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Analysis of diuretic doping agents by HPLC screening and GC–MSD confirmation*

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Abstract: The simultaneous analysis of 14 diuretics in human urine (belonging to five different pharmacological groups) by reversed-phase high-performance liquid chromatography (HPLC) with a diode-array detector was developed by using a gradient elution with acetonitrile and phosphate buffer containing propylamine hydrochloride on a Bondclone-ODS (10 μm) column. The method was applied to the screening test of several diuretics abused by athletes. The confirmation analysis was performed by gas chromatography–mass spectrometry with ion-selective detection (GC–MSD). The characteristic mass fragment ions obtained by electron-impact (EI) ionization (70 eV) provided sufficient identification of these diuretic agents. During the 1990 weight-lifting contest of Asia in Kao-Hsiung, Republic of China, one urine sample was found positive for the diuretic hydrochlorothiazide.

Keywords: *Doping; diuretics (acetazolamide, amiloride, bendrofluazide benzthiazide, bumetanide, chlorothiazide, chlorthalidone, cyclothiazide, furosemide, hydrochlorothiazide, probenecid, spironolactone, triamterene, trichlormethiazide); HPLC–DAD; GC–MSD.*

Introduction

Diuretics are usually used to treat hypertension and edema caused by cardiac and renal failure and myocardial infarction. These agents typically increase the urine flow and the net renal excretion of both sodium and water. Recently diuretics have been used by some athletes as a masking agent to increase the urine volume, thereby diluting the concentration of a banned doping drug. Some compounds may well fall under the detection limit by this dilution. Diuretics are also used to lose weight quickly for placement in lower weight categories in certain sports (e.g. weightlifting, judo or taekwondos). During the Olympic games in Seoul in 1988, four athletes tested positively for the diuretic furosemide.

Another compound that attracted much attention in 1988 was probenecid, a uricosuric agent, which has weak diuretic activity. This compound reduces the excretion of anabolic steroids and antibiotics. It may well reduce the concentration of steroids in urine from 1 to 10%, thus making analysis impossible [1, 2]. However, diuretics and probenecid excreted into urine remained unchanged and thus may be detected as the parent compound without having to consider their metabolites. Since 1988, the International Olympic Committee has banned different groups of diuretics as well as probenecid for the Olympic Winter and Summer Games.

Several analytical methods for examining diuretics in urine have been proposed. These include those based on thin-layer chromatography [3, 4], gas–liquid chromatography (GLC) with an FID detector [5–7], GC–MS [8–10], and HPLC [11–21], and some papers have studied the LC method of several diuretics [22–24]. None of the screening procedures for diuretics published in the literature deal with basic diuretics. Thus acidic and basic extraction procedures have been used. The present study describes a HPLC–DAD screening procedure for the simultaneous separation and identification of 14 diuretics in spiked urine and urine samples. The identity of a positive screen of a urine sample was confirmed with GC–MSD using methylated derivatization of diuretics.

Experimental

Reagents and materials

All reagents were of analytical grade.

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Acetonitrile, ethyl acetate and methanol were HPLC grade (Merck). Water was distilled, deionized and filtered by Milli-Q4 bowl reagent water grade system (Waters). Bendrofluazide, benzthiazide, bumetanide, chlorothiazide, cyclothiazide, furosemide, hydrochlorothiazide, probenecid and trichlormethiazide were USP reference standard materials. Chlorthalidone (Merck), acetazolamide, amiloride, spironolactone, triamterene (Sigma Chemical Co.) and theophylline sodium glycinate (internal standard) were working standards supplied by the manufacturer. Propylamine hydrochloride was purchased from Tokoy chemical synthetic company. Figure 1 shows the chemical structure of diuretics tested in this study.

Stock solution of diuretics

A stock solution of each drug was prepared by dissolving 10 mg in methanol (100 μg ml^{-1}). The solutions were protected from light and stored in the dark at 4°C. Appropriate dilutions were made to prepare the standard solutions.

Internal standard solution

Theophylline sodium glycinate (10.0 mg) was dissolved in 100.0 ml of methanol (100 μg ml^{-1}).

