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COMPREHENSIVE SCREENING PROCEDURE FOR DIURETICS IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and reliable screening procedure using high-performance liquid chromatography for the detection of 23 diuretics (belonging to five different pharmacological groups) in urine has been developed. Two aliquots of 2-ml urine samples were extracted separately under acidic and basic conditions. The acidic and basic extracts were pooled, evaporated to dryness and reconstituted in methanol. The methanolic extact was injected onto a Hewlett-Packard Hypersil ODS C_{18} (5 μm) column (column I) and a Hewlett-Packard LiChrosorb RP-18 (5 μm) column (column II; an alternative column). The same gradient mobile phase was used for both columns. A diode array ultraviolet detector was set to monitor the signal to the integrator (Chem Station) at 230 and 275 nm. Recovery studies of the 23 diuretics were performed under acidic and basic conditions. The overall lower limits for detection on column I using both extraction procedures ranged from 0.5 to 1.5 μg/ml of urine (average 1.0 μg/ml). Amiloride, ethacrynic acid and probenecid could not be detected below 5 μg/ml of urine. No interference from the biological matrix was apparent. Amiloride could be detected in urine 4 h after oral administration of 15 mg of amiloride to a healthy volunteer, when the sample was extracted under alkaline conditions. The suitability of the screening method for the analysis of urine samples was tested by studying the variation with time of chlorthalidone, furosemide, probenecid, acetazolamide, quinethazone, spironolactone, bendroflumethiazide, bumetanide, triamterene and hydrochlorothiazide concentrations in the urine of normal human volunteers after minimum single or multiple (probenecid) doses. The results obtained indicate that the screening method employing either column I or II would be rapid and reliable to be used in doping control and clinical laboratories.

INTRODUCTION

The major indications of diuretics are to enhance renal excretion of salt and water and to lower blood pressure. However, their effects are not limited to sodium and chloride; they may also influence the renal reabsorption and excretion of potassium, calcium, magnesium and other ions. Thiazide and loop diuretics increase urinary potassium excretion and can cause hypokalemia in subjects treated with these medications during long-term maintenance therapy. There-

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fore, thiazides are often prescribed in association with potassium-sparing diuretics in order to maintain appropriate body content of electrolytes. Thiazide, loop and potassium-sparing diuretics, alone or in combination, are used widely in the treatment of hypertension, congestive heart failure and some types of oedema.

In the last few years diuretics have been misused and abused in sports where weight categories are involved, to reduce weight prior to a competition or to deliberately dilute the urine specimen as a tentative attempt to escape the drug test. In addition, the use of diuretics belonging to carbonic anhydrase inhibitors group leads to an alkaline urine so that the excretion of basic doping substances may reduce, resulting in negative analysis [1–4]. The administration of high-ceiling diuretics like furosemide or bumetanide reduces by four- to five-fold range of the urinary concentrations of doping agents [2–4]. The diuretics have also been misused in sports to control water retention, one of the most frequent adverse effects of anabolic steroids. Probenecid (a uricosuric agent, which has a weak diuretic activity) has been included in the analytical scheme because it is known that athletes have used this drug in order to decrease the urinary excretion of anabolic steroids. The Medical Commission of the International Olympic Committee has banned different groups of diuretics as well as probenecid for the Olympic Winter and Summer Games in 1988.

Some analytical problems for the detection and identification of diuretics and their metabolites are due to (i) their wide variety of chemical structures, (ii) their wide variety of functional groups, (iii) their wide differences in pK_a values, (iv) their low volatility and (v) lack of their metabolic studies in several cases.

A number of chromatographic techniques, too numerous to cite here, have been used to detect and quantitate individual diuretics. However, only a few screening procedures have been reported [5–13]. Smith and Hermann [5], Stohs and Scratchley [6] and Honigberg et al. [7] have screened mixtures of pure compounds in pharmaceutical dosage forms. Pilsbury and Jackson [8] have analyzed thiazide diuretics by combination of spectrophotometry with paper chromatography. This procedure deals only with thiazides and lacks the sensitivity and specificity of high-performance liquid chromatography (HPLC). Sohn et al. [9] have used a non-ionic resin column for extaction followed by thin-layer chomatography in the analysis of a limited range of commonly used diuretics. Tisdall et al. [10] have employed an HPLC procedure for screening thiazide diuretics in urine. The principal disadvantages of their procedure are the requirement of two mobile phases in separate 12-min runs, largely limited for detection of thiazide diuretics and the inefficiency to analyse chlorothiazide without derivatization. Shah et al. [11] have developed an HPLC procedure to determine nine thiazides in urine. Again, this procedure could screen thiazide diuretics in urine samples, when these compounds are present alone. Recently Fullinfaw et al. [12] have described an HPLC procedure for detection of twelve potassium-depleting diuretics (ten thiazide and thiazide-type diuretics and two loop diuretics) in urine. Here too, these authors did not study the chromatographic separation of carbonic anhydrase inhibitors, potassium-sparing and uricosuric agents in urine.

This study was undertaken to develop an HPLC screening procedure for determination of 23 diuretics in urine. These compounds are classified according to

their pharmacological properties in five different groups, namely carbonic anhydrase inhibitors, thiazide and thiazide-type, loop, potassium-sparing and uricosuric agents.

EXPERIMENTAL

Reagents and materials

All reagents were of analytical grade. Methanol was of HPLC grade (Caledon, Georgetown, Canada). Acetonitrile and ethyl acetate, distilled-in-glass quality, were purchased from Caledon. Water was distilled, deionized and filtered (10^{-18} Ω resistivity) by Milli-Q 4-bowl reagent-grade water system (Continental Water Systems, Oakville, Canada). Authentic samples of diuretics were graciously supplied by the manufacturers: amiloride, chlorothiazide, hydrochlorothiazide, dichlorphenamide, ethacrynic acid, probenecid (Merck-Frosst, Pointe-Claire, Canada); acetazolamide, quinethazone (Cynamid, Baie d'Urfée, Canada); flumethiazide (Squibb, Montreal, Canada); triamterene (Smith Kline and French, Mississauga, Canada); hydroflumethiazide, bendroflumethiazide (Boots, Nottingham, U.K.); chlorthalidone (Ciba-Geigy, Basel, Switzerland); trichloromethiazide (Schering, Pointe-Claire, Canada); methyclothiazide (Abbott, Montreal, Canada); furosemide (Hoechst, Montreal, Canada); metolazone (Pennwalt, Rochester, MN, U.S.A.); benzthiazide (Robins, Mississauga, Canada); cyclothiazide (Eli Lilly, Indianapolis, IN, U.S.A.); polythiazide (Pfizer, New York, NY, U.S.A.); bumetanide (Leo Ballerup, Denmark); spironolactone, canrenone (Searle, Oakville, Canada). β-Hydroxyethyltheophylline (external standard) was obtained from Sigma (St. Louis, MO, U.S.A.). Propylamine hydrochloride was purchased from Aldrich (Milwaukee, WI, U.S.A.).

Standard solution

The stock solution of each drug was prepared by dissolving 10.0 mg of a compound in 100.0 ml of methanol (100 μg/ml). These solutions were stored in the dark at 4°C.

External standard solution

β-Hydroxyethyltheophylline (10.0 mg) was dissolved in 200.0 ml of methanol (50 μg/ml).

High-peformance liquid chromatography

A Hewlett-Packard (HP) Model 1090 liquid chromatograph equipped with a diode array UV detector was used in this study; the instrument was linked to a data system (HPLC Chem Station, HP Model 35731 B, Avondale, PA, U.S.A.).

Column I: an HP Hypersil ODS (C_{18}), 5-μm column, 200 mm×4.6 mm I.D.

Column II: HP LiChrosorb RP-18 (C_{18}), 5-μm column, 200 mm×4.6 mm I.D. (an alternative column).

The columns were used at ambient temperature (22°C).

Mobile phase

Solvent A was a 0.05 *M* phosphate buffer (pH 3) containing 0.016 *M* propylamine hydrochloride. This was made by dissolving 6.9 g of sodium phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$) and 1.59 g of propylamine hydrochloride in 1 l of distilled, deionized water. This solution was adjusted to pH 3 with concentrated phosphoric acid. It was later filtered over a Millipore 0.45-μm HA filter and degassed with helium for 30 min before use.

Solvent B was acetonitrile. It was filtered over a Millipore 0.5-μm FH filter and degassed with helium for 30 min before use.

A gradient was used to increase the solvent B content from 15% at 2 min to 80% at 20 min. The flow-rate of the mobile phase was kept at 1 ml/min. Using the data system, the detector can follow up to eight different wavelengths at the same time till the end of the run. The detector was set to monitor the signal to the integrator at 230 and 275 nm as these wavelengths were found to be the optimum for the diuretics screened in this study. In addition, spectral data were stored in the data system and this could be plotted at the end of the run.

Extraction procedure

Each urine sample was extracted under acidic and basic conditions.

