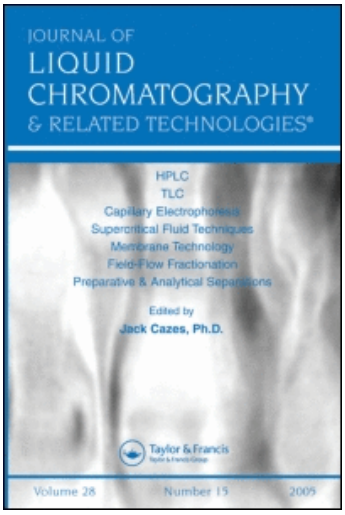

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Solid-Phase Extraction Techniques for Assay of Diuretics in Human Urine Samples

Pilar Campins-falcó[a]; Rosa Herráez-Hernández[a]; Adela Sevillano-cabeza[a]
[a] Departamento de Quimica Analitica Facultad de Quimica, Universidad de Valencia, Burjassot, Valencia, Spain

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SOLID-PHASE EXTRACTION TECHNIQUES FOR ASSAY OF DIURETICS IN HUMAN URINE SAMPLES

PILAR CAMPÍNS-FALCÓ*, ROSA HERRÁEZ-HERNÁNDEZ, AND ADELA SEVILLANO-CABEZA
Departamento de Química Analítica
Facultad de Química
Universidad de Valencia
Burjassot, Valencia, Spain

Summary.- Solid-phase extraction techniques were evaluated for the treatment of urine samples in the analysis of diuretics before injection into an HP-Hypersyl ODS-C18 column. Six different reversed-phase extraction columns were tested, and the results obtained are compared with those obtained in a classical liquid-liquid extraction with ethyl acetate.

The solid-phase extraction procedures are the best overall choice for all the diuretics tested, due to their versatility, the minor time-consuming, and the good recovery percentages obtained. C18 and C8 packings give the highest recoveries for a majority of the diuretics studied. However, CH or PH columns, due to their greater selectivity, can be used if the elution of the matrix is not complete in the washing solution. This could be more suitable

(*) To whom correspondence should be addressed

to obtain satisfactory recoveries of the more polar diuretics such as acetazolamide or hydrochlorothiazide.

INTRODUCTION

The most employed technique for detecting or quantifying diuretics in biological fluids is the reversed-phase liquid chromatography. The analysis of these drugs in serum plasma or urine by HPLC requires sample clean-up procedures to remove proteins, pigments etc, before injection into the columns. The difficulties with direct injection procedures are primarily due to column degradation from irreversible endogenous compounds adsorption, resulting in a decrease in column performance and an increase peak pressure. The most straight-forward method for sample preparation generally uses a liquid-liquid extraction under acidic or basic conditions to recover and concentrate the drug in a suitable organic solvent, and to remove the endogenous compounds that can be harmful to the column packing. These procedures are usually labour-intensive operations and multi-step extractions may be necessary. Large volumes of organic solvents may be needed. Moreover, the immiscibility of the two employed phases can lead to the formation of emulsions obtaining variable recoveries of the analytes. However, the recovery found for a given compound by using an optimized extraction procedure is generally high, about 80-100 % (1). Low recoveries of basic and/or weakly basic diuretics are obtained using acid extraction, whereas acid diuretics show poor extraction under basic conditions.

Fullinfaw et al (2) obtained low recoveries for acidic diuretics such as chlorothiazide or furosemide because a re-extraction at pH 7.5 was needed to remove the strong acidic

urinary endogenous compounds. Cooper et al (3) proposed two extractions under both acidic and basic conditions for screening test in order to increase the recoveries of diuretics.

Problems such as discussed above for the liquid-liquid extraction techniques, have lead to the research of alternative clean-up procedures.

Precipitation by salts such as $ZnSO_4$, $Ba(OH)_2$ and MeCN (or MeOH) has been proposed for the determination of some diuretics in plasma and urine (4)(5).

The literature also describes the employment of special packings such as the internal surface reversed-phase support (ISPR) to eliminate proteins adsorption (6)(7).