Apparatus

High-performance liquid chromatography with diode-array detector (DAD)

A Waters Model 990 liquid chromatography equipped with a diode-array UV detector and a solvent delivery system controller was used for the screening procedure; the instrument was linked to a data system (NEC Power Mate 2). The chromatographic column was Bondclone-

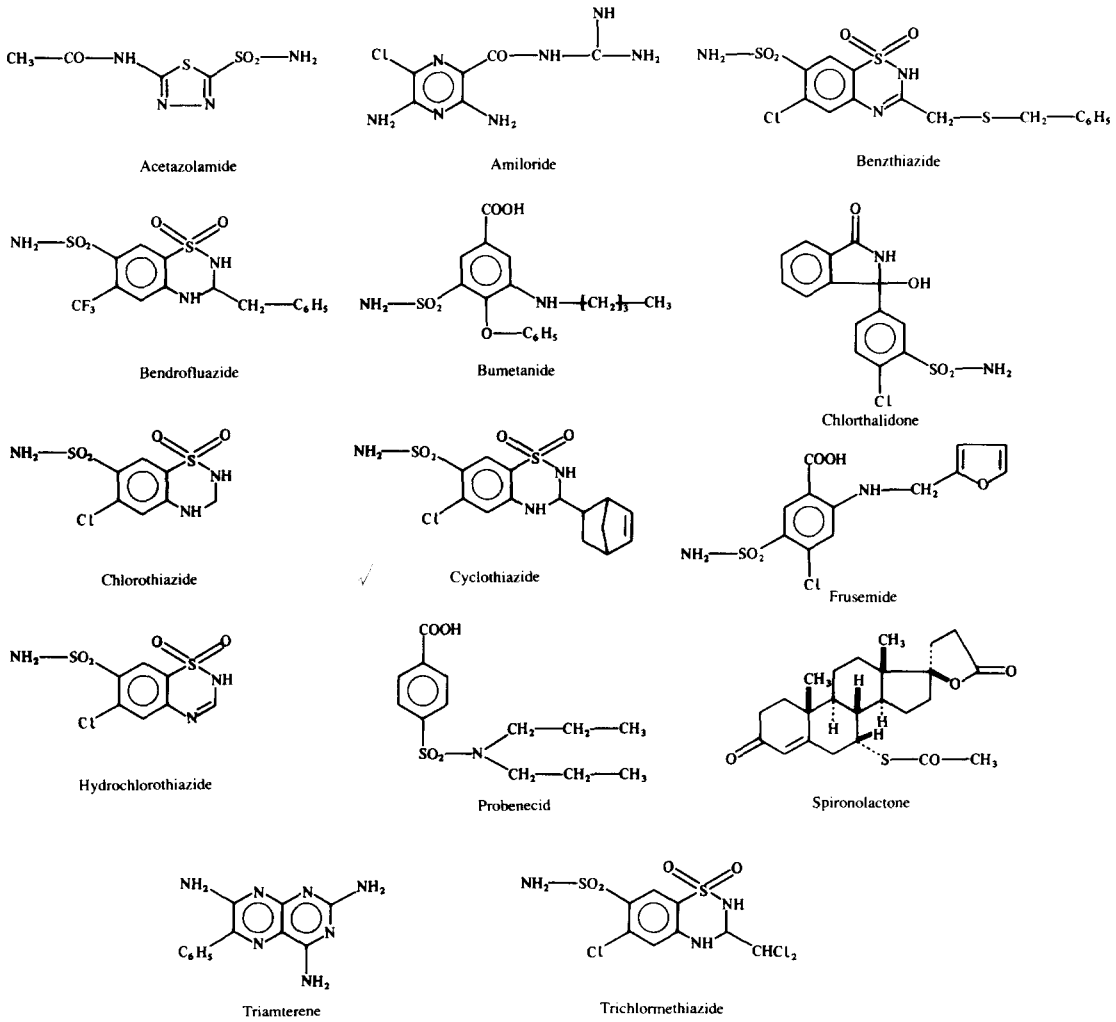


Figure 1
Chemical structures of diuretics.