Acidic extraction. To 2 ml of urine sample was added 0.5 g of solid buffer consisting of monopotassium phosphate (KH_2PO_4)–disodium phosphate (Na_2HPO_4) (99:1, w/w). The final pH of the mixture ranged from 5 to 5.5. It was agitated with vortex action for 15 s. The sample was then extracted with 4 ml of ethyl acetate for 10 min on an Eberbach mechanical agitator. After centrifuging at 600 *g* for 5 min (Model HN-S II, rotor IEC 809, Damon/IEC Division, Needham, MA, U.S.A.), the organic layer was transferred to a 15-ml tube with screw cap and the aqueous layer was discarded. To the organic phase were added 2 ml of 5% aqueous lead acetate solution in order to remove urinary pigments and other extraneous materials. The mixture was then vortexed for 10 s and centrifuged as above. The aqueous phase was discarded and the organic phase was preserved for further elaboration (fraction AE).

Basic extraction. To 2 ml of urine sample was added 0.5 g of solid buffer consisting of sodium bicarbonate–potassium carbonate (3:2, w/w). The final pH of the mixture ranged from 9 to 9.5. It was then vortexed for 15 s, 4 ml of ethyl acetate were added, and the mixture was shaken mechanically on an Eberbach agitator for 10 min and centrifuged as above. The aqueous phase was discarded and the organic phase was pooled with fraction AE obtained from the acidic extraction. The combined extracts were evaporated to dryness at 50°C with a slow stream of nitrogen. The residue was then reconstituted with 300 μl of external standard solution, and 5 μl were injected into the liquid chromatograph.

Recovery studies

To 2 ml of blank urine were added 300 μl of methanolic solution of each drug (100 μg/ml) equivalent to 15 μg/ml of urine. The samples were then subjected to the complete extraction procedure described above. The percentage recovery

was estimated by comparing the peak areas obtained with the respective peak areas of a standard methanolic solution containing 100 μg/ml of each drug.

Human studies

Urinary excretion studies were performed in normal healthy volunteers using minimum single or multiple doses. Urine samples were collected at appropriate time intervals post-dose. At each urine collection, the sample volume was measured.

Detection limit

The stock solutions containing 100 μg/ml of each drug were prepared in methanol. The following aliquots of the stock solutions were added to 2 ml of blank urine samples: (1) 10 μl of canrenone, furosemide, hydrochlorothiazide, hydroflumethiazide, methyclothiazide and polythiazide (0.5 μg/ml); (2) 20 μl of acetazolamide, bendroflumethiazide, benzthiazide, bumetanide, chlorothiazide, chlorthalidone, cyclothiazide, dichlorphenamide, flumethiazide, metolazone, spironolactone, triamterene and trichloromethiazide (1 μg/ml); (3) 30 μl of quinethazone (1.5 μg/ml); (4) 100 μl of amiloride, ethacrynic acid and probenecid (5 μg/ml). The spiked urine samples were subjected to acidic and basic extraction procedures as previously described to estimate sensitivity and the minimum quantities detectable in the volunteer specimens.

RESULTS

Chromatography

Fig. 1 illustrates a chromatogram obtained from a methanolic solution containing a mixture of 23 diuretics and the external standard at a concentration of 100 μg/ml using column I. The detector was set at 230 nm. In this system, furosemide coelutes with metolazone and ethacrynic acid with bumetanide at retention times of 12.165 and 15.247 min, respectively. The peaks were plotted by the Chem Station in relation to the UV absorbance of the most intense peak. Spironolactone could be detected better at 230 nm.

Fig. 2 shows their corresponding UV spectra including β-hydroxyethyltheophylline as an external standard.

Fig. 3 shows the same type of chromatogam with the detector wavelength set at 275 nm. There is coelution of triamterene with flumethiazide, furosemide with metolazone and ethacrynic acid with bumetanide at retention times of 7.678, 12.153 and 15.247 min, respectively. Most of the diuretics examined in this study have a better UV absorbance at 230 nm (except canrenone, which could be detected better at 275 nm). Generally, the urinary endogenous compounds absorb less at 275 nm. At this wavelength, there is less interference with other exogenous urinary compounds, while retaining high sensitivity for the diuretics. Therefore, there is more facility for the interpretation of the results at 275 nm.

Since the above three pairs of diuretics could not be resolved distinctly using column I, column II was tried to improve the separation of these compounds. The same mobile phase was employed as mentioned previously. Figs. 4 and 5 represent

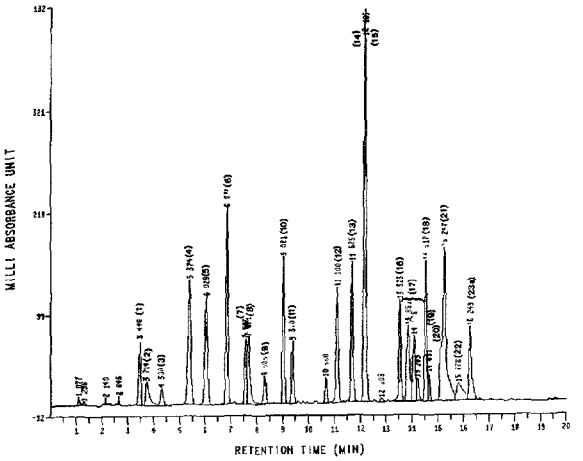


Fig. 1. Chomatogram obtained with a standard methanolic solution containing a mixture of diuretics and the external standard using column I at a detector wavelength of 230 nm. Each peak corresponds to 0.5 μg. The peaks were plotted by the Chem Station in relation to the UV absorbance of the most intense peak (overlapped peak of drugs 14 and 15). Peaks: 1 = β-hydroxyethyltheophylline; 2 = amiloride; 3 = acetazolamide; 4 = chlorothiazide; 5 = hydrochlorothiazide; 6 = quinethazone; 7 = triamterene; 8 = flumethiazide; 9 = hydroflumethiazide; 10 = chlorthalidone; 11 = dichlorphenamide; 12 = trichloromethiazide; 13 = methyclothiazide; 14 = furosemide; 15 = metolazone; 16 = benzthiazide; 17 = cyclothiazide; 18 = polythiazide; 19 = bendroflumethiazide; 20 = ethacrynic acid; 21 = bumetanide; 22 = probenecid; 23a = spironolactone. Canrenone could not be detected at this wavelength.

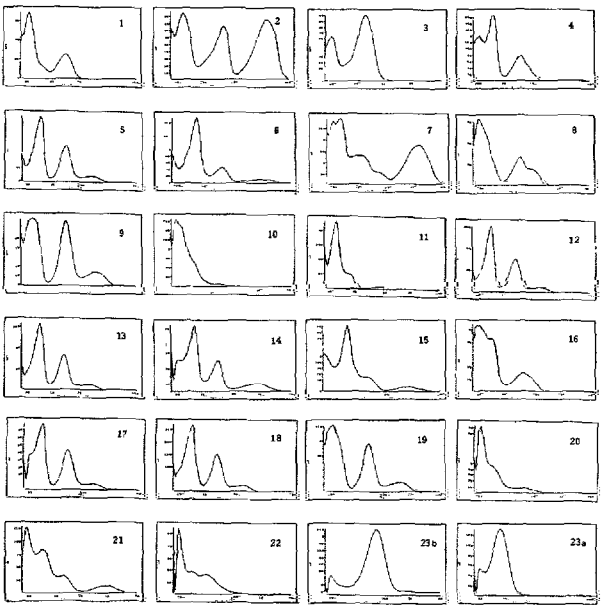


Fig. 2. UV spectra of the compounds listed in the legend to Fig. 1 (23b = canrenone).

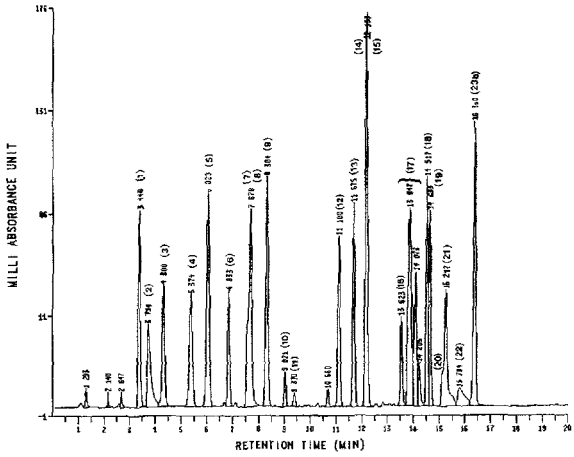


Fig. 3. Chromatogram obtained with a standard methanolic solution containing a mixture of diuretics and the external standard using column I at detector wavelength of 275 nm. Each peak corresponds to 0.5 μg. The peaks were plotted by the Chem Station in relation to the UV absorbance of the most intense peak (overlapped peak of drugs 14 and 15). Spironolactone could not be detected at this wavelength (canrenone = peak 23b). Other experimental conditions were the same as mentioned in the text.

