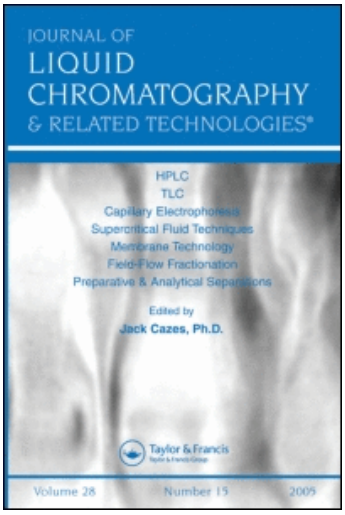

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IMPROVED SCREENING PROCEDURE FOR DIURETICS

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ABSTRACT

An improved screening procedure for commonly used diuretics in urine samples is described. The diuretics mixture assayed is quickly and satisfactorily resolved using an HP-Hypersil ODS-C18 column and a mobile phase consisting of acetonitrile/phosphate buffer (pH = 3) in gradient elution mode. Alternatively, acetate buffer (pH = 4) is utilized to improve resolution and sensitivity with more acidic compounds such as ethacrynic acid and probenecid.

INTRODUCTION

Diuretics have important applications in diseases such as congestive heart failure and hypertension. Diuretics estimation in biological fluids is of interest due to the potential role of drug monitoring in optimizing patient care, and

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because these drugs have been used and abused to reduce body weight in sports that involve weight categories. Diuretics have also been used to deliberately increase urine volume in attempts to evade drug testing, and to control water retention in athletes --one of the most common adverse effects of anabolic steroids. Thus, the specific identification of diuretics is very important in clinical laboratories and doping controls.

Several methods are available for the detection and the determination of individual diuretics (1), although only a few screening procedures have been reported in recent years. Furthermore, these are mostly limited to screen mixtures consisting of pure compounds in pharmaceutical preparations (2)-(8).

Earlier HPLC studies for the detection of diuretics in biological fluids were developed by Tisdall et al (9)., but only for thiazide-type compounds. The method requires two mobile phases and derivatization to analyze chlorothiazide. Shah et al. (10) used different reversed-phase columns, (monomeric MCH and polymeric CH), to analyze thiazide diuretics in urine samples.

Smith et al. (11) adapted the mobile phase of acetonitrile 1 % - aqueous acetic acid proposed by Tisdall et al. (9), in a detailed examination of the reproducibility of the method for thiazide diuretics and in evaluating the robustness of different approaches for recording retention times.

When screening involves other types of diuretics, gradient elution mode operation is required to achieve sufficient resolution in short times of analysis; the detector system used is generally a powerful diode-array to distinguish between partially overlapping chromatographic peaks. Fullinfaw et al. (12) and Cooper et al. (13) proposed such procedures. They

employed aqueous phosphate buffer/acetonitrile as mobile phase, but with different gradient profiles and temperatures. A more detailed systematic study to optimize the chromatographic conditions in screening procedures has been reported by De Croo et al. (14), who examined the effect of eluent composition, organic modifier, pH and temperature on the capacity factors of a number of diuretic drugs (thiazides, loop and potassium-sparing diuretics) using isocratic elution.

In the present study we evaluate the effects of a number of variables that affect the chromatographic separation in gradient elution mode of representative diuretics belonging to the four pharmacological groups, namely carbonic anhydrase inhibitors, thiazide and thiazide-type, loop, potassium - sparing. Probenecid, an uricosuric agent, has also been included in this study because it has a weak diuretic activity and has been used in sports to decrease urinary excretion of anabolic steroids. An improved screening procedure is proposed using coupled HPLC/ UV-Vis approach.

MATERIALS

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-Hypersil ODS-C18 (5 µm, 250 mm x 4 mm ID). Signal detection was set for between 200 and 400 nm every 640 ms, and all the assays were performed at room temperature.

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 ß-hydroxymethyltheophylline (Sigma).

Propylamine hydrochloride (Fluka), diethylamine (Analyticals-Carlo Erba), sodium dihydrogen phosphate monohydrate (Merk), sodium acetate trihydrate (Probus), 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.

METHODS

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°C.

