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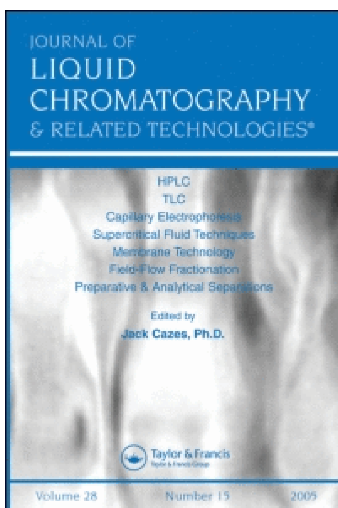

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A COLUMN SWITCHING TECHNIQUE FOR THE SCREENING OF DIURETICS IN URINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method using column switching and reversed-phase high performance liquid chromatography with diode array detection was developed for the screening of 16 diuretics, probenecid and caffeine in urine. First, a 40-μl sample is injected into a short ODS or ODP extraction column with water as eluent and polar matrix components are eluted to waste. The retained compounds are then eluted in backflush mode into the analytical column by means of a column switching valve and a gradient of 0.05 M phosphate buffer (pH 3.1) and acetonitrile. Wavelengths 230, 270 and 360 nm are used for the detection. Recoveries for spiked urine samples were near 100% and relative retention times showed good repeatability. Detection limits were 0.5-2.0 μg/ml.

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INTRODUCTION

Diuretics promote the excretion of water and electrolytes by the kidneys and are widely used therapeutically for hypertension and the oedema associated with cardiac or renal failure[1]. Diuretics have also been misused in sports and since the 1988 Olympic Games have been included in the list of banned compounds . In sports they are used to reduce weight before competition and to increase the volume of urine, in an attempt to dilute the concentration of other doping agents and achieve a negative analysis[2].

Several chromatographic methods are available for investigating individual diuretics in urine[3]. Recently, Herraez-Hernandes et al.[4] have extensively reviewed HPLC methods for their screening and determination in biological fluids. The heterogeneity of diuretics - the wide varieties in chemical structures, functional groups and pK_a values - causes problems in the determination and especially in the sample preparation. In most screening procedures, the drugs are recovered and concentrated by liquid-liquid extraction into ethyl acetate under acidic or basic conditions [2,5,6,7]. Solid phase extractions (SPE) on disposable cartridges also are used[2]. Campins-Falco et al.[8] tested different reversed phase SPE materials for the extraction of diuretics in urine and, in general, C18 and C8 packings gave better recoveries than liquid-liquid extraction with ethyl acetate.

Problems such as low recoveries and time-consuming extraction steps have increased the interest in methods for biological samples not requiring sample preparation. Newer methods used to analyse drugs,

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including diuretics, in biological fluids include direct injection of biological samples by micellar chromatography, column switching and the use of special columns [9,10,11]. Recently, Domingo et al.[12] employed micellar liquid chromatography to determine several diuretics in urine. However, most of the compounds investigated were overlapped by urine matrix components and quantitation was not possible.

This paper reports a fast and sensitive method for the screening of diuretics, probenecid and caffeine in urine. A column switching technique employing a short extraction column was used to minimize sample preparation before injection into the analytical column. Recoveries from the ODS and ODP materials used in the extraction step were compared, a polymer-based reversed phase material was investigated as a stationary phase for the analytical column.

EXPERIMENTAL

Chemicals

Acetazolamide, amiloride, bendroflumethiazide, benzthiazide, bumetanide, caffeine, carbamazepine, chlorthalidone, chlorothiazide, clopamide, dichlorphenamide, ethacrynic acid, furosemide, hydrochlorothiazide, metyrapone, probenecid, spironolactone, triamterene, trichlormethiazide and theophylline were obtained from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile was obtained from Rathburn (Walkerburn, Scotland), HPLC-grade concentrated phosphoric acid was obtained from Fisher

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(Fair Lawn, NJ, USA), and sodium dihydrogen phosphate 1-hydrate $NaH_2PO{\cdot}H_2O$ and disodium dihydrogen pyrophosphate $Na_2H_2P_2O_4$ were from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

Standard Solutions and Urine Samples

Standard stock solutions of diuretics, probenecid, caffeine and carbamazepine (internal standard) were prepared by dissolving 10 mg of the drug in 10 ml methanol (1000 μg/ml), except for triamterene which was prepared by dissolving 10 mg in 40 ml methanol (250 μg/ml). The solutions were protected from light and stored at 4°C. Drug-free urine was obtained from healthy volunteers. Working standard solutions in water and spiked urine samples were prepared by adding appropriate amounts of stock solutions. Urine samples were then buffered to pH 4.9 or 6.0 by mixing them 1:1 with 0.2 M $Na_2H_2P_2O_4$ buffer solution in which the pH was adjusted with phosphoric acid or sodium hydroxide.