ODS (300 × 3.9 mm i.d., 10 μm). The mobile phase was a gradient system of phosphate buffer (0.05 M sodium hydrogen phosphate monobasic hydrate containing 1.59 g of propylamine hydrochloride, pH 3.0) and acetonitrile. The solvent gradient program was as follows: initial acetonitrile was 15% for 1.5 min; it was increased to 45% at 7 min, to 59% at 12 min and 80% at 21 min; the flow rate was 1.0 ml min^{-1} and the column temperature was ambient. The diode-array detector was set to monitor the signal at 275 and 230 nm; spectral data between 200 and 350 nm were stored in the computer memory.

Gas chromatography–mass spectrometry

A Hewlett–Packard GC–MSD system which consisted of a model 5890B GC and a 5790B mass selective detector. HP UX chemstation, and 9144 disc drive was used, together with a HP fused-silica capillary column coated with cross-linked methylsilicone (HP-1 12 m × 0.2 mm i.d., 0.33 μm film thickness). The GC temperature program was set as follows: initial temperature was 200°C, increased by 15°C min^{-1} to 280°C, and 10°C min^{-1} to 300°C. The injector temperature was 290°C, the detector temperature 290°C, and the ion source 200°C. The injector was operated in the splitless mode. The carrier gas was helium (99.999%) at 0.9 ml min^{-1}. The mass spectrometer was operated at 70 eV in the electron-impact mode.

Extraction procedure

Each urine sample was extracted under acidic and basic conditions.

Acidic extraction. A 2.0 ml urine sample was pipetted into a 15-ml tube with a 0.5 g solid buffer [potassium hydrogen phosphate monobasic–sodium hydrogen phosphate dibasic (99:1), pH 5.0]. It was agitated with vortex action for 15 s, and then 4 ml of ethyl acetate was added. The tube was shaken mechanically for 10 min and centrifuged at 2000*g* for 15 min. The organic layer was transferred to a second tube. To the organic phase were added 2.0 ml of 5% aqueous lead acetate solution and the mixture was vortexed for 30 s, then centrifuged as above. The organic phase was retained for further work-up (as AE fraction).

Basic extraction. A 2.0 ml urine sample was pipetted into a 15 ml tube with a 0.5 g solid buffer [sodium bicarbonate–potassium carbonate (3:2), pH 9.0]. It was agitated with vortex action for 15 s and then 4 ml of ethyl acetate was added. The tube was shaken mechanically for 10 min and centrifuged at 2000*g* for 15 min. The organic layer was transferred to a second tube. The organic phase were added to the AE extraction. The combined extracts were evaporated to dryness with a slow stream of nitrogen. The dried residue was reconstituted with 300 μl methanol, and 5 μl was injected into the liquid chromatograph.

Recovery study

To 2.0 ml of blank urine was added 300 μl of a methanolic solution of each drug (100 μg ml^{-1}) equivalent to 15 μg ml^{-1} of urine. The sample was then subjected to the complete extraction procedure described above except it was not combined each extract. The peak area ratio obtained from extracted drug was compared to that of standard solution containing the same final concentration of each drug. The percentage of recovery of each drug by the two extraction methods is listed in Table 1.

Detection limit

Stock solutions containing 100 μg ml^{-1} of each drug were prepared in methanol. The following aliquots of stock solution were added to 2 ml blank urine samples: (1) 10 μl; (2) 20 μl; (3) 30 μl; (4) 100 μl. The spiked urine samples were subjected to acidic and basic extraction procedures as previously described to estimate the sensitivity and minimum quantities detectable in the urine samples.