Mobile Phase

Phosphate buffer (solvent A of the gradient), was prepared by dissolving the appropriate amount of sodium dihydrogen phosphate monohydrate and propylamine hydrochloride in 500 mL of distilled and deionized water. The pH was adjusted by adding a minimum amount of concentrated phosphoric acid. An additional solvent A, acetate buffer, was prepared from sodium acetate trihydrate and acetic acid. All the solutions were prepared daily, filtrated with a nylon membrane, 0.45 μm (Teknokroma) and degassed with helium before use. Acetonitrile was used as solvent B of the gradient.

Urine Samples

Urine samples were spiked with the appropriate amount of diuretic standard solutions; following liquid-liquid extraction according to (13), the samples were filtered with nylon filters 25 mm, 0.45 μm (Teknokroma). Finally, 5 μL of each sample were injected into the column using a Hamilton micro-syringe.

RESULTS AND DISCUSSION

The diuretics screening procedure employed by Cooper et al. (13) was initially tested for synthetic samples; 0.5 μg of each

diuretic was injected, along with 0.25 μg of the internal standard. The mobile phase was acetonitrile/0.05 M phosphate buffer (pH=3) containing 0.016 M propylamine hydrochloride. A gradient was used to increase the acetonitrile content from 15 % at 0 - 2 min, to 80 % at 20 min. The mobile phase flow-rate was kept at 1 mL/min. The gradient profile of Fullinfaw's method (12) was also reproduced, but operating at room temperature and with a mobile phase flow-rate of 1 mL/min (acetonitrile/0.01 M phosphate buffer at pH=3). The gradient shapes in both cases are shown in Figure 1. Fullinfaw's gradient is steeper than Cooper's gradient, although strength at the end of the gradient is greater in the latter procedure. Figure 2 shows the diuretics mixture chromatograms in both cases.

The retention times are longer than those obtained by Fullinfaw et al. (12), as the chromatogram is carried out at room temperature and with 1 mL/min flow-rate, the peak shapes are similar. This screening procedure was proposed for thiazide, thiazide-type and loop diuretics, i. e., it is less general than Cooper's procedure. The chromatogram reflected in Figure 2a is similar to that reported in (13); analysis time was 17 min. Cyclothiazide yields two elution peaks, probably due to the presence of stereoisomers (9)(13).

In turn, the ethacrynic acid and bumetanide chromatographic overlap, their retention times being 15.70 and 15.91 min, respectively. The broad ethacrynic acid peak (acidic diuretic) presents tailing, and the retention time varied,was found to vary considerably with concentration and mobile phase pH. Retention time varied between 15.9 to 15.0 min for injected amounts in the 0.075-5 μg range. When the amount of injected ethacrynic acid is under 0.5 μg, this diuretic coelutes with bumetanide (Figure 3).

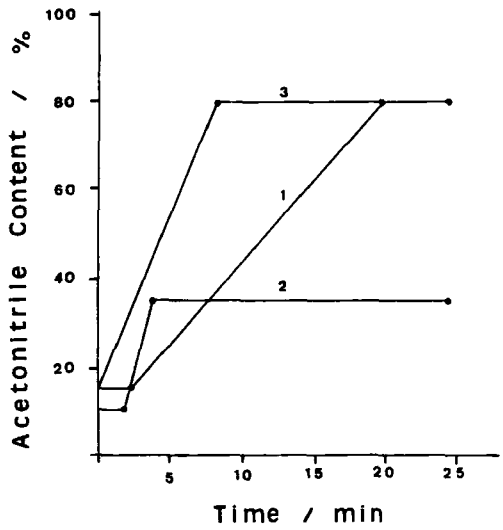

FIGURE 1
Gradient shape of the Cooper et al. (1), Fullinfaw et al. (2) and new proposed (3) screening procedures.

Between 0.5 and 1 μg overlapping is less, and the two agents can be distinguished. Finally, at over 1.25 μg, ethacrynic acid coelutes with bendroflumethiazide -peaks overlapping being less if the amount present is greater than 2.5 μg.

Figure 4 shows the variation in retention time of ethacrynic acid (for an injected amount of 0.5 μg) with the pH of the phosphate buffer. The variation is seen to be highly significant. pH control in this procedure is very important when detecting this diuretic.

Following these results, we systematically evaluated different experimental conditions to develop an improved screening procedure, with an optimized analysis time and detection of the acidic compounds (e. g. ethacrynic acid and probenecid).