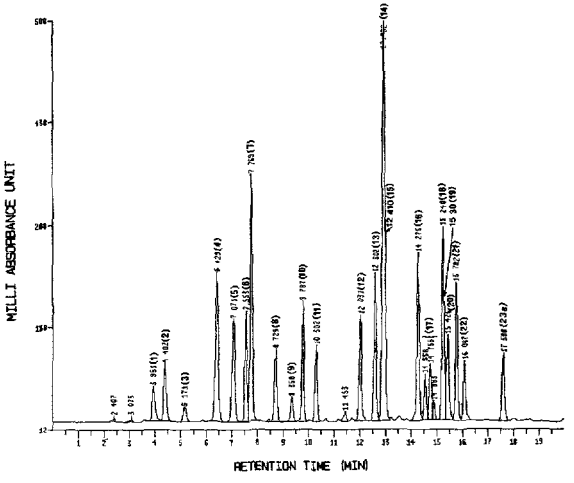


Fig. 4. Chromatogram obtained with a standard methanolic solution containing a mixture of diuretics and the external standard using column II at a detector wavelength of 230 nm. Each peak corresponds to 0.5 μg. The peaks were plotted by the Chem Station in relation to the UV absorbance of the most intense peak (overlapped peak of drugs 14 and 15). Peaks: 1 = amiloride; 2 = β-hydroxyethyltheophylline; 3 = acetazolamide; 4 = chlorothiazide; 5 = hydrochlorothiazide; 6 = triamterene; 7 = quinethazone; 8 = flumethiazide; 9 = hydroflumethiazide; 10 = chlorthalidone; 11 = dichlorphenamide; 12 = trichloromethiazide; 13 = methyclothiazide; 14 = furosemide; 15 = metolazone; 16 = benzthiazide; 17 = cyclothiazide; 18 = polythiazide; 19 = ethacrynic acid; 20 = bendroflumethiazide; 21 = bumetanide; 22 = probenecid; 23a = spironolactone.

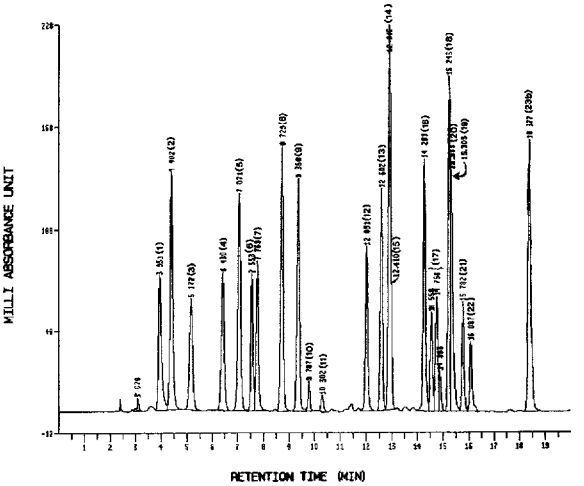


Fig. 5. Chromatogram obtained with a standard methanolic solution containing a mixture of diuretics and the external standard using column II at a detector wavelength of 275 nm. Each peak corresponds to 0.5 μg. The peaks were plotted by the Chem Station in relation to the UV absorbance of the most intense peak (overlapped peak of drugs 14 and 15). Peak 23b = canrenone. Other experimental conditions were the same as mentioned in the text.

the chromatograms obtained from a methanolic solution containing a mixture of the 23 diuretics at a concentration of 100 μg/ml using column II at 230 and 275 nm, respectively. Again with this column, furosemide could not be separated from metolazone, but ethacrynic acid and triamterene were well resolved from bumetanide and flumethiazide, respectively. However, there was incomplete resolution between polythiazide, ethacrynic acid and bendroflumethiazide using column II.

Moreover, by matching the UV spectra of furosemide with metolazone, ethacrynic acid with either bumetanide or bendroflumethiazide and triamterene with flumethiazide, it can be seen that they are easily distinguishable (Fig. 2).

Column II has an advantage over column I as it gives sharp symmetrical peaks with acidic diuretics like ethacrynic acid, furosemide, bumetanide and probenecid.

Cyclothiazide gave several peaks on elution with columns I and II. This is probably due to the presence of stereoisomers, as already observed by Tisdall et al. [10] and De Croo et al. [13].

Figs. 6, 7 and 8 illustrate chromatograms obtained from extracts of blank urine samples from three subjects containing β-hydroxyethyltheophylline as an external standard. These three types of chromatographic profile were observed in most extracts of blank human urine. Fig. 6 shows that the chromatogram is free of background peaks. Fig. 7 indicates the presence of two peaks from endogenous compounds in the chromatogram at retention times of 5.69 and 10.00 min, but the UV spectra of these peaks did not match with any diuretic screened in this study. Fig. 8 demonstrates the presence of theophylline and caffeine peaks at retention times of 3.12 and 4.36 min, respectively. Theophylline did not interfere with the separation of diuretics due to its shorter retention time. Caffeine and acetazolamide have very close retention times, but they are easily distinguished by their UV spectra.

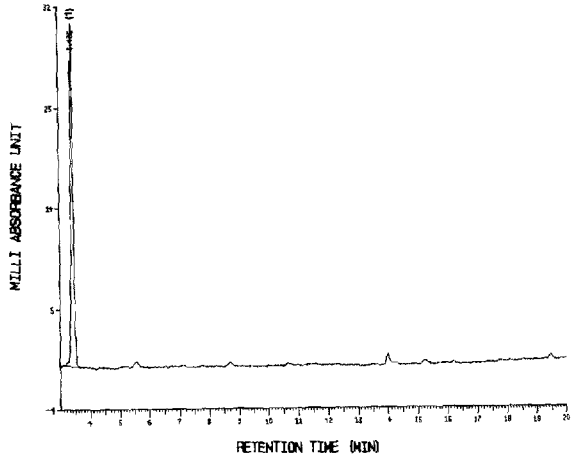


Fig. 6. Chromatogram obtained from an extract of blank urine sample from subject 1. Peak 1 = β-hydroxyethyltheophylline. Column I; detector set at 275 nm.

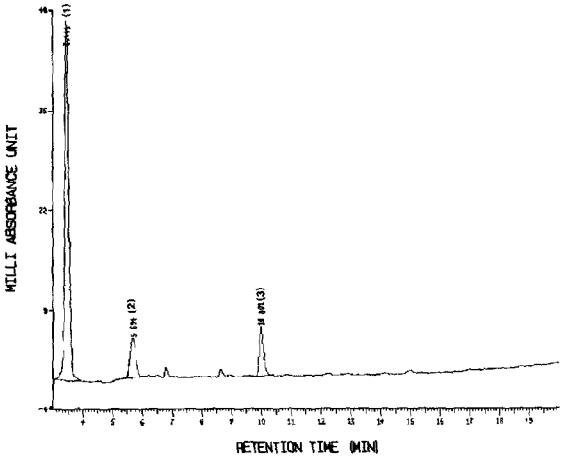


Fig. 7. Chromatogram obtained from an extract of blank urine sample from subject 2. Peaks: 1 = β-hydroxyethyltheophylline; 2 = endogenous compound; 3 = endogenous compound. Column I; detector set at 275 nm.

Fig. 9 shows a chromatogram of a urinary extract of an athlete on non-diuretic medications. Although the retention times of acetaminophen (peak 2, 3.45 min), acetylsalicylic acid (peak 4, 7.16 min) and diflunisal (peak 5, 14.09 min) are very close to the diuretics mentioned in Table I, these compounds do not interfere with the screening procedure as their UV spectra are different from diuretics investigated in this study. These non-diuretic medications were identified by gas chromatography–mass spectrometry (GC–MS). In addition to these medications, the athlete was also taking albuterol, chlorpheniramine, phenylpropanol-

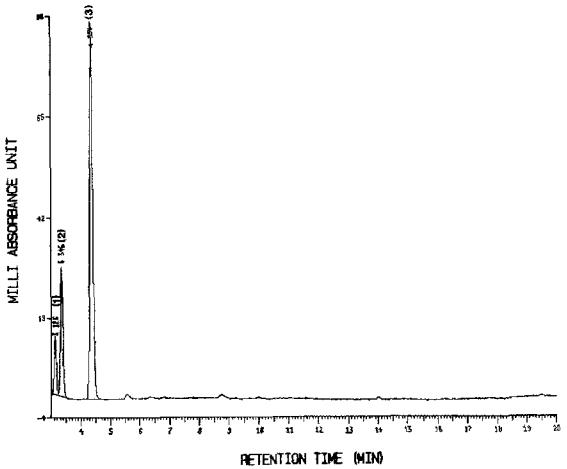


Fig. 8. Chromatogram obtained from an extract of blank urine sample from subject 3. Peaks: 1 = theophylline; 2 = β-hydroxyethyltheophylline; 3 = caffeine. Column I; detector set at 275 nm.

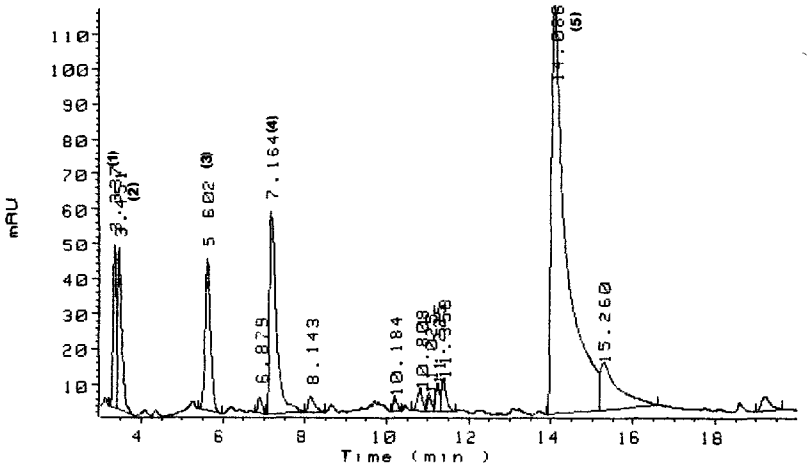


Fig. 9. Chromatogram obtained from a urinary extract of an athlete on non-diuretic medications. Peaks: 1 = β-hydroxyethyltheophylline; 2 = acetaminophen; 3 = endogenous compound; 4 = acetylsalicylic acid; 5 = diflunisal. Column I; detector set at 275 nm.

amine, oxymetazoline and piroxicam. These drugs were not detectable under the experimental conditions used in this study. This fact demonstrates the selectivity and reliability of the screening procedure.