Table 1
Extraction recovery of the screening procedure. In all cases 15 μg ml^{-1} of each diuretics was added

Diuretic	Recovery (mean ± SD, n = 3) (%) Acidic extraction	Basic extraction
Acetazolamide	87 ± 1.0	0
Amiloride	7 ± 4.6	23 ± 4.6
Bendrofluazide	98 ± 1.1	64 ± 5.4
Benzthiazide	83 ± 0.8	88 ± 5.0
Bumetanide	90 ± 1.5	43 ± 5.0
Chlorothiazide	94 ± 2.4	17 ± 3.7
Chlorthalidone	42 ± 1.0	72 ± 4.2
Cyclothiazide	97 ± 0.2	58 ± 7.6
Furosemide	85 ± 2.2	36 ± 3.0
Hydrochlorothiazide	96 ± 0.2	82 ± 3.7
Probenecid	81 ± 4.0	16 ± 0.6
Spironolactone	65 ± 4.7	40 ± 5.0
Triamterene	24 ± 0.6	88 ± 4.0
Trichlormethiazide	85 ± 1.2	70 ± 4.5

Derivatization

A sample extracted as described above was evaporated to dryness. The residue was dissolved with 200 μl of acetone, 20 μl of methyl iodide and 100 mg of potassium carbonate was added, and the solution was then heated in an oven at 60°C for 2 h. A 2 μl aliquot of methylated product was injected into the GC–MSD.

Results and Discussion

Screening with HPLC–DAD

Figure 2 illustrates a chromatogram obtained from a methanolic solution containing a mixture of 14 diuretics and internal standard at a concentration 100 μg ml^{-1}. The detector was set at 275 nm; under this condition spironolactone could hardly be detected but a better result was obtained at 230 nm. Under the conditions of this study, most of the diuretics tested have a better UV absorbance at 230 nm. Generally the urinary endogeneous compounds absorbed less at 275 nm. At this wavelength, there is less interference with other exogenous urinary compounds, while retaining high sensitivity for the diuretics.

The mixed diuretics were separated within 21 min. An individual diuretic agent could be detected by its retention time and UV spectrum. The retention time of benzthiazide and cyclothiazide were very close (16.39 and 16.62 min) and were not well resolved. However, the UV maxima and spectra were different; therefore their identification was possible. Cyclothiazide gave two peaks on a Bondclone-ODS column; this was probably due to the presence of its diastereoisomers as already reported by Tisdall *et al.* and De Croo *et al.* [20, 21, 23].

Probenecid, with a long retention time, showed a broad and asymmetrical peak shape, seemingly due to silanophillic interactions between the nonendcapped silanol group of the packing materials and the polar groups of probenecid. Figure 3 shows corresponding UV spectra including that of theophylline sodium glycinate, the internal standard. Figure 4A illustrates the chromatogram obtained from extracts of a blank urine sample spiked with theophylline sodium glycinate as internal standard; peaks 2, 3 and 4 arise from endogeneous products. Figure 4B illustrates the chromatogram of a positive result for hydrochlorothiazide in an athlete's urine sample; peaks 3 and 4 are due to endogeneous products.