Instrumentation

The HPLC system consisted of a Hewlett-Packard model 1090 instrument equipped with a 1040 diode array detector (DAD), a computer, a disc drive unit, an integrator, a printer (Hewlett-Packard, Avondale, U.S.A.), an LKB 2150 HPLC pump (Bromma, Sweden) and a Valco 6-port valve. Volume of the sample loop was 40-μl. The complete column-switching system is depicted in Figure 1.

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Chromatographic Conditions

The column used for the analytical separation was Shiseido SG-120 (polymer-based C18, 200 x 4.6 mm, 5 μm). Hypersil octadecylsilica (ODS) (20 x 4 mm, 5 μm) and Asahipak octadecylpolymer (ODP) (10 x 4.6 mm, 5 μm) were used as extraction columns.

The eluent for the extraction of polar matrix compounds (pump 1) was HPLC-grade water. The eluent for the backflushing and analytical separation (pumps 2 and 3) consisted of a gradient of 0.05 M phosphate buffer (pH 3.1) and acetonitrile. The buffer was prepared by dissolving 6.9 g of $NaH_2PO_4 \cdot H_2O$ in 1000 ml of water. The pH was adjusted with phosphoric acid. Both eluents were filtered and degassed before use. The following gradient was used for the backflushing analytical separation: initial acetonitrile 7%, increased to 15% at 3.5 min, to 50% at 12 min and maintained at 50% to 23 min. The flow rate of the extraction eluent and the gradient was 1 ml/min and the column temperature was ambient. The diode array detector was set to monitor the signal at 230, 270 and 360 nm. Spectral data between 190 and 400 nm were stored in the computer memory and could be plotted at the end of the run.

Column Switching Procedure

Step 1 (0-3 min). The valve is in position 1 (Figure 1). The urine sample is injected into the extraction column with water as eluent from pump A. The drugs of interest are retained by the extraction column while polar matrix components are eluted to waste.

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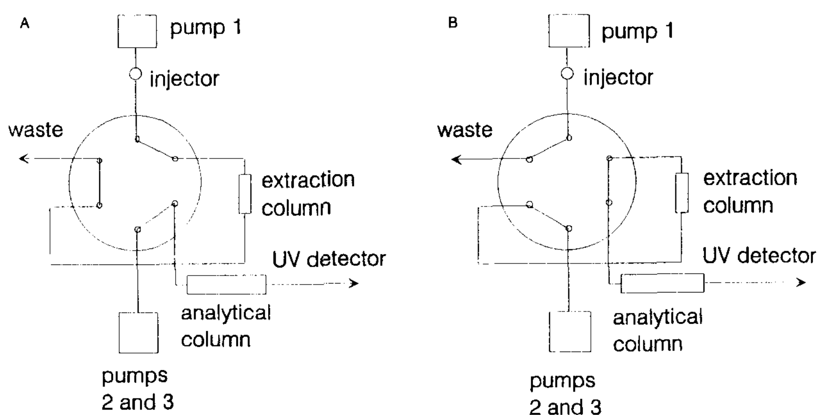

FIGURE 1. Schematic representation of the column switching system. The valve is in position 1 (A) and in position 2 (B).

Step 2 (3-23 min). The valve is switched to position 2. A gradient of the mobile phase from pumps B and C is passed through the extraction column in backflush mode and elutes the retained components into the analytical column, where they are separated.

Step 3 (23-33 min). The valve is switched to position 1. The extraction column and the analytical column are allowed to stabilize for 10 minutes before the next injection.

Recovery

Analytical recoveries were calculated by comparing the peak heights of standards or spiked samples passed through both the extraction and analytical columns with the peak heights of standard of the same concentration injected directly onto the analytical column.