Fig. 10 illustrates a chromatogram obtained from an extract of urine sample 4 h after oral administration of 15 mg of amiloride (pK_a 8.7) to a healthy volunteer using the described extraction procedure. Amiloride (peak A) elutes at a retention time of 3.67 min. Amiloride is well resolved from theophylline (peak 1), external standard (peak 2), caffeine (peak 3) and an endogenous compound (peak 4). When the same urine sample was extracted under basic conditions and its extract injected 4 h later, a peak (6) at a retention time of 6.6 min appeared to

TABLE I

DIURETICS, THEIR PHARMACOLOGICAL CLASSIFICATION, RETENTION TIMES (t_R) AND RELATIVE RETENTION TIMES (RRT) USING COLUMNS I AND II

Compound	Diuretic type	Column I		Column II	
		t_R (min)	RRT[a]	t_R (min)	RRT[a]
β-Hydroxyethyltheophylline	–	3.45	1.00	4.40	1.00
Amiloride	Potassium-sparing	3.71	1.08	3.95	0.90
Acetazolamide	Carbonic anhydrase inhibitor	4.30	1.25	5.17	1.18
Chlorothiazide	Thiazide	5.37	1.56	6.43	1.46
Hydrochlorothiazide	Thiazide	6.03	1.75	7.07	1.61
Quinethazone	Thiazide-type	6.83	1.98	7.77	1.77
Triamterene	Potassium-sparing	7.68	2.23	7.55	1.71
Flumethiazide	Thiazide	7.68	2.23	8.73	1.98
Hydroflumethiazide	Thiazide	8.30	2.41	9.36	2.13
Chlorthalidone	Thiazide-type	9.02	2.62	9.79	2.22
Dichlorphenamide	Carbonic anhydrase inhibitor	9.37	2.72	10.30	2.34
Trichloromethiazide	Thiazide	11.10	3 22	12.03	2.73
Methyclothiazide	Thiazide	11.68	3.39	12.60	2.86
Furosemide	Loop	12.16	3.53	12.92	2.93
Metolazone	Thiazide-type	12.16	3.53	12.92	2.93
Benzthiazide	Thiazide	13.52	3.92	14.28	3.24
Cyclothiazide	Thiazide	13.85	4.02	14.76	3.35
Polythiazide	Thiazide	14.52	4.21	15.24	3.46
Bendroflumethiazide	Thiazide	14.63	4.25	15.33	3.48
Ethacrynic acid	Loop	15.10	4.38	15.31	3.48
Bumetanide	Loop	15.25	4.43	15.78	3.59
Probenecid	Uricosuric agent	15.78	4.58	16.09	3.65
Spironolactone	Potassium-sparing	16.25	4.72	17.60	4.00
Canrenone	Potassium-sparing	16.36	4.72	18.38	4.18

[a]Relative to β-hydroxyethyltheophylline.

arise as an artifact product (Fig. 11). The UV spectra of amiloride (A) and its artifact product (B) resemble each other (Fig. 12).

When the same urine sample was extracted under acidic conditions, neither amiloride nor its artifact product could be detected (Fig. 13).

Similarly, triamterene, another weakly basic diuretic (pK_a 6.2) and its metabolite hydroxytriamterene could not be extracted from human urine under acidic conditions.

Recently, Forrest et al. [14] indicated that amiloride is light-sensitive, and their working standard solutions were prepared daily in subdued light. A standard methanolic solution of amiloride was tested for its stability against daylight and temperature (22 °C) in our laboratory. A slight and negligible decomposition of this drug was observed after one day but the amount of the artifact product formed was not significant.

The stability of amiloride in the urinary extract obtained after applying the described extraction procedure from the same volunteer under similar laboratory light and temperature conditions was comparable to its standard methanolic so-

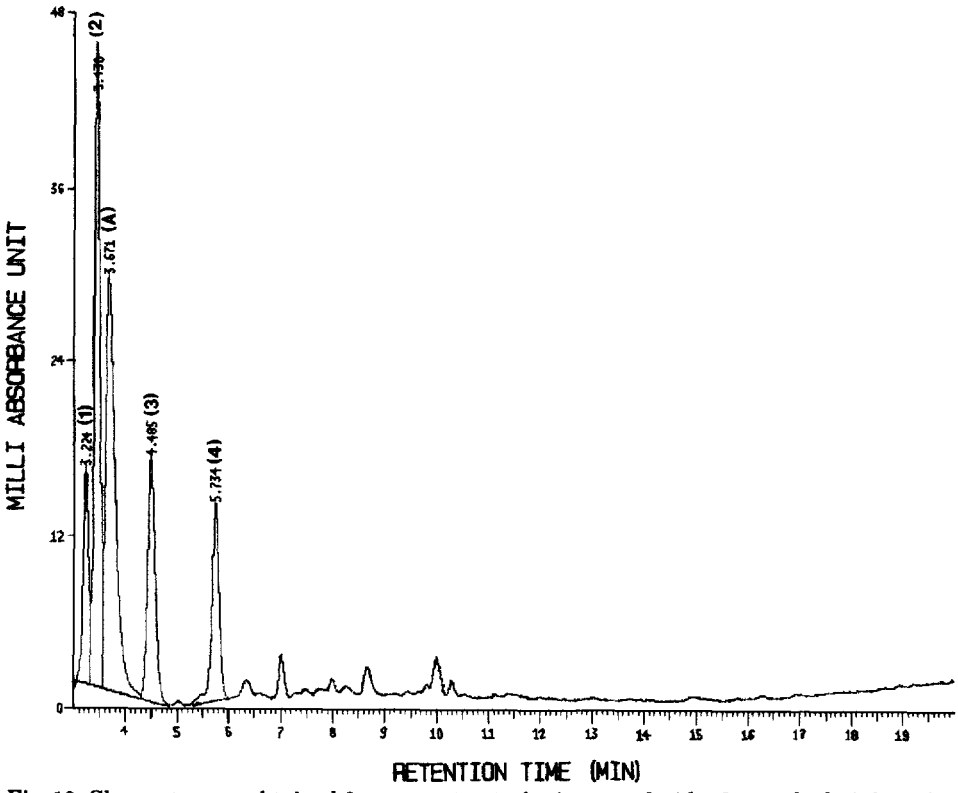


Fig. 10. Chromatogram obtained from an extract of urine sample 4 h after oral administration of 15 mg of amiloride to a healthy volunteer. The sample was extracted according to the described procedure. Peaks: 1 = theophylline; 2 = β-hydroxyethyltheophylline; A = amiloride; 3 = caffeine; 4 = endogenous product. Column I; detector set at 275 nm.

lution. This extraction procedure can be applied for screening amiloride without major decomposition, if a urinary extract is analysed within a day. Besides the factor of light, the pH value of the medium also plays a role for the stability of amiloride. It seems that amiloride is more stable at acidic or neutral than basic pH medium (Figs. 10 and 11). Amiloride was found to be stable in an eighteen-months-old urine sample of a volunteer, when the sample was preserved at −20°C in the dark.

Unfortunately, all the screening procedures for diuretics published in the literature to date do not deal with the basic diuretics [5–12]. Therefore acidic and basic extraction procedures have been used in this study. The analysis of urinary samples containing diuretics by acidic and basic extractions by HPLC are not time-consuming as the extracts are finally pooled at the end of the extraction procedure.

De Croo et al. [13] studied the retention characteristics of amiloride, triamterene and other thiazide, loop and potassium-sparing diuretics in different liquid chromatographic systems but they did not apply their findings for the analysis of these compounds in biological fluids.