Micellar liquid chromatography using direct injection without any prior sample preparation has been employed to determine chlorthalidone in human plasma (8). Although it can be combined with an extraction before injection into the column, the authors indicate that it is not a practical proposition. Furthermore, the micellar system shows a loss of chromatographic efficiency due to poor mass transfer.

Sentell et al (9) have suggested that micellar liquid chromatography applied to the determination of bumetanide can be satisfactory if adequate surfactant concentrations are used in the mobile phase, specially in conjunction with a micellar concentration gradient.

Solid-phase extractions on disposable cartridges have also been reported in the literature for the analysis of diuretics such as acetazolamide (10) or amiloride (11), but they are not of general use. Solid-phase extraction techniques perform a similar function to trace-enrichment columns. They use sorbents of the same type as those used in analytical columns, but with a much greater

particle size. The sorbent is held at the bottom of a syringe-like column between two inner frits. The biological fluid passes through the material, and the drug is bound to the sorbent. Then, this is washed using deionised water or a buffer solution to remove endogenous compounds. Finally the drug is eluted in a small volume of a suitable organic solvent. The consumed time is much less than that needed to process the sample by a liquid extraction procedure.

This work shows the possibilities of the solid-phase extraction technique for the screening of diuretics including drugs of all the pharmacological groups, namely carbonic anhydrase inhibitors, thiazide and thiazide-type, loop, potassium - sparing and uricosuric agents.

The packing materials employed are of normal use in reversed-phase liquid chromatography: C18, C8, C2, ciclohexyl (CH), phenyl (PH) and cyano (CN). The results obtained are compared with those found by a classical liquid-liquid extraction procedure with ethyl acetate, proposed by Cooper et al (3).

EXPERIMENTAL CONDITIONS

Reagents. All the reagents were of analytical grade. Methanol and acetonitrile were of HPLC grade (Scharlau). Water was distilled, deionized and filtered in nylon membranes, 0.45 µm (Teknokroma). Diuretics standard solutions were prepared by dissolving in methanol pure compounds: amiloride hydrochloride (ICI-Pharma), acetazolamide (Cyanamid Ibérica), hydrochlorothiazide (ICI-Pharma), triamterene (Sigma), chlorthalidone (ICI-Pharma), furosemide (Lasa), cyclothiazide (Boheringer Ingelheim), bendroflumethiazide (Sigma), bumetanide (Boheringer Ingelheim), ethacrynic acid

(Sigma), probenecid (Sigma) and spironolactone (Searle Ibérica S.A.). The internal standard was β-hydroxymethyltheophylline (Sigma).

Propylamine hydrochloride (Fluka), sodium dihydrogen phosphate monohydrate (Merk), disodium phosphate ($Na_2HPO_4.12H_2O$) (Probus), sodium bicarbonate (Probus), potassium carbonate (Probus), lead acetate (Fluka) and ethyl acetate, HPLC grade (Scharlau), were also used.

Standard solutions. The standard solution of each diuretic was prepared by dissolving 50 mg of the pure compound in 25 mL of methanol (2000 µg/mL); triamterene standard solution was prepared by dissolving 100 mg of the pure compound in 250 mL of methanol (400 µg/mL). The internal standard was prepared by dissolving 250 mg of the pure compound in 250 mL of methanol (1000 µg/mL). All the solutions were stored in the dark at 2^0C.

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

The column was an HP-Hypersyl ODS-C18 (5 µm, 250 mm x 4 mm ID). The detector was set to obtain the signal between 200 and 400 nm each 640 ms and all the assays were carried out at ambient temperature.

Solid-phase materials.- Six different Bond-Elut columns (Scharlau) (100 mg/1 mL) were evaluated for the extraction of diuretics from urine samples: C18, C8, C2, ciclohexyl, phenyl and cyanopropyl.