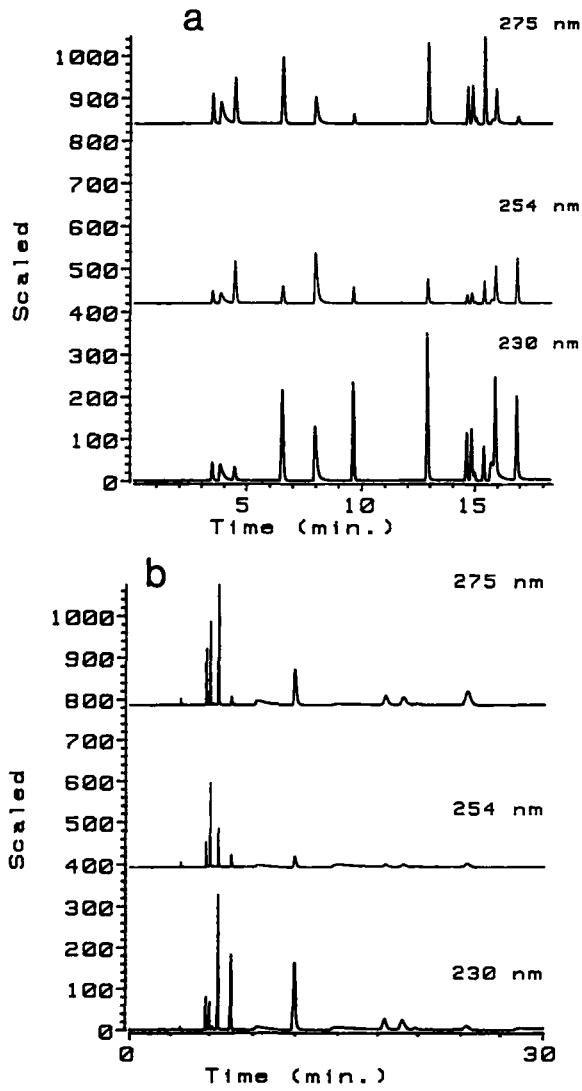

FIGURE 2
Chromatograms at different wavelengths for a mixture of diuretics eluted using gradients 1 (2a) and 2 (2b). The amount injected of each diuretic was 0.5 μg. Retention times (min): internal standard 3.47, 5.58; amiloride 3.40, 9.32; acetazolamide 4.52, 5.87; hydrochlorothiazide 6.61, 6.48; triamterene 8.01, 15.96; chlorthalidone 9.66, 7.43; furosemide 12.92, 12.02; ciclothiazide 14.66 and 18.52 (peak I), and 14.66, 19.81 (peak II); bendroflumethiazide 14.41, 24.42 for gradients elution 1 and 2, respectively; ethacrynic acid 15.10; bumetanide 15.91 and spironolactone 16.67 for gradient 1.

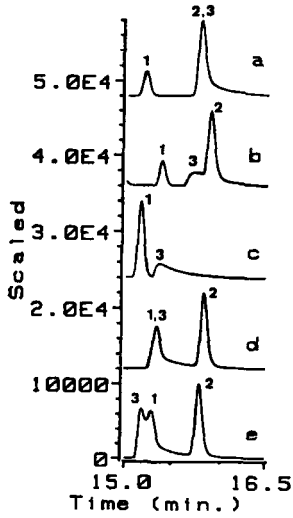

FIGURE 3
Normalized chromatograms at 230 nm for different mixtures of bendroflumethiazide (peak 1), bumetanide (peak 2) and ethacrynic acid (peak 3). The injected amounts were 0.075:0.075:0.075 (3a), 0.5:0.5:0.5 (3b), 3:0:1 (3c), 1.25:1.35:1.25 (3d) and 1.25:1.25:2.50 μg, respectively.

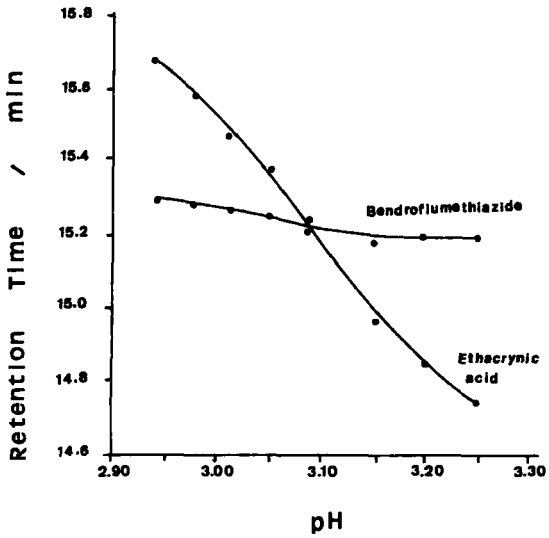

FIGURE 4
Retention times of bendroflumethiazide and ethacrynic acid vs pH of the phosphate buffer using gradient elution 1. The amounts injected of each diuretic were 1.25 μg.