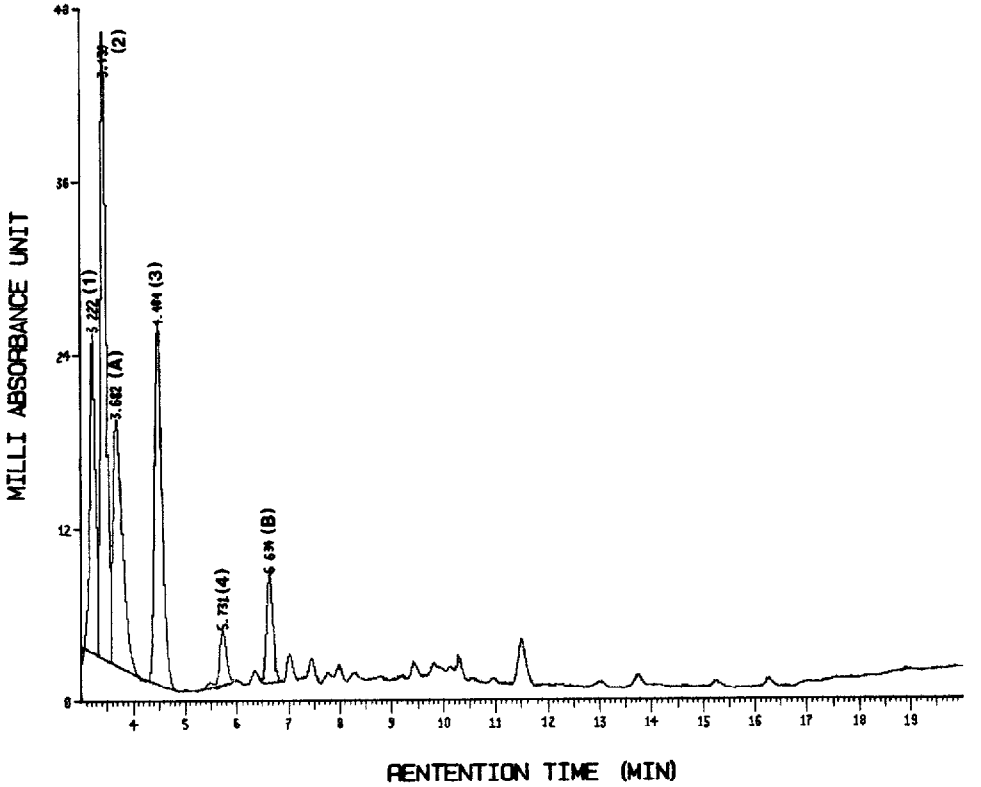


Fig. 11. Chromatogram obtained from an extract of urine sample 4 h after oral administration of 15 mg of amiloride to a healthy volunteer. The sample was extracted under basic conditions and analyzed 4 h later. Peaks: 1 = theophylline; 2 = β-hydroxyethyltheophylline; 3 = caffeine; 4 = endogenous product; A = amiloride; B = artifact product of amiloride. Column I; detector set at 275 nm.

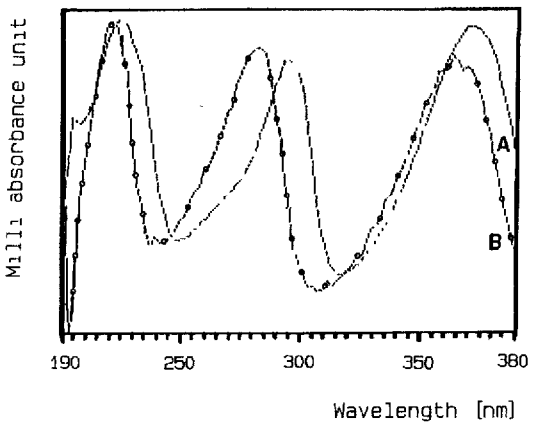


Fig. 12. UV spectra of peaks A (amiloride) and B (artifact product of amiloride) plotted by the Chem Station at the end of a chromatographic run.

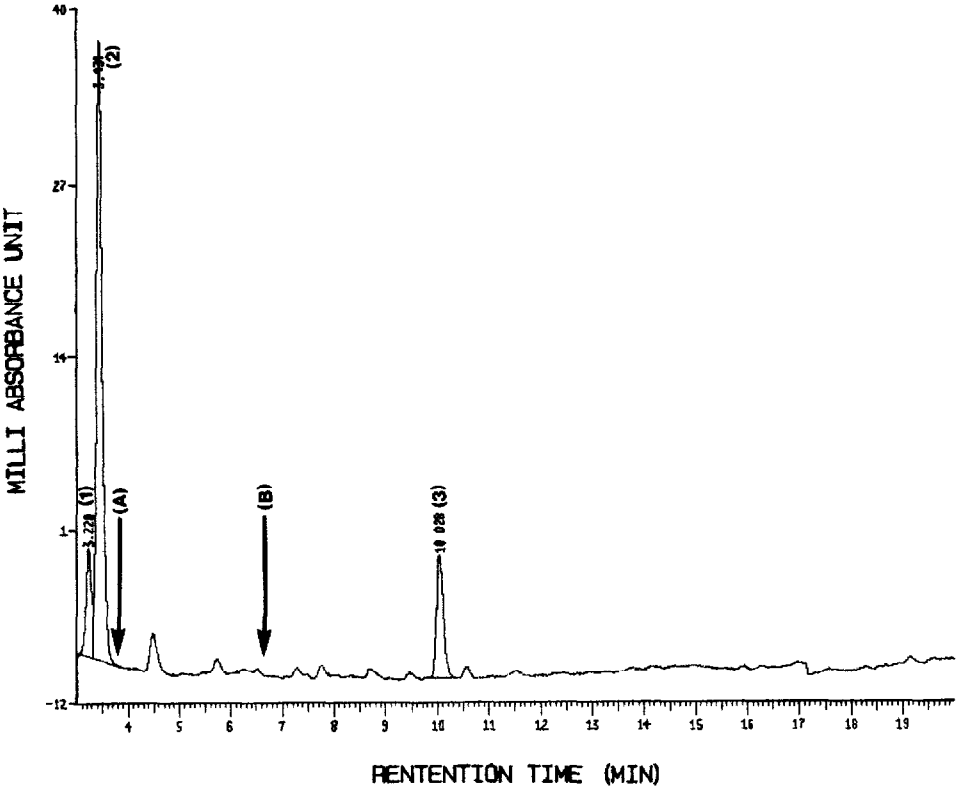


Fig. 13. Chromatogram obtained from an extract of urine sample 4 h after oral administation of 15 mg of amiloride to a healthy volunteer. The sample was extracted under acidic conditions. Peaks: 1 = theophylline; 2 = β-hydroxyethyltheophylline; 3 = endogenous product. The arrows A and B show the absence of peaks at the retention times of amiloride and its artifact product, respectively. Column I; detector set at 275 nm.

Figs. 14–18 illustrate chromatograms of positive findings detected in the urine samples of athletes as examples using this screening procedure. Fig. 14 shows the presence of acetazolamide (peak 2). It is well separated from external standard (peak 1) and three other peaks from endogenous compounds (peaks 3, 4 and 5). Fig. 15 demonstrates a positive screen of furosemide (peak 3), which is well resolved from theophylline (peak 1) and external standard (peak 2). Fig. 16 illustrates the presence of hydrochlorothiazide (peak 4), which is well separated from theophylline (peak 1), external standard (peak 2), caffeine (peak 3) and a peak from an endogenous compound (peak 5). Fig. 17 shows a positive screen of probenecid (peak 4), which is well resolved from external standard (peak 1) and its metabolites (peaks 2, 3 and 5). The identity of metabolites was confirmed by GC–MS. Fig. 18 demonstrates the presence of triamterene (peak 6), which is well separated from theophylline (peak 1), external standard (peak 2), caffeine (peak 3) and three other peaks from endogenous compounds (peaks 4, 5 and 7). In all these cases, the urinary extracts of positive urine samples did not show background peaks that could interfere with this screening procedure.

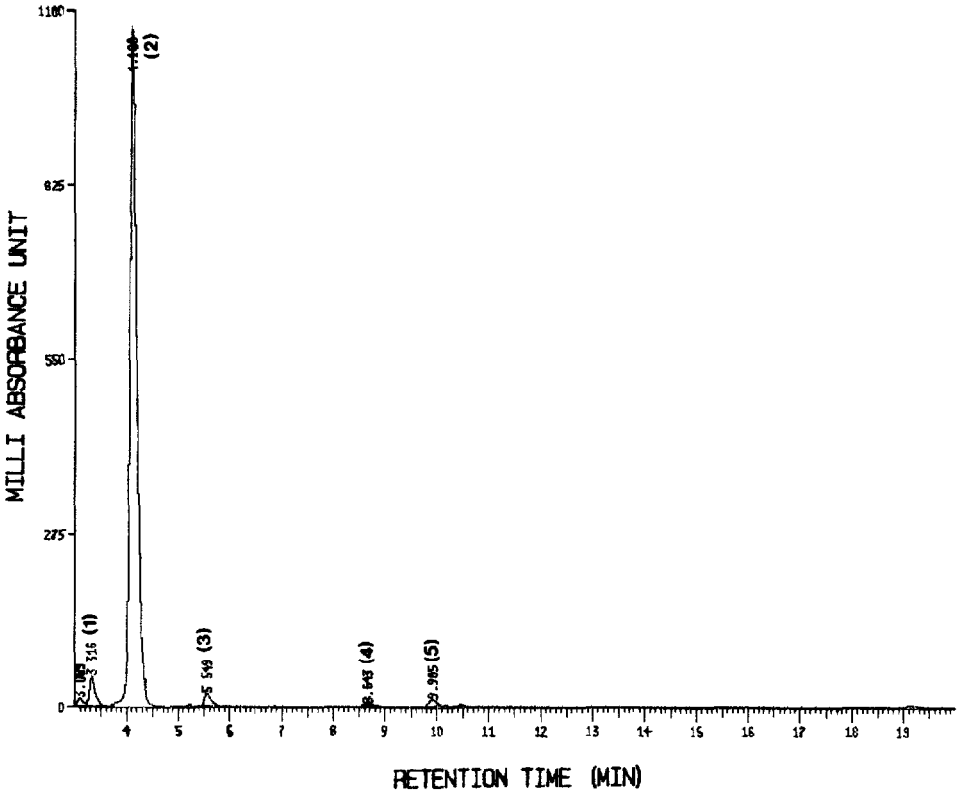


Fig. 14. Chromatogram representing a positive screen of acetazolamide of an athlete's urine sample. Peaks: 1 = β-hydroxyethyltheophylline; 2 = acetazolamide; 3, 4 and 5 = endogenous products. Column I; detector set at 275 nm.