Chromatography. Mobile phase: a gradient phosphate buffer/ acetonitrile with an increasing acetonitrile content from 15 % at zero min to 80 % at 8 min was used. The phosphate buffer was prepared by dissolving 3.45 g of sodium dihydrogenphosphate monohydrate in 500 mL of distilled and deionized water, after addition of 0.7 mL of propylamine hydrochloride. The pH was then adjusted to 3.00 by addition of the minimum quantity of concentrated phosphoric acid. The solution was prepared daily, filtrated with a nylon membrane (0.45 µm) and degassed with He before use. The flow was set to 1 mL/min. The chromatographic signal was monitored at 230, 254 and 275 nm.

Extraction procedure.

Liquid-liquid extractions.- According with the procedure proposed by Cooper et al (3), 2 mL of urine spiked with 300 µL of methanolic solution being 100 µg/mL in each diuretic, were extracted with 4 mL of ethylacetate under acidic and basic conditions (pH of 5-5.5, and 9-9.5, respectively). The organic fraction was evaporated to dryness and the residue was then reconstituted with 300 µL of internal standard solution (containing 50 µL/mL of β-hydroxymethyltheophylline). This solution was then filtered with nylon filters 25 mL, 0.45 µm (Teknokroma), and 5 µL were injected into the column with a Hamilton microsyringe.

Solid-phase extractions.- The solid-phase extraction columns were conditioned previously by drawing with 500 µL of methanol, followed by 300µL of distilled water. Samples of urine (2 mL) containing 300 µL of methanolic solution of each diuretic (100 µg/mL) were transferred to the columns, and washed to eliminate the biological

matrix with different volumes of distilled water. Diuretics were eluted from the columns with 500 µL of methanol. The resulting solutions were then evaporated, regenerated and filtered as described for liquid-liquid extractions, and 5 µL were injected into the analytical column.

Recoveries.- The percent recoveries from a particular extraction, were calculated comparing the peak areas obtained for diuretics in the spiked samples (referred to the internal standard), with the respective peak areas obtained for direct injection of methanolic solutions containing 100 µg/mL of each diuretic and 50 µg/mL of internal standard.

RESULTS AND DISCUSSION

The injection of 5 µL of the methanolic standard solutions containing 100 µg/mL gives the reference peak areas and the retention time of the different diuretics assayed (see Table 1). Ciclothiazide gave two elution peaks probably due to the presence of stereoisomers (3)(12). The peaks corresponding to bumetanide and ethacrynic acid were overlapped and then, they were always assayed individually.

Liquid-liquid extraction. Figure 1 illustrates the chromatograms obtained from acidic and basic extracts of blank urine samples of a normal healthy volunteer according to the liquid-liquid procedure proposed in (3).

The number and amount of biological matrix components in the organic phase is greater if the pH of the aqueous phase is acid, as can be seen in Figure 1. However, their retention times are not

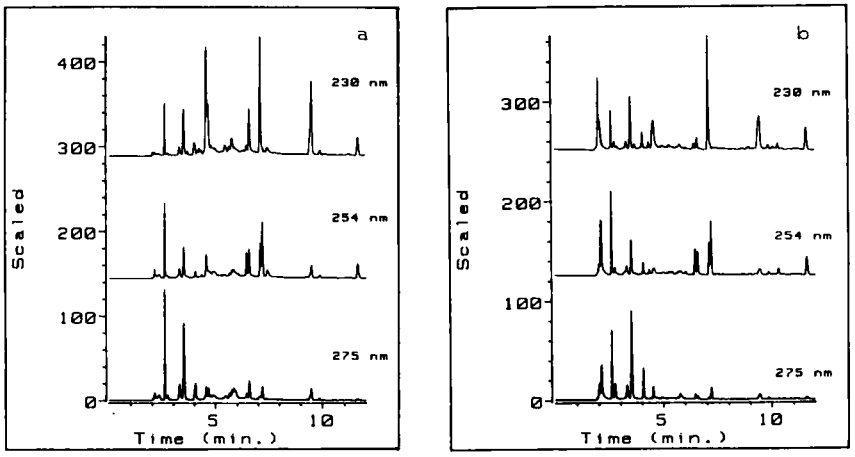

FIGURE 1. Chromatograms at different wavelengths from blank urine samples after a liquid-liquid extraction under acidic (1a) and basic (1b) conditions. Peak at 3.49 min corresponds to the internal standard.