Gradient Elution

Acetonitrile-water mixtures are reportedly the best eluents for diuretics. Consequently, we have always employed them as solvents, together with the mobile phase composition at the beginning and end of the gradient proposed by Cooper et al. (13). Gradient steepness was varied to shorten the time of analysis and accentuate peak intensity. Initially, the time required to obtain 80 % of acetonitrile (end of the gradient), was shortened from 20 to 12 and 8 min. The retention time of the more retained diuretics decreased accordingly. The agents eluted at retention times of under 8 min were not modified. With the aim of modifying these chromatographic peaks, the beginning of the gradient was started at zero min. The shape of gradient 3 in Figure 1 yields a time of analysis of under 10 min, while exhibiting the same resolution as in the screening procedure reported by Cooper et al. (13) but greater peak intensities. Times of under 8 min for the end of the gradient produced overlapping bendroflumethiazide and cyclothiazide peaks. In addition, the strength of the solvent mixture at the beginning of the gradient was varied from 15 % to 20 and 25 % but the results offered no advantage. We thus selected the third gradient shown in Figure 1. The resulting chromatograms at the three selected wavelengths are shown in Figure 5. Different variables were studied subsequently studied under these conditions.

pH and buffer solution

pH is not a critical variable when utilizing the proposed gradient, as reflected in Figure 6, where the variation of

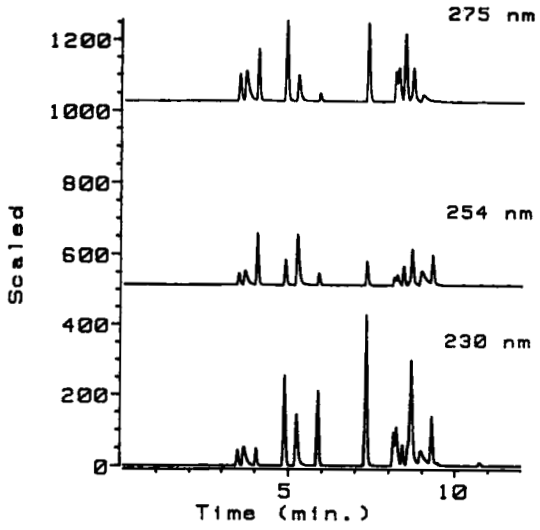

FIGURE 5
Chromatograms at different wavelengths obtained for a mixture of diuretics eluted using gradient 3. The injected amount of each diuretic was 0.5 μg. Peak at 3.49 min corresponds to the internal standard. (See Table 1).

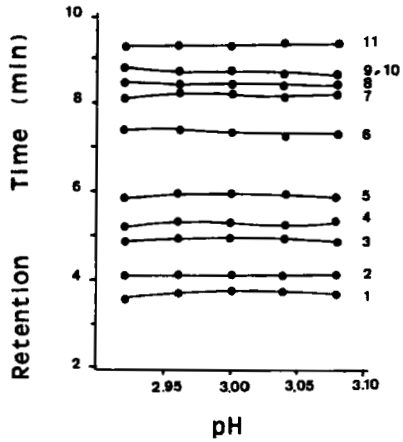

FIGURE 6
Retention time vs pH of the phosphate buffer for the studied diuretics in the proposed gradient elution procedure: 1, amiloride; 2, acetazolamide; 3, hidrochlorothiazide; 4, triamterene; 5, chlorthalidone; 6, furosemide; 7, ciclothiazide; 8, bendroflumethiazide; 9, ethacrynic acid; 10, bumetanide; 11, spironolactone. The injected amount of each diuretic was 0.5 μg.

