Conclusions

The column-switching technique can be of interest for the sample clean-up of medium or high-polarity drugs, when solid-phase extraction cartridges do not give satisfactory results. With appropriate selection of the washing solvent and system design, we have determined acetazolamide with satisfactory precision and accuracy at therapeutic levels, the total analysis time being very short.

5. Acknowledgements

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