TABLE 1. Retention time of the diuretics assayed.

DIURETIC	RETENTION TIME (min)
Amiloride	3.71
Acetazolamide	4.06
Hydrochlorothiazide	4.92
Triamterene	5.31
Chlorthalidone	5.90
Furosemide	7.31
Ciclothiazide (peak I)	8.16
(peak II)	8.25
Bendroflumethiazide	8.44
Ethacrynic acid	8.56
Bumetanide	8.66
Probenecid	8.96
Spironolactone	9.46

TABLE 2. Recovery percentages of the diuretics after liquid-liquid extraction under acidic and basic conditions.

DIURETIC	PERCENTAGE RECOVERED (%)	
	Acidic conditions	Basic conditions
Amiloride	0	23.8
Acetazolamide	80.4	0
Hydrochlorothiazide	71±9	63±8
Triamterene	26±1	74±10
Chlorthalidone	85±4	75±8
Furosemide	41.7±0.5	25±4
Ciclothiazide	53±2	62±6
Bendroflumethiazide	56±8	60±9
Bumetanide	74±9	39±3
Ethacrynic acid	72±3	49.3
Probenecid	73.2	45.0
Spironolactone	69±6	62±5

very close to those presented by the diuretics assayed (see Table 1), and also their UV spectra do not match with any diuretic screened in this study.

The precision and recovery results of each drug are shown in Table 2. The recovery of the acidic diuretics such as furosemide, bumetanide, ethacrynic acid and probenecid is poor when the pH of the aqueous phase is basic. Acetazolamide is not extracted in such conditions. Triamterene, a weakly basic diuretic is recovered in a low percentage, and even amiloride is not recovered if the extraction is carried out at acidic conditions. This diuretic also presents a low recovery in basic conditions, therefore when the amount of amiloride in biological fluids is small, its identification can be very difficult. The extraction in acidic conditions is more effective in most cases as can be seen in Table 2.

These results are comparable to those obtained by Cooper et al (3), although in some cases the recovery percentages are lower.

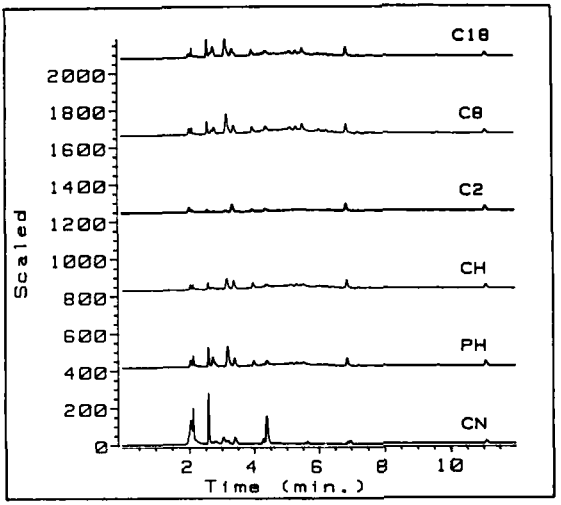

FIGURE 2. Chromatograms at 230 nm from blank urine samples extracted in a C18 and in a C2 solid-phase extraction columns. Peak at 3.49 min corresponds to the internal standard.

Solid-phase extraction. Six different packing materials with different polarities were tested: C18, C8, C2, CH, PH and CN. Figure 2 illustrates the chromatograms of blank urine samples obtained with the solid-phase extraction columns tested. The wash step was carried out with 2 mL of distilled water. The chromatograms for a complete mixture of diuretics obtained in the different columns studied can be observed in Figure 3.

The percent recoveries obtained for the diuretics appear in Table 3 for the different packing tested. The precision of the method is similar to that shown by the liquid-liquid extraction. However, the recoveries are greater.