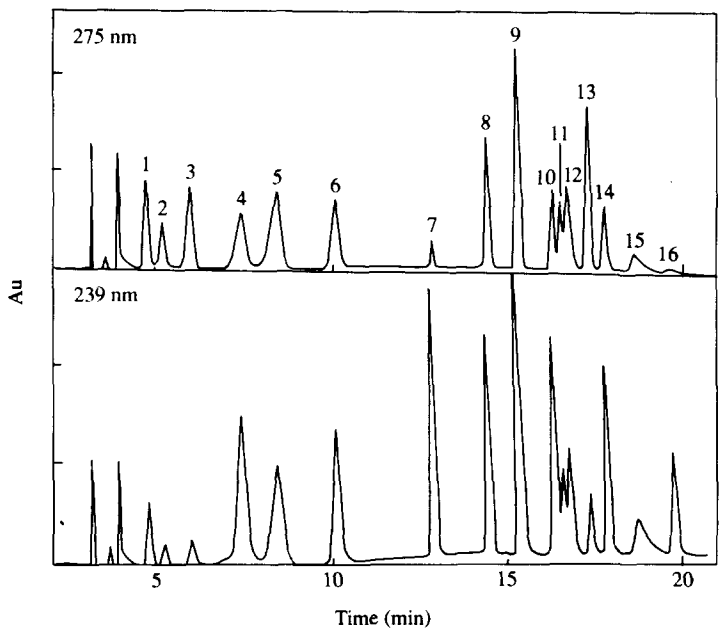


Figure 2
HPLC chromatogram obtained with a standard methanolic solution containing a mixture of diuretics and internal standard using Bondclone-ODS (300 × 3.9 mm, 10 μm) at detector wavelengths of 275 and 230 nm. Each peak corresponds to 500 ng. Peak identities: (1) amiloride; (2) TSTD (theophylline sodium glycinate); (3) acetazolamide; (4) chlorothiazide; (5) hydrochlorothiazide; (6) triamterene; (7) chlorthalidone; (8) trichlormethiazide; (9) furosemide; (10) benzthiazide; (11, 12) cyclothiazide; (13) bumetanide; (14) bendrofluazide; (15) probenecid; (16) spironolactone. Spironolactone and other diuretics show better absorbance at 230 nm than that at 275 nm.

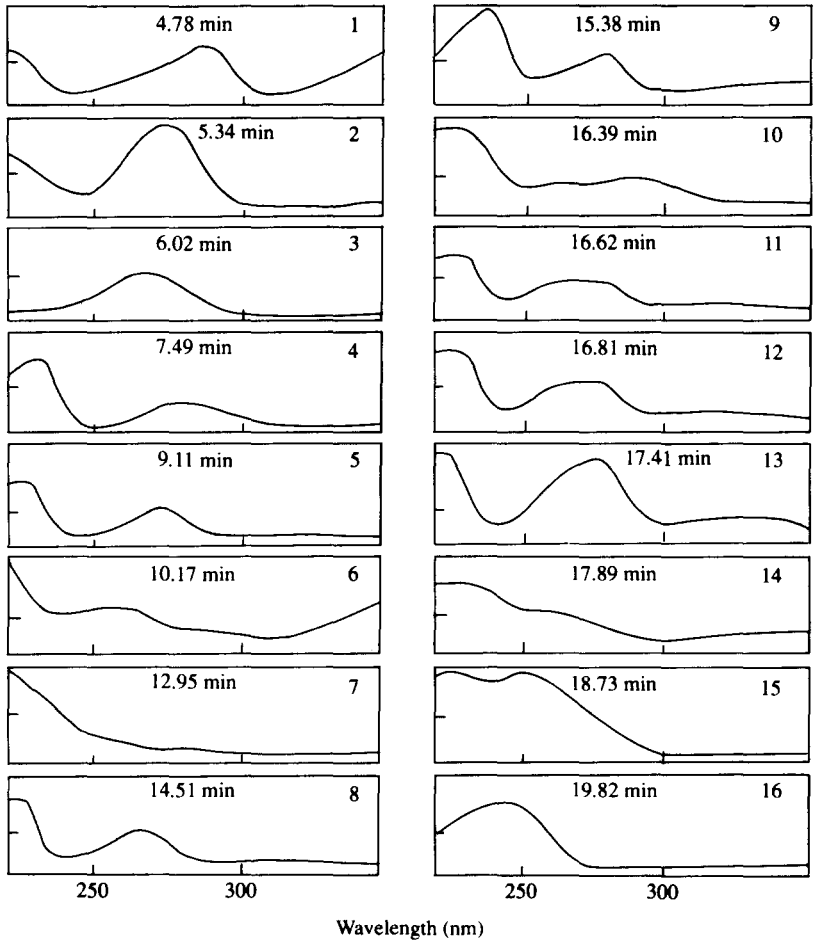


Figure 3
UV spectra of standard diuretics and internal standard. Peak identity: same as Fig. 2.