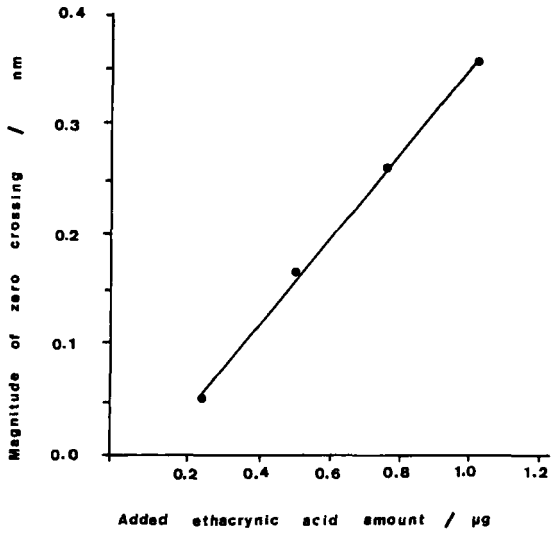

FIGURE 7
Plot of the zero-crossing for different amounts of ethacrynic acid in presence of 0.5 µg of bumetanide.

retention time of the different diuretics tested with the phosphate buffer pH is shown. Within the range studied, this variable did not modify the elution order of the compounds tested. This offers an advantage over Cooper's gradient. However, the acidic drug peaks were also broad, probably because the working pH and pK_a values were similar (3.5 and 3.4 for ethacrynic acid and probenecid respectively).

Ethacrynic acid retention time likewise did not depend on concentration, and it was always seen to coelute with bumetanide. The zero-crossing graph proposed by Grant et al. (15), obtained from the first-derivative plot of the spectrum at a mixture retention time (8.7 min), was directly related to the amount of ethacrynic acid involved (Fig. 7). The mixture spectra are thus proportional to the relative concentration of both diuretics.

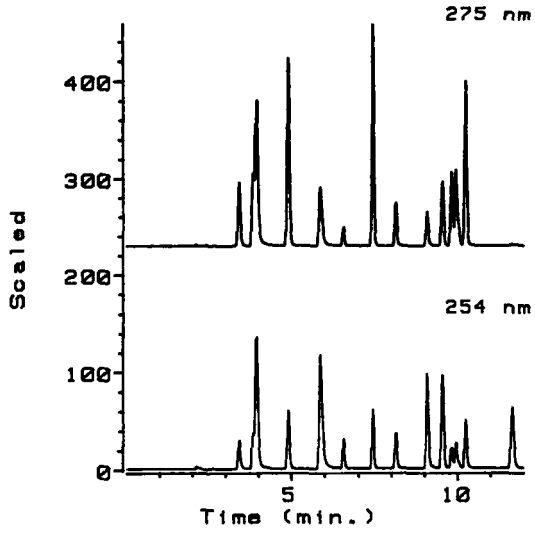

FIGURE 8
Chromatograms at 254 and 275 nm of a mixture of diuretics eluted with acetate buffer using gradient elution 3. The injected amount of each diuretic was 0.5 µg. (See Table 1).

Under such conditions, modification of the ethacrynic acid peak with the concentration is therefore negligible. Phosphate buffer pH values of over 3 improved the ethacrynic peak, but mixture resolution was poor.

Another buffer was tested at a higher pH. A solution of sodium acetate 0.05 M containing 0.016 M of propylamine hydrochloride at a pH value of 4 (adjusted with acetic acid) was chosen as solvent A for gradient 3 in Figure 1. The diuretics mixture yielded the chromatogram shown in Figure 8. Other gradient designs were tested as in the case of phosphate buffer, but the same elution program for both gave the best results.

Due to the absorption characteristics of the acetate buffer, the latter is not suitable for obtaining analytical signals at wavelengths under 250 nm.

TABLE 1

Retention Times of Diuretics Eluted with Phosphate and Acetate Buffers, Using Gradient 3.

DIURETIC	RETENTION TIME (min)	
	Phosphate buffer	Acetate buffer
Amiloride	3.71	3.93
Acetazolamide	4.06	3.95
Hydrochlorothiazide	4.92	4.90
Triamterene	5.31	5.86
Chlorthalidone	5.90	6.56
Furosemide	7.31	7.42
Ciclothiazide (peak I)	8.16	9.80
(peak II)	8.25	9.84
Bendroflumethiazide	8.44	10.24
Ethacrynic acid	8.56	8.11
Bumetanide	8.66	9.53
Probenecid	8.96	9.07
Spironolactone	9.46	11.66

Under the above conditions the acidic compounds eluted at relatively shorter retention times, and the resulting peaks were symmetrical and narrow (Figure 8). Only amiloride and acetazolamide overlapped, although they may be distinguished spectrophotometrically. Higher pH values yielded poorer results. Table 1 shows the retention time for the compounds tested with the two buffer solutions assayed. The gradient with acetate buffer required a longer time, but the analysis time was still shorter than that required by previously reported procedures.