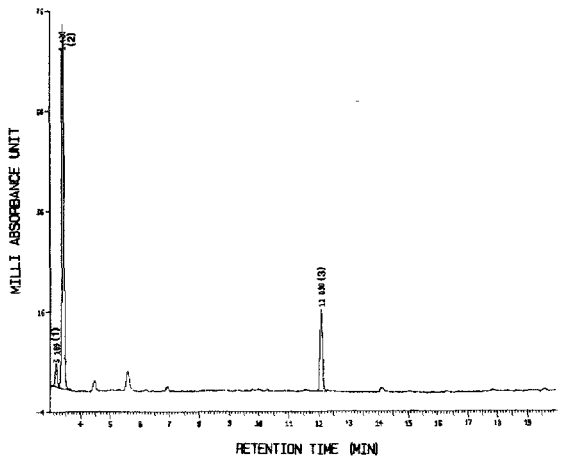


Fig. 15. Chromatogram representing a positive screen of furosemide of an athlete's urine sample. Peaks: 1 = theophylline; 2 = β-hydroxyethyltheophylline; 3 = furosemide. Column I; detector set at 275 nm.

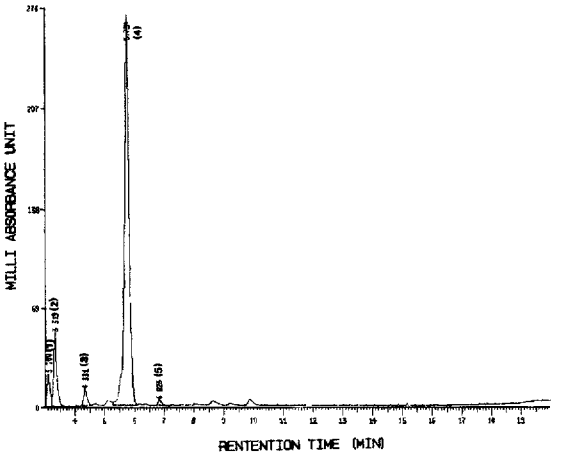


Fig. 16. Chromatogram representing a positive screen of hydrochlorothiazide of an athlete's urine sample. Peaks: 1 = theophylline; 2 = β-hydroxyethyltheophylline; 3 = caffeine; 4 = hydrochlorothiazide; 5 = endogenous product. Column I; detector set at 275 nm.

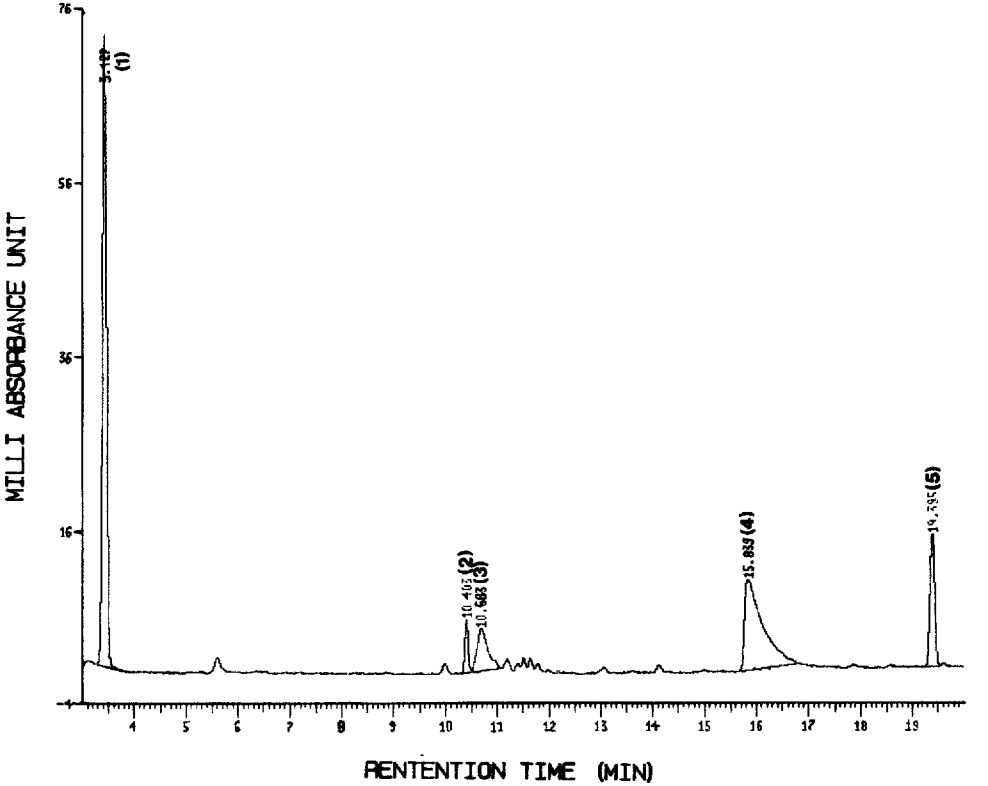


Fig. 17. Chromatogram representing a positive screen of probenecid of an athlete's urine sample. Peaks: 1 = β-hydroxyethyltheophylline; 2 and 3 = metabolites of probenecid; 4 = probenecid; 5 = metabolite of probenecid. Column I; detector set at 275 nm.

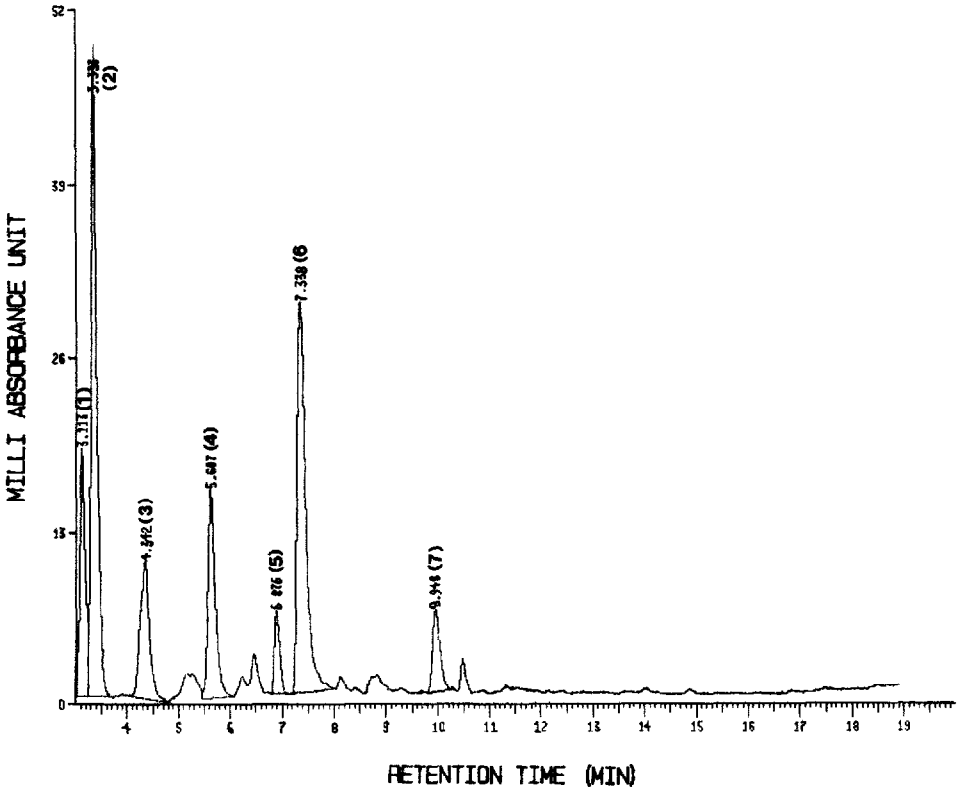


Fig. 18. Chromatogram representing a positive screen of triamterene of an athlete's urine sample. Peaks: 1 = theophylline; 2 = β-hydroxyethyltheophylline; 3 = caffeine; 4 and 5 = endogenous products; 6 = triamterene; 7 = endogenous product. Column I; detector set at 275 nm.

Table I shows the list of diuretics studied, their pharmacological classification and their retention and relative retention times using columns I and II.

Recovery studies

A urine sample was spiked in triplicate to contain 15 μg/ml of each of the drugs using acidic and basic extraction procedures. The reproducibility and recovery results of each drug are given in Table II. The low recoveries of amiloride and triamterene using acidic extraction are due to their basic and weakly basic pK_a values of 8.7 and 6.2, respectively. With the exception of these two drugs, the percentages of recovery of other compounds vary from 53 to 100%. The overall values of standard deviations vary from $\pm$0.6 to $\pm$9.0. The percentage recovery of amiloride is again low (25% $\pm$2.3) using basic extraction, whereas it is high for triamterene (81% $\pm$ 1.5). More acidic drugs, e.g. ethacrynic acid, chlorothiazide, flumethiazide, furosemide and bumetanide, show poor extraction recovery under basic conditions. Acetazolamide and probenecid could not be extracted at all under basic conditions. The recoveries of other compounds vary from 53 to 97%. The overall values of standard deviations vary from $\pm$0.6 to $\pm$6.2.