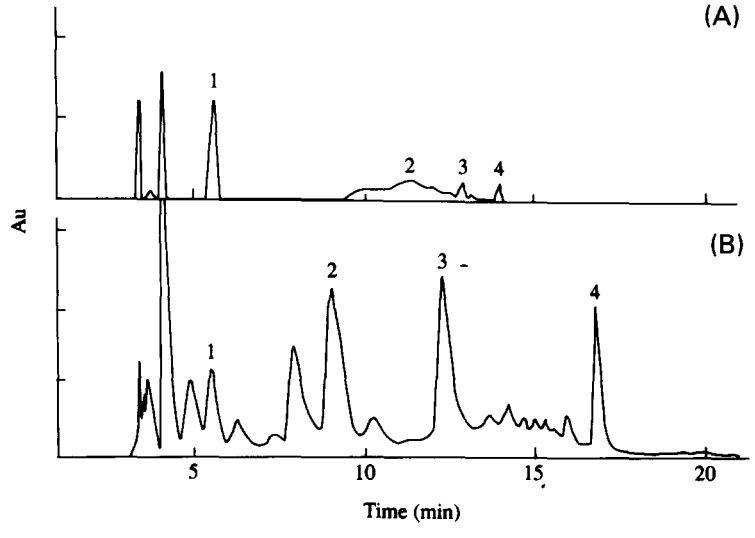


Figure 4
(A) Chromatogram obtained from an extract of internal standard spiked into blank urine. Peak 1 is (ISTD) theophylline sodium glycinate, peaks 2, 3 and 4 are endogenous compounds. (B) Chromatogram obtained from an extract of a positive screening of hydrochlorothiazide from an athlete's urine. Peak 1: Theophylline sodium glycinate, peak 2: hydrochlorothiazide, peaks 3 and 4 are endogenous compounds. Column: Bondclone-ODS, detector set at 275 nm.

Recovery study

It was necessary to pretreat the specimens in order to extract the drug of interest and to remove the interference from the matrix. Pretreatment with a proton-accepting solvent such as ethyl acetate, diethyl ether or methyl isobutyl ketone at a suitable pH or protein precipitation have been described [24]. In this paper, ethyl acetate was chosen because it is nonflammable and created little interference in the extraction process.

For the recovery study, samples of blank urine were spiked in triplicate with 15 μg ml^{-1} of each diuretic of interest and worked up using the acidic and basic extraction procedures. The recovery of each drug is given in Table 1. The lower recovery of amiloride and triamterene using acidic extraction was due to their basic and weakly basic pK_a values of 8.7 and 6.2, respectively. With the exception of those drugs, the percentage recovery of other compounds varied from 42 to 98%. The values of standard deviations varied from ±0.2 to ±4.7. The recovery of amiloride was also low using basic extraction, whereas it was high for triamterene. More acidic drugs (e.g. probenecid, chlorothiazide and furosemide) showed poor extraction recovery under basic conditions. The recovery of other compounds varied from 40 to 88%. The overall values of standard variations varied from ±0.6 to ±7.6.

Confirmation with GC–MSD

For the diuretic agents which contain sulphonamide, carboxyl, amine, amide or hydroxy groups (and therefore are non-volatile and polar), poor GC response was observed. It was necessary to derivatize the parent drug to improve volatility and thermal stability. Under these conditions, hydrochlorothiazide was methylated at all the *N*-bonded hydrogen atoms (NH adjacent to an aromatic ring as in furosemide remained unmethylated).

Figure 5 shows the total ion chromatogram (TIC) obtained from a spiked urine extract of hydrochlorothiazide and a sample positive for hydrochlorothiazide in the urine. Both TICs shows the same retention time.

Hydrochlorothiazide contains one chlorine atom. Its fragment ions [m+] are accompanied by an [m + 2] ion because of the isotopic effect of chlorine. Its mass spectrum has a large molecular ion peak (*m/z* 353) and (*m/z* 355) after methylation. As shown in Scheme 1 [25] the methylated derivative of hydrochlorothiazide yields a base peak *m/z* 310 from loss of CH_3NCH_2, and contains the isotopic pattern at *m/z* 312. The ion peak of *m/z* 288 is formed by the loss of SO from the $[M - 1]^+$ ion and contains the isotopic pattern of *m/z* 290. Figure 6 show the mass spectrum (MSD) obtained from the extract of standard hydrochlorothiazide spiked into blank urine and that of a