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RESULTS AND DISCUSSION

Optimization of the Extraction Step

1. Blank Urine and Standard Solutions

Two short reversed phase columns, ODS and ODP, were used for the extraction step. First, the retention of the drugs and urine matrix components on the two columns was investigated by using water as eluent. With flow rate of 1 ml/min, the minimum extraction time for removal of most polar and UV-absorbing urine matrix components from the column was 2.5 minutes. The length of the extraction step needed to retain the drugs in the column was optimized by studying recoveries after different extraction times. With both columns, when the extraction time was 4 minutes and the flow rate was increased to 2 ml/min, recoveries for standard solutions of all compounds except acetazolamide, chlorothiazide and hydrochlorothiazide ranged from 90 to 110 %. The high recoveries indicated that the compounds were well retained. However, with the ODS column and a flow rate of 1 ml/min, acetazolamide and chlorothiazide eluted after 3 minutes. With the ODP column, acetazolamide, chlorothiazide and hydrochlorothiazide were not concentrated at the front of the column and excessive band broadening was observed when extraction time was increased. After 3 minutes extraction time (flow rate of 1 ml/min), recoveries of these compounds on ODP material were 30-50% when calculated from peak heights and 80-90% when calculated from peak areas. With urine samples, recoveries were calculated from peak heights because of matrix components eluted nearby; the chosen length of the extraction step on ODS and ODP columns was 2.7 and 3.0 minutes, respectively.

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2. Spiked Urine Sample

Direct injection of spiked urine samples to the LC system proved to be unsatisfactory because recoveries varied with the pH of the sample. Normally, the pH of urine is between 5 and 8 [13]. When the pH was near 8 the recoveries were lower from both extraction columns. However, when the pH was near 5, more matrix components were retained by both columns and additional peaks from the urine matrix appeared in the chromatogram. In addition, chlorothiazide and acetazolamide were not retained by the ODP column when the pH exceeded 6.3, and chlorothiazide was not retained by the ODS column when the pH exceeded 5.0. As a consequence, it was necessary to buffer the urine sample to pH 6.0 and 4.9, respectively, when the ODP and ODS columns were used. Recoveries for the spiked urine samples are shown in Table 1. The enrichment of some compounds as a narrow band at the front of the extraction column can be seen from the high recoveries.

Analytical Separation and Detection with DAD

Polymer-based reversed phase material and a gradient of acidic phosphate buffer and acetonitrile were used for the analytical separation. Figures 2 and 3 illustrate the chromatograms of a standard solution containing 16 diuretics, probenecid, caffeine and carbamazepine (internal standard) with the detector set at 230 nm. All compounds can be separated under the chromatographic conditions described. The chromatograms also reveal the different behaviour of the ODS and ODP extraction columns. Band broadening of acetazolamide, chlorothiazide

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TABLE 1.

Recoveries and relative standard deviations (n=6) for spiked urine samples using ODP and ODS extraction columns. In all cases 8 μg/ml of each of drug was added.

Diuretic	Recovery (mean±S.D.,n=6) (%)	
	ODP	ODS
Amiloride	108±4	114±4
Acetazolamide	72±3	81±14
Caffeine	107±4	115±4
Chlorothiazide	36±1	92±3
Hydrochlorothiazide	35±3	107±4
Triamterene	98±4	111±4
Metyrapone	116±5	115±8
Chlorthalidone	109±3	124±6
Dichlorfenamide	102±4	113±3
Clopamide	109±7	124±4
Trichlormethiazide	93±5	110±4
Carbamazepine (ISTD)	111±4	124±4
Furosemide	80±2	109±3
Benzthiazide	97±6	112±4
Bendroflumethiazide	94±4	95±2
Ethacrynic acid	86±5	81±2
Bumetanide	93±3	95±3
Probenecid	95±3	102±3
Spironolactone	97±5	100±5

and hydrochlorothiazide in the ODP column is clearly evident in the chromatogram of figure 3. Table 2 shows the retention times and relative retention times of the investigated diuretics by the screening procedure.

Three wavelengths, 230, 270 and 360 nm, were used to monitor the eluting compounds. Most of the diuretics investigated have a strong

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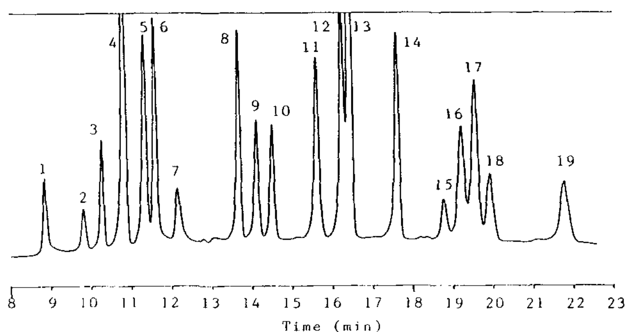

FIGURE 2. Chromatogram of a standard solution containing diuretics, caffeine and carbamazepine (ISTD) obtained after extraction in an ODS column. Detection at 230 nm and 0.080 a.u.f.s. Other chromatographic conditions: see Experimental. Peaks: 1 = amiloride; 2 = acetazolamide; 3 = caffeine; 4 = chlorothiazide; 5 = hydrochlorothiazide; 6 = triamterene; 7 = metyrapone; 8 = chlorthalidone; 9 = dichlorfenamide; 10 = clopamide; 11 = trichlormethiazide; 12 = carbamazepine (ISTD); 13 = furosemide; 14 = benzthiazide; 15 = bendroflumethiazide; 16 = ethacrynic acid; 17 = bumetanide; 18 = probenecid; 19 = spironolactone.