TABLE II

EXTRACTION RECOVERY OF THE SCREENING PROCEDURE

In all cases 15 μg/ml of each of the diuretics was added.

Diuretic	Recovery (mean ± S.D., $n=3$) (%)	
	Acidic extraction	Basic extraction
Acetazolamide	83 ± 0.6	0
Amiloride	10 ± 1.0	25 ± 2.3
Bendroflumethiazide	76 ± 4.0	87 ± 3.6
Benzthiazide	89 ± 1.7	85 ± 4.4
Bumetanide	93 ± 5.0	31 ± 1.5
Canrenone	91 ± 2.3	92 ± 0.6
Chlorothiazide	78 ± 3.2	7 ± 1.0
Chlorthalidone	86 ± 3.8	93 ± 2.1
Cyclothiazide	69 ± 1.1	86 ± 0.6
Dichlorphenamide	100 ± 7.2	97 ± 3.0
Ethacrynic acid	64 ± 3.5	3 ± 1.0
Flumethiazide	85 ± 2.3	20 ± 1.0
Furosemide	69 ± 2.1	24 ± 6.2
Hydrochlorothiazide	87 ± 1.7	88 ± 1.0
Hydroflumethiazide	61 ± 1.0	91 ± 2.6
Methyclothiazide	92 ± 2.5	93 ± 2.0
Metolazone	92 ± 3.6	82 ± 4.0
Polythiazide	86 ± 2.6	93 ± 3.6
Probenecid	93 ± 4.0	0
Quinethazone	53 ± 2.1	53 ± 1.0
Spironolactone	82 ± 9.0	95 ± 3.8
Triamterene	9 ± 1.7	81 ± 1.5
Trichloromethiazide	89 ± 2.3	69 ± 3.5

Application of the method to human studies

The human studies, approved by the Ethics Committee of the Institute, were conducted on healthy male volunteers. Volunteers were given individually single oral doses of chlorthalidone (25 mg), furosemide (40 mg), probenecid (two oral doses of 500 mg after an interval of 12 h for two consecutive days), acetazolamide (250 mg), quinethazone (50 mg), spironolactone (25 mg), bendroflumethiazide (5 mg), bumetanide (5 mg), triamterene (100 mg) and hydrochlorothiazide (25 mg). The medications were administered after an overnight fast. Fig. 19A–J shows examples of the urinary excretion profiles of chlorthalidone, furosemide, probenecid, acetazolamide, quinethazone, spironolactone, bendroflumethiazide, bumetanide, triamterene and hydrochlorothiazide, respectively. C_{max} and T_{max} values can be obtained directly from the individual observed concentration versus time profiles. For chlorthalidone, $C_{max}=5.2$ μg/ml, $T_{max}=2$ h. For furosemide, $C_{max}=15.7$ μg/ml, $T_{max}=8$ h. For probenecid, $C_{max}=82.5$ μg/ml (after the first dose), $T_{max}=6$ h (after the first dose). For acetazolamide, $C_{max}=292.6$ μg/ml, $T_{max}=2$h. For quinethazone, $C_{max}=11.4$ μg/ml, $T_{max}=2.5$ h. For spironolactone, $C_{max}=1.4$ μg/ml, $T_{max}=7.8$ h. For bendroflumethiazide, $C_{max}=2.8$ μg/ml, $T_{max}=4$

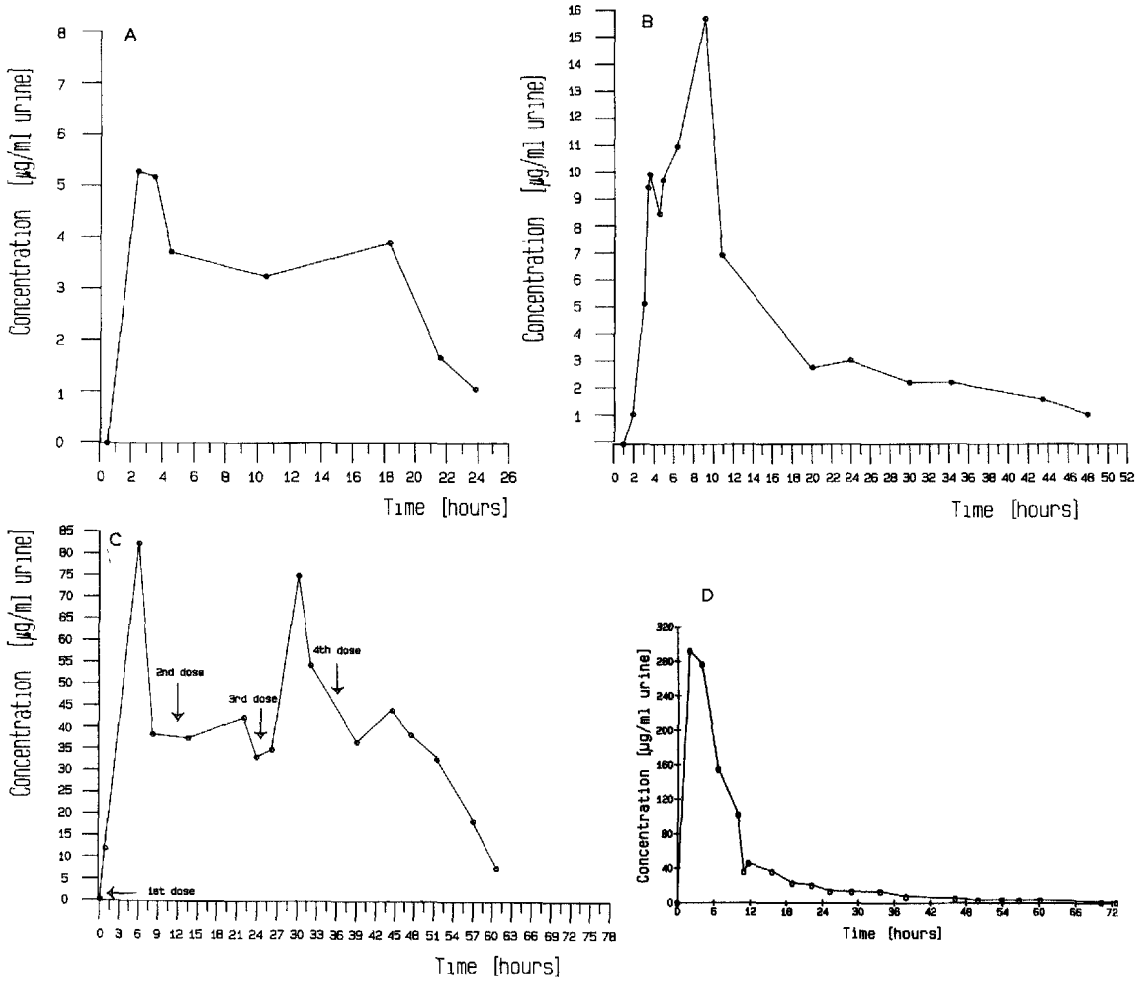
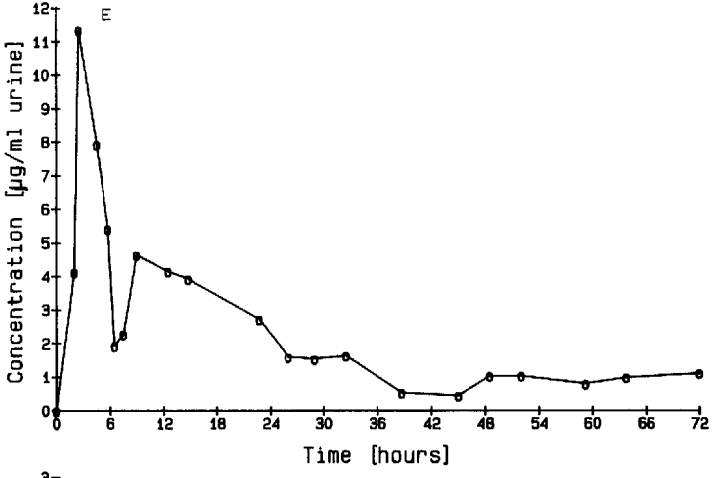
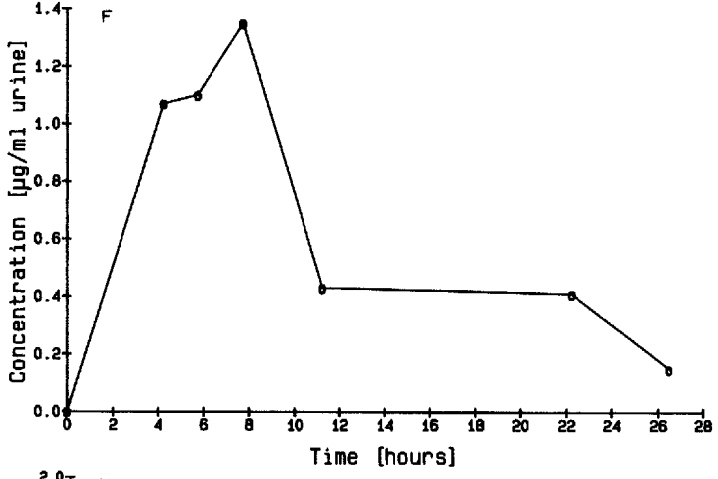
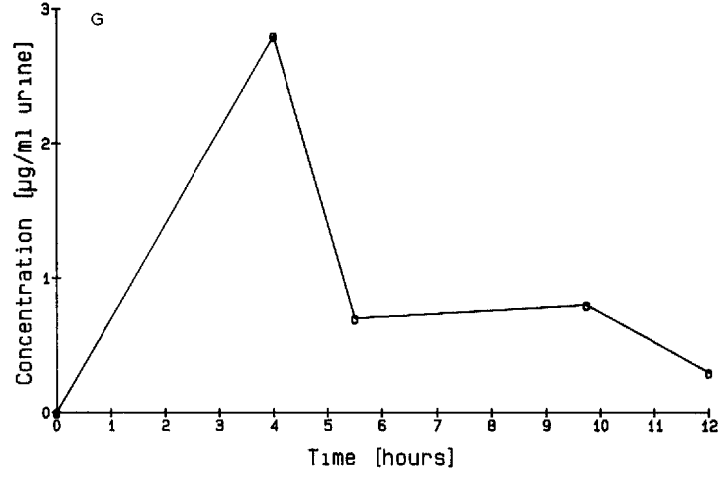