Ionic Strength and Competing Base Influence

Diuretics retention time was not modified when 0.01 M phosphate buffer was used as solvent A of the gradient, but the peak intensity was less than that achieved with 0.05 M solutions.

In the case of acetate buffer, this concentration affected resolution -particularly for triamterene and chlorthalidone.

Diethylamine assayed as competing base for both buffers gave poorer results than propylamine.

Urine Samples

Real samples analysis were performed on 2 mL of urine spiked to contain 0.5 μg of each diuretic. Each urine sample was extracted under acidic and basic conditions as described in (13). The extracts were evaporated with a slow nitrogen stream. The residues were then reconstituted with 300 μL of internal standard solution, and 5 μL were injected into the column. The recovered percentages were estimated as being similar to those reported in (13). Figure 9 shows chromatograms obtained from the blank urine extraction. Several peaks corresponding to endogenous compounds appeared, but their UV spectra failed to match any of the drugs screened; overlapping was small or negligible, as can be seen in Figure 10, where the chromatograms of the spiked urine under both, acidic and basic conditions, are shown.

The compounds evaluated in this study have better absorbance at 230 nm, with the exception of acetazolamide and bendroflumethiazide, and interference of the urinary endogenous compounds is not critical at this wavelength. We therefore evaluate the detection limit at this wavelength using phosphate buffer and standard solutions of this diuretics in methanol. This value was established as the concentration which gave an analytical signal three times greater than the background noise. The sensitivities of acetazolamide and bendroflumethiazide, can

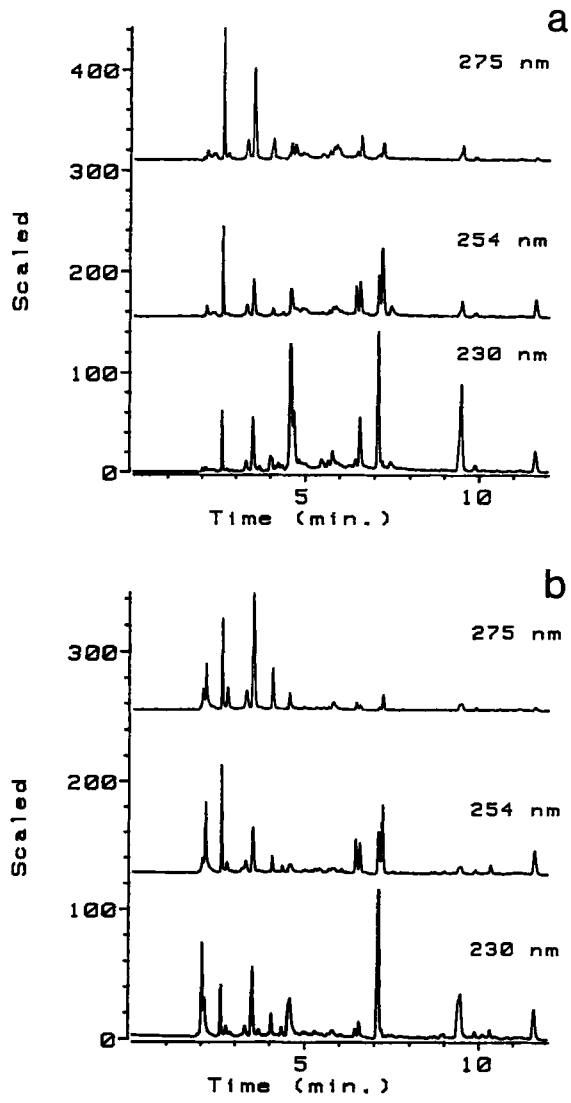

FIGURE 9
Chromatograms at different wavelengths of blank urine samples extracted under acidic (9a) and basic (9b) conditions.