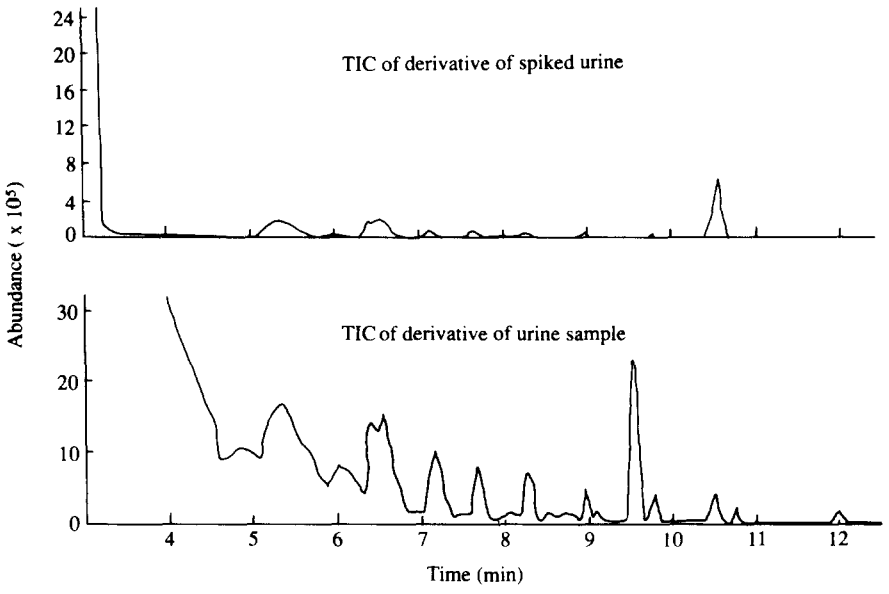
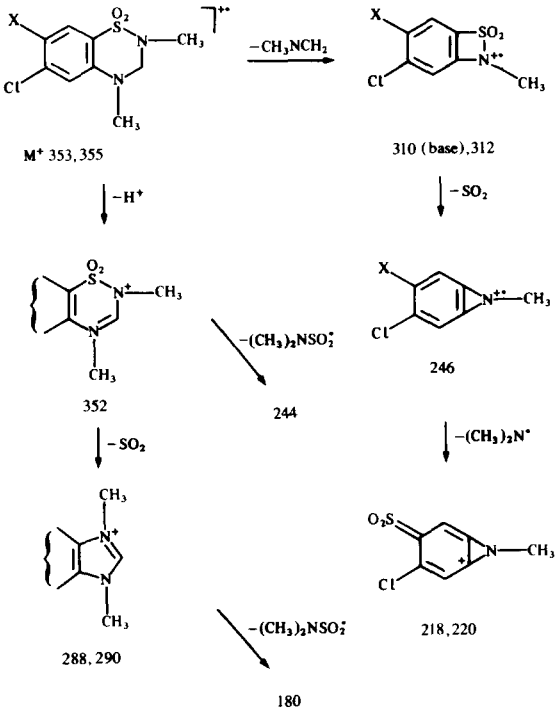


Figure 5
Total ion chromatogram obtained from the derivative of the residue of spiked urine extract (standard hydrochlorothiazide) and the urine sample extract.



Scheme 1
Fragmentation of hydrochlorothiazide derivative, X = $(CH_3)_2NSO_2$.

positive screening of hydrochlorothiazide urine sample. The characteristic mass fragment ions [27] for the methylated compound are listed in Table 2.

Urinary excretion studies of hydrochlorothiazide have been performed by Fullinfaw *et al.* [22] and Cooper *et al.* [26]. Table 3 showed the sensitivity of the screening procedure using Bondclone-ODS. The lower limit of detection ranges from 0.5 to 1.5 μg ml^{-1} in urine. Amiloride could not be detected below 5.0 μg ml^{-1} of urine. Since the limit of detection of hydrochlorothiazide is 0.5 mg ml^{-1}, this diuretic can be detected at this level within 44 h after administration using this procedure.