Even in the more apolar packings - C18, C8 and C2 - the chromatograms show minor background peaks (corresponding to the

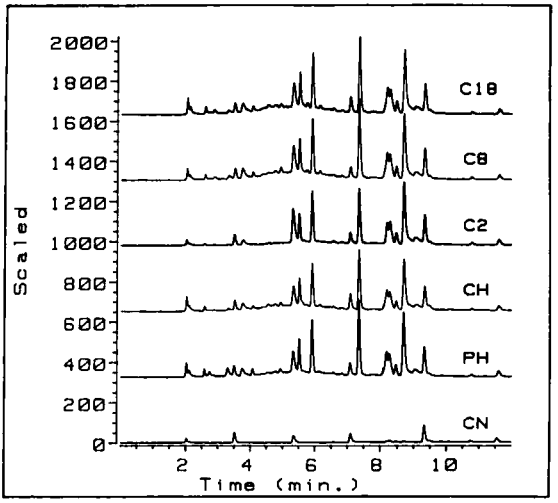

FIGURE 3. Chromatograms at 230 nm from urine samples spiked with a mixture of diuretics and extracted in the different solid-phase extraction columns. Peak at 3.49 min corresponds to the internal standard.

TABLE 3. Recovery percentages of diuretics in the different solid-phase extraction columns tested. The volume of water used in the wash step was 2.0 mL.

DIURETIC	PERCENTAGE RECOVERED (%)					
	C18	C8	C2	CH	PH	CN
Amiloride	103±7	104±10	59±8	85±9	83±9	5
Acetazolamide	<16	0	0	0	0	0
Hydrochlorothiazide	27±5	29±6	8±3	19±6	21±3	0
Triamterene	98	94	94±17	68	66	20
Chlorthalidone	114±8	115±2	126±4	102±15	105±3	<1
Furosemide	85±12	103±10	76±6	81±14	84±5	3
Ciclothiazide	102±12	100±7	108±6	80±10	83±8	10
Bendroflumethiazide	98±5	98±12	109±3	86±7	89±2	19
Bumetanide	93	96	91	100	92	-
Ethacrynic acid	83	102	114	74	84	-
Probenecid	87±5	95±12	134±7	75±6	103±4	0
Spironolactone	92±8	94±8	106±12	81±9	88±4	52

apolar endogenous compounds), than those obtained by a liquid-liquid extraction in an acidic medium, and comparable to those found under basic conditions.

The C18, C8 and C2 packings proportionate the highest recoveries, generally greater than 80 %. Significative differences between these columns are not observed, except for amiloride which shows lower values in a C2 column and acetazolamide, which is only retained in a C18 column. The percentages obtained for ethacrynic acid and probenecid are slightly greater in a C2 packing.

These percentages are comparable to that obtained with CH and PH packings for the more apolar diuretics, which are eluted in the chromatographic process at greater retention times (see Table 1). However, with the CH or PH solid-phase extraction columns, the percentages obtained for the more polar diuretics -which are eluted in short retention times- are lower than those obtained with the C18, C8 or C2 packings.

The CN columns are not appropriate for these drugs for the low recovery percentages obtained, generally lower than 20 %. Acetazolamide, hydrochlorothiazide and probenecid are not retained with this material.

Chlorthalidone gives values significantly greater than 100 % when C18, C8 or C2 columns are employed. This could be explained for the contribution of the matrix. Most of the components of the matrix are eluted between 5 and 6 min, being the retention time of chlorthalidone 5.90 min. This effect is less significative for the CH or PH columns, where the retention of the matrix is minor.

The values obtained for ciclothiazide are also greater than 100 %, if the elution peak at 8.16 min is considered, but the recoveries are good when the peak at 8.25 min is used for the calculation.