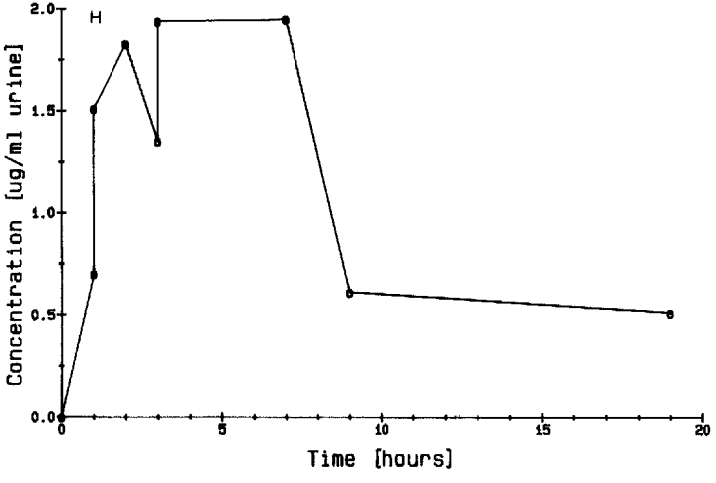
Fig. 19.

(Continued on p. 84)


E
Concentration [µg/ml urine]
Time [hours]


F
Concentration [µg/ml urine]
Time [hours]


G
Concentration [µg/ml urine]
Time [hours]


H
Concentration [ug/ml urine]
Time [hours]

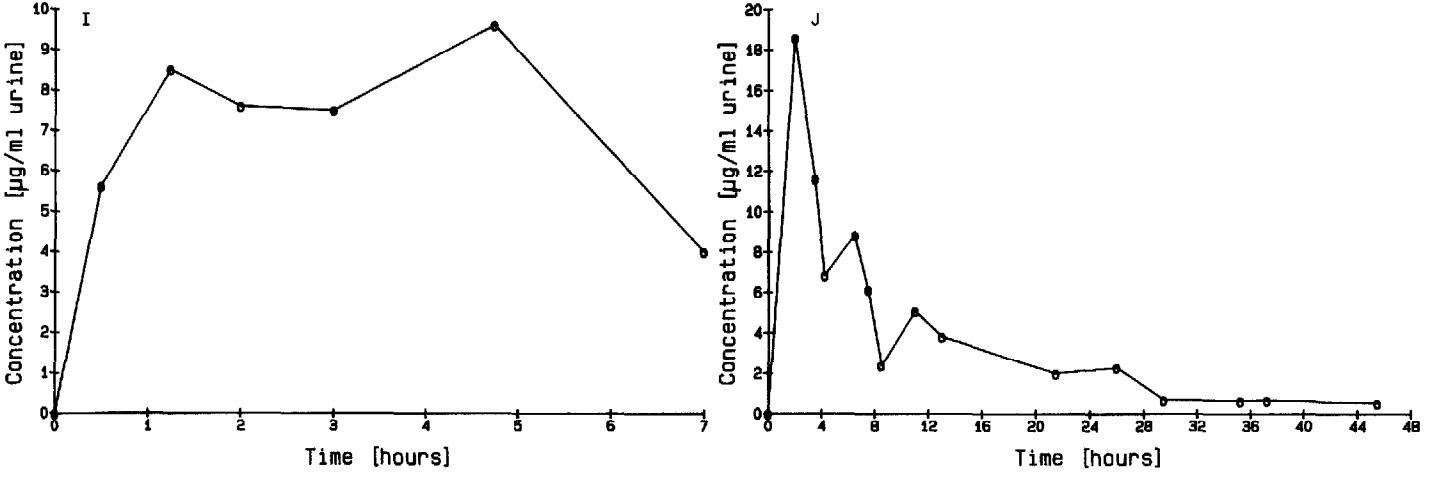


Fig. 19. (A) Urinary excretion-time profile of chlorthalidone. (B) Urinary excretion-time profile of furosemide. (C) Urinary excretion-time profile of probenecid. (D) Urinary excretion-time profile of acetazolamide; (E) Urinary excretion-time profile of quinethazone; (F) Urinary excretion-time profile of spironolactone. (G) Urinary excretion-time profile of bendroflumethiazide. (H) Urinary excretion-time profile of bumetanide. (I) Urinary excretion-time profile of triamterene. (J) Urinary excretion-time profile of hydrochlorothiazide.

h. For bumetanide, $C_{max}=1.9$ μg/ml, $T_{max}=7$ h. For triamterene, $C_{max}=9.6$ μg/ml, $T_{max}=4.8$ h. For hydrochlorothiazide, $C_{max}=18.6$ μg/ml, $T_{max}=2$ h. The above mentioned ten diuretics could be detected for as long as 72 h post administration.

If administration doses of some diuretics are very low or the metabolism of certain diuretics is very rapid, then larger amounts of urine samples were used to pursue the urinary excretion–time profiles of these drugs. The urine volume can be reduced, if either acidic or basic extraction is adopted as the signal-to-noise ratio is reduced significantly thereby lowering the detection limit of the drugs. The detection limit of diuretics given in Table III is for the screening procedure, in which case it was fixed till the drug peak could generate a full UV spectrum using the diode array detector.

Detection limit

Table III shows the sensitivity of this screening procedure using column I. The overall lower limits of detection using both extraction procedures range from 0.5 to 1.5 μg/ml of urine (average 1.0 μg/ml) depending on the type of diuretic. Amiloride, ethacrynic acid and probenecid could not be detected below 5 μg/ml of urine.

TABLE III

DETECTION LIMITS IN THE DIURETIC SCREENING PROCEDURE

Drug	Detection limit (μg/ml)	
	Acidic extraction	Basic extraction
Acetazolamide	1.0	0
Amiloride	0	5.0
Bendroflumethiazide	1.0	1.0
Benzthiazide	1.0	1.0
Bumetanide	1.0	1.0
Canrenone	0.5	0.5
Chlorothiazide	1.0	0
Chlorthalidone	1.0	1.0
Cyclothiazide	1.0	1.0
Dichlorphenamide	1.0	1.0
Ethacrynic acid	5.0	0
Flumethiazide	1.0	1.0
Furosemide	0.5	0.5
Hydrochlorothiazide	0.5	0.5
Hydroflumethiazide	0.5	0.5
Methyclothiazide	0.5	0.5
Metolazone	1.0	1.0
Polythiazide	0.5	0.5
Probenecid	5.0	0
Quinethazone	1.5	1.5
Spironolactone	1.0	1.0
Triamterene	0	1.0
Trichloromethiazide	1.0	1.0

DISCUSSION

This procedure is being currently used in our laboratory for screening urine samples of Canadian and American amateur athletes. Using the described procedure, we are able to screen with reliability these diuretics, which are on the banned lists of the Medical Commissions of the International Olympic Committee and the National Collegiate Athletic Association of the United States.

In our routine screening procedure, column I is regularly used for the detection of these compounds. It is adequate enough to give highly satisfactory results for the detection of most of the diuretics. However, column II can be used as an alternative column in case of identification of potentially positive samples.

This HPLC method is very practical as no derivatization steps are necessary. At the end of extraction procedures, the acidic and basic extracts are pooled so that the chromatographic running time is reduced to half. Fullinfaw et al. [12] suggested a clean-up wash with phosphate buffer (pH 7.5). This step was avoided as it led to poor recoveries of several drugs as noticed by the same authors [12]. If a chromatogram with several extraneous peaks results, the diode array detector can obtain UV spectra of these peaks in that region. It can be noted that the following drugs did not interfere in the screening procedure as their UV spectra were different from spectra of diuretics investigated in this study: acetaminophen, acetylsalicylic acid, caffeine, diflunisal, fenoprofen, ibuprofen, indomethacin, methocarbamol, naproxen, phenylbutazone, sulindac, tetracycline, theobromine, theophylline, tolmetin, trimethoprim and verapamil. The above-mentioned drugs were found in the urine samples of the athletes during the screening procedure.

The results obtained indicate that this screening method for the detection of different groups of diuretics in urine is reliable and rapid enough to be used in doping control and clinical laboratories. Work is in progress to confirm the identity of these diuretics in human urine by GC–MS.

ACKNOWLEDGEMENTS

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