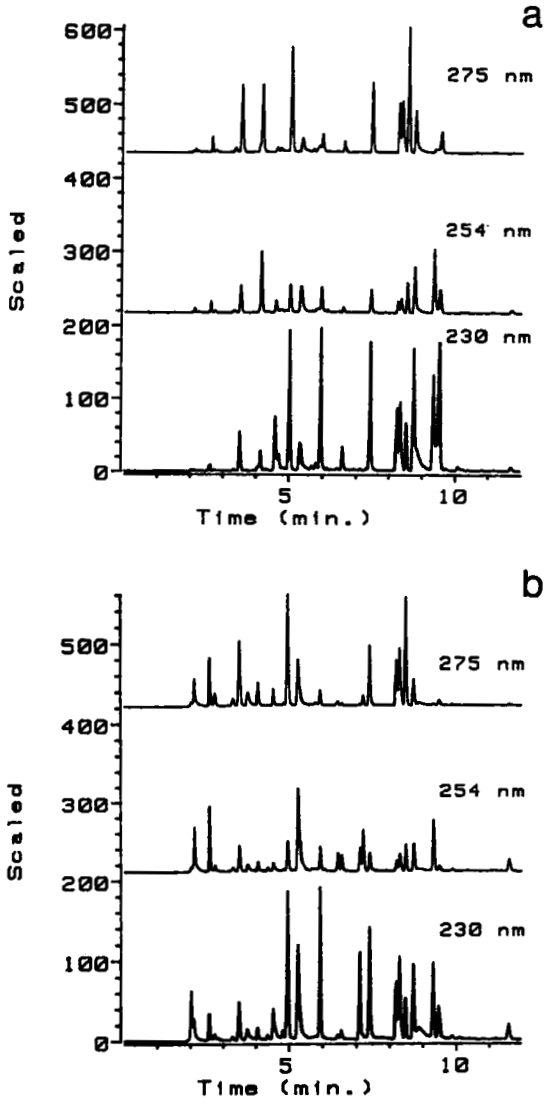

FIGURE 10
Chromatograms of urine samples spiked with 0.5 μg of each diuretic extracted under acidic (10a) and basic (10b) conditions.

TABLE 2

Limits of Detection at 230 nm for Diuretics Eluted with Phosphate Buffer Using Gradient 3.

DIURETIC	LIMIT OF DETECTION	
	Concentration (μg/mL)	Injected amount (ng)
Amiloride	0.2	1
Acetazolamide	0.5	3
Hydrochlorothiazide	0.01	0.05
Triamterene	0.2	1
Chlorthalidone	0.04	0.2
Furosemide	0.02	0.1
Ciclothiazide	0.08	0.4
Bendroflumethiazide	0.5	3
Ethacrynic acid	1.0	5
Bumetanide	0.04	0.2
Probenecid	1.0	5
Spironolactone	0.1	0.5

be increased by measuring the analytical signal at higher wavelengths.

Afterwards, an urine sample was spiked with a known amount of diuretics which correspond to their detection limit -bearing in mind the recovery percentage- and extracted as described above. Amiloride and acetazolamide were only detected in acidic and basic extracts, respectively. All the diuretics were identified from their spectra. Thus, the results in Table 2 may be extended to urine samples.

In view of the published urinary excretion-time profiles (12)(13), the procedure may be considered valid, and the values obtained were found to be lower than those required in (13). Another sample was treated in the same way, but solvent A of the mobile phase was acetate buffer, with a selected wavelength of 250 nm. In this case bumetanide could not be distinguished in either acidic or basic extract. Identification was generally more

difficult when using a phosphate buffer/acetonitrile gradient at concentrations close to the detection limit, as the spectrum was modified at wavelengths lower than 250 nm due to acetate absorption - particularly with diuretics involving shorter retention times.

In conclusion, the use of a mobile phase consisting of acetonitrile/phosphate buffer (pH=3) in gradient elution mode (from 15 % acetonitrile content at 0 min to 80 % at 8 min), offers good resolution in the screening of diuretic mixtures in urine samples. The time of analysis was short (under 10 min), and sensitivity was better than that previously reported.

Substitution of the phosphate buffer with acetate buffer (pH=4) diminished sensitivity; however, the latter was sufficient for most of the compounds tested, considering their excretion time profiles in urine. Resolution of bumetanide and ethacrynic acid was complete, and the peak shape of acidic drugs such as ethacrynic acid and probenecid was improved, thus facilitating identification. This is particularly important in detecting ethacrynic acid, as screening procedures which use phosphate buffers do not provide appropriate results (12).

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