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Review

# Detection of diuretic agents in doping control

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**Abstract**

Since the inclusion of diuretics in the list of banned substances in sports in 1988, a large number of screening and confirmation procedures to detect the presence of these substances in urine samples have been developed. In this paper, a review of the analytical methodology described to analyze diuretics is presented. The paper has been focused on the needs of doping control and mainly screening procedures including sample preparation and liquid or gas chromatographic separation have been considered. More relevant papers using capillary zone electrophoresis have been also considered. Mass spectrometry is mandatory in doping control for confirmation purposes, and finally, mass spectrometric techniques described for diuretics have been reviewed.

*Keywords:* Diuretics; Reviews

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## 1. Introduction

Diuretics are drugs widely used in clinical practice mainly in the treatment of hypertension and in different kinds of edema [1,2]. Diuretics increase the renal excretion of water and electrolytes, as a consequence of its disturbing action on the ionic transport in the nephron. They act by interfering the

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tubular reabsorption of sodium and this leads to an increase in its renal excretion which is accompanied by water elimination.

Diuretics may be classified according to their chemical structure (Fig. 1), their mechanism and primary site of action in the nephron, and their diuretic potency (Table 1) [1–5]. Those diuretics with primary action in the proximal tubule include the carbonic anhydrase inhibitors such as acetazolamide and diclofenamide which are sulphonamide derivatives; their diuretic efficacy is low, causing the excretion of less than 5% of filtered sodium. Maximum efficacy (excretion of more than 15% of filtered sodium) is reached with those drugs which have their major activity in the ascending limb of the loop of Henle by inhibition of the sodium–potassium–chloride electroneutral system; this group includes sulphonamide derivatives such as furosemide, bumetanide and piretanide, and phenoxyacetic acids such as etacrynic acid. In the early portion of the distal tubule, sodium chloride reabsorption is impaired by benzothiadiazine diuretics and related compounds (chlorthalidone, indapamide) as their primary site of action; they are considered medium efficacy diuretics (excretion of 5–10% of the filtered sodium). The major site of action of the named potassium-sparing diuretics (spironolactone and amiloride and triamterene) is the late distal tubule and the collecting duct where they inhibit the exchange of sodium for potassium and hydrogen; these drugs have low diuretic efficacy and differ chemically and in their mechanism of action. There is also a group of low efficacy diuretics whose mechanism of action is based on the osmotic effect (osmotic diuretics).

The use of diuretics has been forbidden by the Medical Commission of the International Olympic Committee [36] because it was shown that they were misused in sports for two main reasons: to achieve acute weight losses before competition, in sports where weight categories are involved, and to mask the ingestion of other doping agents by reducing their concentration in urine. This effect may be accomplished either by increasing the urine volume, or by increasing the urinary pH (carbonic anhydrase inhibitors) and, thus, reducing the excretion in urine of basic doping agents.

Weight loss produced by diuretics has been described by different authors [37–40]. Caldwell et al. [38,39] described a 4.1% reduction of the body-weight after a furosemide dose of 1.5 mg/kg in weightlifters, wrestlers, judoka and boxers. Armstrong et al. [40] induced a 2% of weight loss with furosemide in distance runners. The increase of the urine volume after intake of diuretics can be an indirect marker of the weight loss: increases of urine volume between 800 and 1600 ml in 3–4 h can be achieved after therapeutic doses of furosemide, bumetanide or piretanide [15,41].

The effects of diuretics on the excretion of other drugs has been extensively studied by Delbeke and Debackere [41–45]. Acetazolamide administration reduces the excretion of basic doping agents, as mephentermine, phentermine, ethylamphetamine and amphetamine, due to an increase of the urinary pH [41–43]. Urine concentrations of these compounds can be reduced below the detection limits of the routine antidoping tests. Moreover, longer half-lifes in the body can produce an increase in the metabolism of some compounds [42]. High efficacy diuretics, such as furosemide and bumetanide, only act by dilution on the urine without effects on drug disposition [41–43,45].

The family of diuretics includes compounds with wide differences in molecular structures (Fig. 1) and, in consequence, in physico-chemical properties. Partition coefficients octanol–water and $pK_a$ values for some diuretics are presented in Table 2 to illustrate this variability. Taking into account the chemical nature of their functional groups, diuretics may be classified in four sub-groups as indicated in Table 2.