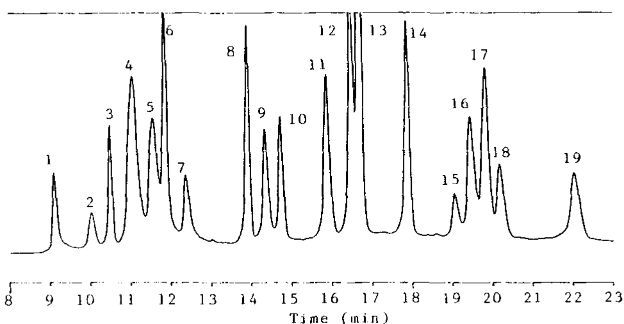

FIGURE 3. Chromatogram of a standard solution containing diuretics, caffeine and carbamazepine (ISTD) obtained after extraction in an ODP column. Detection is at 230 nm and 0.080 a.u.f.s. Other chromatographic conditions and the elution order are the same as in Figure 2.

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TABLE 2.

Retention times (t_R) and relative retention times (RRT) for the whole screening procedure using ODP and ODS extraction columns.

Diuretic	ODP		ODS	
	t_R (min)	RRT	t_R (min)	RRT
Amiloride	9.1	0.55	8.9	0.55
Acetazolamide	10.0	0.61	9.8	0.60
Caffeine	10.5	0.64	10.3	0.64
Chlorothiazide	11.0	0.67	10.8	0.67
Hydrochlorothiazide	11.6	0.70	11.3	0.70
Triamterene	11.8	0.72	11.6	0.72
Metyrapone	12.4	0.75	12.2	0.75
Chlorthalidone	13.9	0.84	13.7	0.85
Dichlorfenamide	14.3	0.87	14.1	0.87
Clopamide	14.7	0.89	14.5	0.90
Trichlormethiazide	15.9	0.96	15.6	0.96
Carbamazepine (ISTD)	16.5	1.00	16.2	1.00
Furosemide	16.7	1.02	16.4	1.01
Benzthiazide	17.9	1.08	17.6	1.09
Bendroflumethiazide	19.1	1.16	18.8	1.16
Ethacrynic acid	19.5	1.18	19.2	1.19
Bumetanide	19.8	1.20	19.5	1.20
Probenecid	20.2	1.23	19.9	1.23
Spironolactone	22.1	1.34	21.8	1.35

absorbance at 230 or 270 nm, but in general and as reported earlier [5,6], interference from matrix components was less at 270 nm. Amiloride and triamterene were detected at 360 nm. Figures 4 and 5 illustrate the chromatograms of a urine blank obtained after extraction with the ODS and ODP precolumns, respectively. In both cases caffeine metabolite

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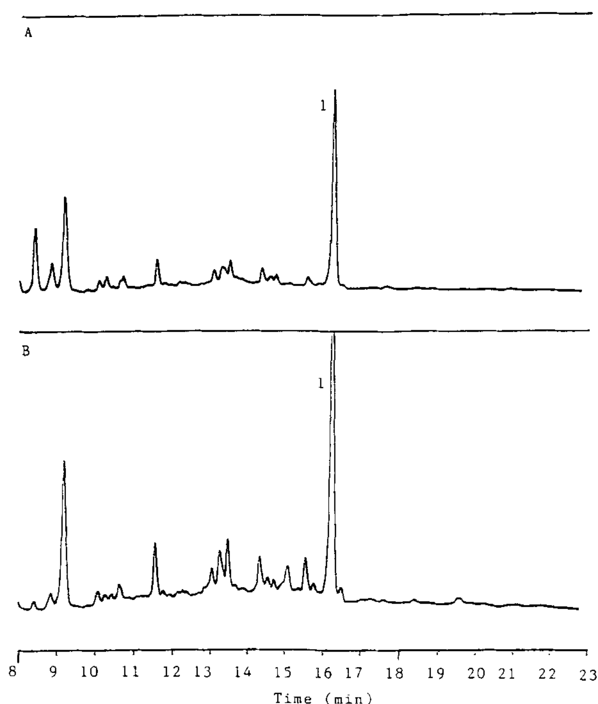