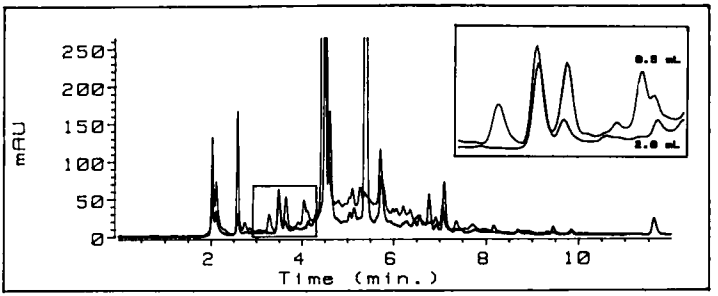

FIGURE 4. Diference between chromatograms from urine spiked with acetazolamide and extracted in a C18 column, using 2.0 and 0.5 mL of water in the wash step.

All the solid-phase extraction columns show low recoveries (less than 30 %) for hydrochlorothiazide, and acetazolamide only can be detected if a C18 column is used, even when 150 µg of this diuretic are added to the sample. These results are due to the wash step, as these diuretics are rather polar (see retention times in Table 1), and can be eluted with the 2 mL of water.

Then, these recoveries can be improved decreasing the volume of water used. In Figure 4 can be observed the chromatograms obtained in a C18 solid-phase extraction column when washing with 2.0 and 0.5 mL of water. Table 4 shows the results obtained when the wash step is carried out using 0.5 mL of water. Under these conditions acetazolamide shows, in all packings tested, percentages greater than 60 %, except in a C2 column. The values for hydrochlorothiazide are also greater than those obtained using 2.0 mL of water (three times greater in a C2 column). In all cases, the background peaks corresponding to the urinary endogenous compounds increase. This affects the recoveries for chlorthalidone, which are

TABLE 4. Recovery percentages of diuretics in the different solid-phase extraction columns tested. The volume of water used in the wash step was 0.5 mL.

DIURETIC	PERCENTAGE RECOVERED (%)				
	C18	C8	C2	CH	PH
Amiloride	110±3	72±3	54±13	109	89±5
Acetazolamide	63±6	71±4	26±6	61±2	68
Hydrochlorothiazide	45.5±0.7	57±2	27±7	53.5±0.7	49
Triamterene	120±3	139	104±20	128	96
Chlorthalidone	146±6	138±1	138	145±5	116±5
Furosemide	92±2	92±6	70	87±4	78±8
Ciclothiazide	81±3	80±4	88	76±2	72
Bendroflumethiazide	102±10	92±5	103	89±2	79±8
Bumetanide	101	79	64	74	59
Ethacrynic acid	89	100	113	96	93
Probenecid	127	119±9	99	120±6	97
Spironolactone	89±6	83±3	98	81	71±9

too high. Triamterene (t_R = 5.31 min) also gives recovery percentages greater than 100 % for a majority of the packings tested.

Reduction of volumes of water used in the wash step does not significantly modify the recoveries of the more apolar diuretics.

CONCLUSIONS

The employment of solid-phase extraction columns for samples treatment proportionates excellent results in the analysis of diuretics in urine samples by reversed-phase liquid chromatography. The recovery percentages obtained with apolar packings, such as C18 or C8, are generally greater than 80 % for a majority of the diuretics tested. These recoveries are clearly better than those obtained with a liquid-liquid extraction procedure with ethyl acetate.

The recoveries obtained for the more polar diuretics can be improved decreasing the volume of water used in the wash step, although it can increase the retention of the components of the matrix. The solid-phase extraction columns more polar, such as, CH or PH could be an alternative, because they are more selective and retain less endogenous compounds, while the retention of diuretics is not significantly affected.

In all cases, the chromatograms obtained show less number of background peaks (and their intensity is minor) than those obtained with a liquid-liquid extraction under acidic conditions, being this medium necessary to recover the most diuretics tested.

Solid-phase extraction techniques are rapid (the time consumed by a liquid-liquid extraction is approximately three times greater than that required by a solid-phase extraction), simple and give good recoveries for all diuretics tested. Furthermore an unique extraction is effective for all of them. Therefore, these techniques are advantageous over liquid-liquid extraction in the analysis of diuretics and their mixtures, or in screening procedures.

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