Metabolism and urinary excretion of diuretics have to be considered for doping control purposes. The main metabolic pathways and the percentages of the dose excreted unchanged in urine described for some diuretics after pharmacokinetic studies in humans using different routes of administration and different doses are listed in Table 1. Most of diuretics are excreted unchanged in urine to variable extent: from 99% of the dose for acetazolamide to 4–12% for triamterene. Therefore, procedures to screen for diuretics in human urine can be mainly developed to detect the parent compounds. Nevertheless, some compounds such as spironolactone are nearly completely metabolized. Spironolactone is

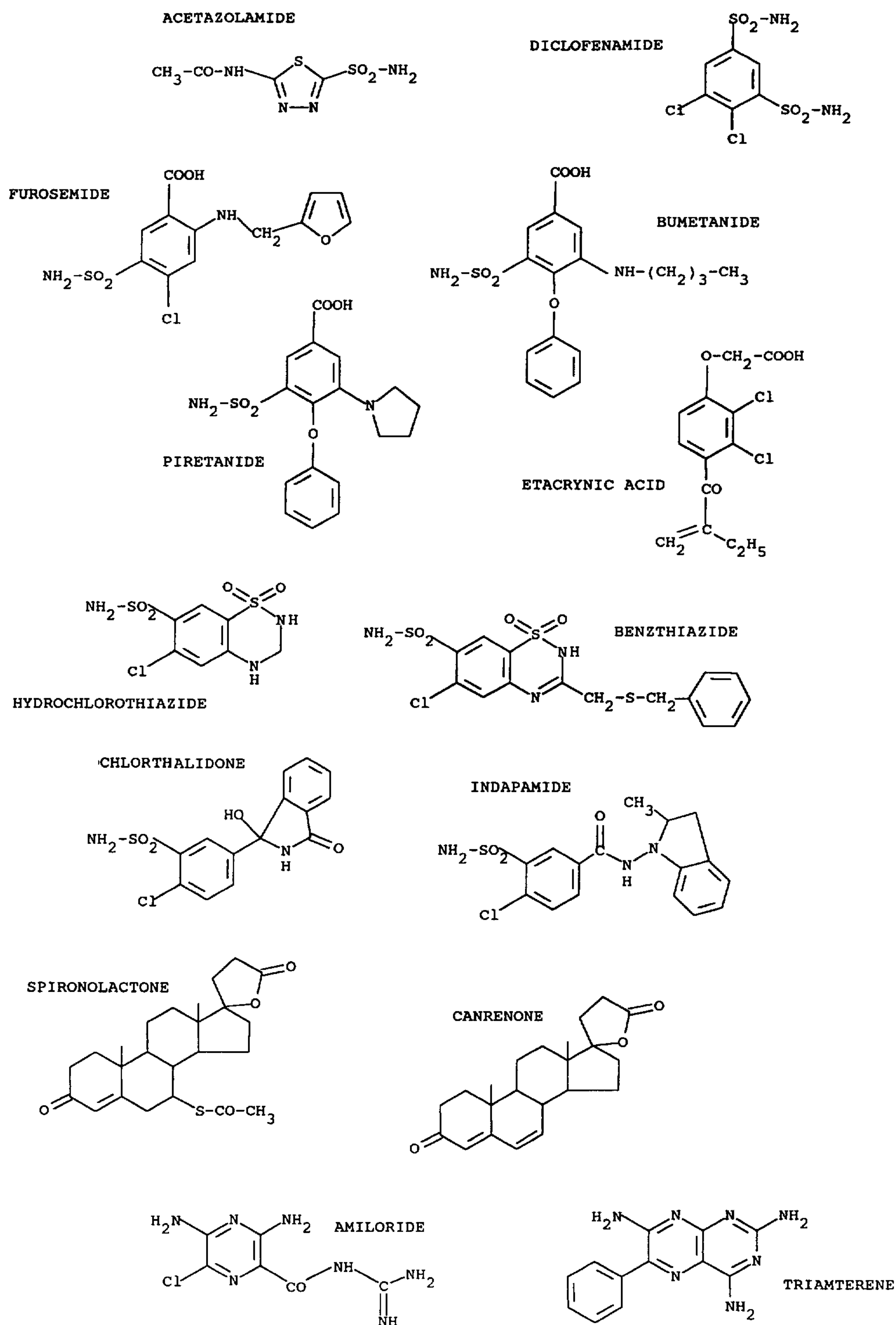


Fig. 1. Structures of some diuretics.

Table 1
Pharmacological classification of diuretics, percentage of the dose excreted unchanged in urine and main metabolic pathways