Conclusions

The HPLC–DAD screening procedure successfully separated 14 diuretics in urine. The separation was rapidly achieved within 21 min by using a gradient elution with acetonitrile and pH 3.0 phosphate buffer solution containing propylamine hydrochloride on a Bondclone-ODS column. The percentage

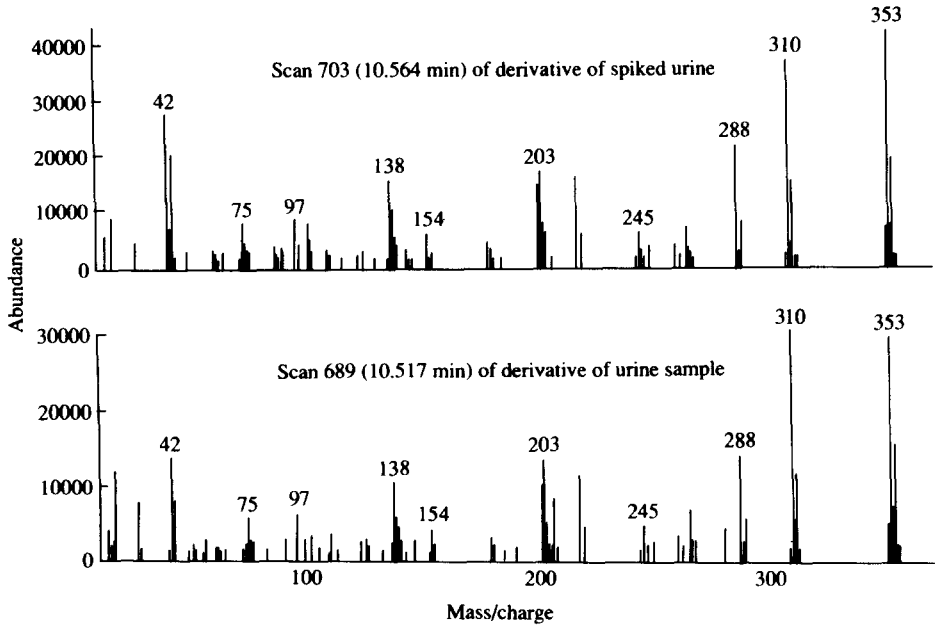


Figure 6
Mass spectrum obtained from methyl derivative of the residue of spiked urine extract (standard hydrochlorothiazide) and the urine sample extract.

Table 2
Retention time (t_R) and characteristic fragment ions of methylated diuretic and urine sample

Methylated compound	t_R (min)	Characteristic mass fragment ions (*m/z*)								Molecular weights
Hydrochlorothiazide	10.59	218	220	288	290	310	312	353	355	353
Urine sample	10.52	218	220	288	290	310	312	353	355	353

Table 3
Detection limits in the diuretics screening procedure

Drug	Detection limit (μg ml^{-1}) Acidic extraction	Basic extraction
Acetazolamide	1.0	0
Amiloride	0	1.5
Bendrofluazide	1.0	1.0
Benzthiazide	1.0	1.0
Bumetanide	1.0	1.0
Chlorothiazide	1.0	0
Chlorthalidone	1.5	1.5
Cyclothiazide	1.0	1.0
Furosemide	0.5	0.5
Hydrochlorothiazide	0.5	0.5
Probenecid	5.0	5.0
Spironolactone	1.0	1.0
Triamterene	0	1.0
Trichlormethiazide	1.0	1.0

recoveries using acidic and basic extractions were better than that of the method described by Fullinfaw [22]. The procedure also minimized the loss during different pH conditions and reduced the chromatographic running time by half by combining two extractions. The procedure thus was reliable due to higher recovery (98 and 88%) under both extraction methods. Methylated derivatives of hydrochlorothiazide were separated on a capillary GC column (HP-1) under these conditions. Compounds were identified by retention time and characteristic mass fragment ions using GC–MSD.

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