FIGURE 4. Chromatogram of urine blank. ODS extraction column. Detection at 230 nm (lower) and 270 nnm (upper) and at 0.080 a.u.f.s. Other chromatographic conditions are the same as in Figure 2. Peaks: 1 = carbamazepine.

theophylline coeluted with amiloride. Since theophylline does not absorb at 360 nm, however, amiloride could be quantified at this wavelength. In some runs endogenous compounds coeluted with acetazolamide, chlorothiazide and chlorthalidone and these compounds could not be identified from their UV spectra.

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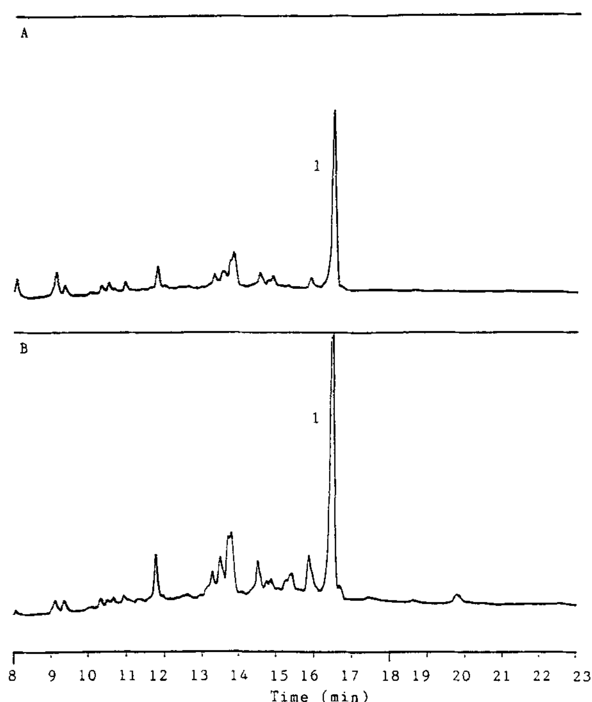

FIGURE 5. Chromatogram of urine blank. ODP extraction column. Detection at 230 nm (lower) and 270 nnm (upper) and at 0.080 a.u.f.s. Other chromatographic conditions are the same as in Figure 2. Peaks: 1 = carbamazepine.

Validation

The precision and accuracy of the method were evaluated by performing six replicate analyses of a spiked urine sample containing 8 μg/ml of each of the drugs investigated. Relative standard deviations for recoveries given in Table 1 ranged from 2 to 17% (mean 4.5%). Standard deviations for retention times and relative retention times, given in Table

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TABLE 3.

Detection limits (S/N=3) and wavelengths used for the detection in the screening procedure for spiked urine samples.

Diuretic	Detection limit (μg/ml)	Wavelength (nm)
Amiloride	0.5	360
Acetazolamide	1.0	270
Caffeine	1.0	270
Chlorothiazide	1.0	230
Hydrochlorothiazide	1.0	270
Triamterene	0.5	360
Metyrapone	2.0	270
Chlorthalidone	1.0	230
Dichlorfenamide	1.0	230
Clopamide	1.0	230
Trichlormethiazide	1.0	230
Furosemide	1.0	230
Benzthiazide	0.5	230
Bendroflumethiazide	0.5	270
Ethacrynic acid	0.5	230
Bumetanide	0.5	230
Probenecid	1.0	230
Spironolactone	1.0	230

2, were 0.004-0.026 min (mean 0.012 min) and 0.0000-0.0015 (mean 0.0005). Detection limits (S/N=3) and optimum wavelengths are given in Table 3. Greater sensitivity for standard solutions can be achieved by increasing the injection volume. However, larger injection volumes of spiked urine samples increases the amount of interfering urine matrix components in the chromatogram.

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CONCLUSION

The proposed method for the screening of diuretics, probenecid and caffeine in urine offers definite advantages over liquid-liquid extraction and SPE methods. The total analysis time is shorter and no transfer or evaporation of the sample is required. A large number of injections, 30-40, can be made into the same extraction column, so that the analysis is economical. No deterioration of the analytical column was observed during the study. Although we used a manual column switching valve the entire method can be automated with computer-programmable equipment. Reproducible retention times, recoveries and sensitivity show that the method is applicable to routine analysis.

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