| Compound | Unchanged (%) | Reference | Main metabolic pathways | Reference |
|---|---|---|---|---|
| **1. HIGH EFFICACY DIURETICS**: Loop diuretics | | | | |
| Bumetanide | 35–44 | [6] | Hydroxylation in butyl side chain | [7] |
| | 55 | [7] | N-debutylation | |
| | 27–37 | [8] | Conjugation with glucuronic acid | |
| | 58–69 | [9] | | |
| Etacrynic acid | ND[a] | | Glutathion conjugation | [2] |
| Furosemide | 48 | [10] | Loss of the side chain linked to the | [10–13] |
| | 20–88 | [11] | group (analytical artifact?) | |
| | | | Conjugation with glucuronic acid | |
| Piretanide | 45 | [14] | Hydroxylation of the pyrrolidine ring | [15,16] |
| | 60–74 | [15] | Unknown pathways | |
| **2. MEDIUM EFFICACY DIURETICS**: Benzothiadiazides and related compounds | | | | |
| Bendroflumethiazide | 30 | [17] | Unknown | |
| Benzthiazide | 10 | [18] | - | |
| Chlorthalidone | 34–53 | [19] | Unknown | |
| | 25–70 | [20] | | |
| Hydrochlorothiazide | 70–95 | [21] | - | |
| | 65–72 | [22] | | |
| | 50–60 | [23] | | |
| | 30–64 | [24] | | |
| | 46 | [25] | | |
| Indapamide | 7 | [26] | Breakdown of the amide bond | [26,27] |
| | | | Conjugation with glucuronic acid and sulphate | |
| **3. LOW EFFICACY DIURETICS**: | | | | |
| 3.1. Potassium-sparing diuretics | | | | |
| Spironolactone | 0 | [28–30] | Loss of side chain in position 7 | [28,29] |
| | | | Oxidation in the side chain | |
| | | | Hydroxylation | |
| | | | Conjugation | |
| Amiloride | 33–43 | [24] | - | |
| | 36 | [25] | | |
| Triamterene | 4–12 | [24] | Hydroxylation of the benzene ring | [31–34] |
| | 7 | [31] | Conjugation with sulphate | |
| | 5 | [32,33] | | |
| | 4–5 | [34] | | |
| 3.2. Carbonic anhydrase inhibitors | | | | |
| Acetazolamide | 98 | [35] | - | |

[a] ND: no quantitative data.

Table 2
Classification of diuretics according to their acid–basic behaviour

| Acid/basic behaviour | Compound | p$K_a$ | [Reference] | log *P* |
|---|---|---|---|---|
| 1. *Basic diuretics* | Amiloride | 8.7 | [4] | ND[a] |
| | Triamterene | 6.2 | [4] | 0.98 |
| 2. *Neutral diuretics* | Canrenone | - | | 2.68 |
| | Spironolactone | - | | 2.78 |
| 3. *Weakly acidic diuretics* | Acetazolamide | 7.4, 9.1 | [47] | −0.26 |
| | | 7.2, 9.0 | [48] | |
| | Diclofenamide | 7.4, 8.6 | [48] | 1.03 |
| | Bendroflumethiazide | 9.0 | [49,50] | 1.19 |
| | Benzthiazide | 6 | [50] | 1.46 |
| | Chlorthalidone | 9.35 | [51] | ND |
| | Hydrochlorothiazide | 9.5, 11.3 | [49,50] | −0.07 |
| 4. *Strongly acidic diuretics* | Bumetanide | 3.6, 7.7 | [52] | 0.12 |
| | Etacrynic acid | 3.5 | [3] | −0.81 |
| | Furosemide | 3.8, 7.5 | [52] | −0.83 |
| | Piretanide | 4.1 | [3] | ND |

Values of p$K_a$ and log *P* [46] of some diuretics. log *P* is defined as the logarithm of the partition coefficient octanol–water, except for: spironolactone and canrenone (octanol–phosphate buffer pH 7.4); diclofenamide, bumetanide and etacrynic acid (partition coefficient diethyl ether–phosphate buffer pH 7.4 transformed to octanol–aqueous phase according to the equation proposed by Hansch and Leo [46]); and bendroflumethiazide and benzthiazide (octanol–phosphate–citrate buffer pH 6.5).
[a] ND: no data.

excreted in urine as canrenone, which has also diuretic activity, and other sulphur containing metabolites.

The objective of this paper is to review the analytical methodology described to detect diuretics in urine keeping in mind the requirements of doping control. Screening methods reported since 1980 allowing the analysis of a high number of diuretics have been mainly considered in sample preparation and liquid chromatography (LC) sections. Taking into account the need of derivatization of most of diuretics before gas chromatography (GC) and the role of this separation technique for screening analyses and also for confirmation of specific compounds, particular attention has been paid to the derivatization procedures described for both screening purposes and single-compound analysis. More relevant papers using the relatively new separation technique, capillary zone electrophoresis (CZE), have been also included. Due to the need of mass spectrometry (MS) for confirmation purposes in doping control, mass spectrometric techniques described to analyze diuretics have been finally reviewed.

## 2. Sample preparation

The analysis of drugs present in biological samples requires some form of sample preparation. A large number of procedures for the detection and quantitation of particular diuretics have been described. However, the number of screening methods for the whole group is limited and they have been mainly reported after the restriction of the use of diuretics in sports.

Liquid–liquid extractions have been the most widely used techniques to isolate unmodified diuretics from urine. Procedures optimized to the analysis of acidic diuretics have been described by Tisdall et al. [53] and Fullinfaw et al. [54]. In both cases, the urines were acidified (pH 5 or pH 4.1, respectively) and extracted with ethyl acetate; a clean-up of the organic phase with a phosphate solution (pH 8 or pH 7.5) was performed before evaporation to dryness. Extraction recoveries between 50 and 90% were described except for the more acidic compounds that were lost in the clean-up step. A treatment of the urine sample with $NaBH_4$

was proposed by Tisdall et al. [53] to convert chlorothiazide to hydrochlorothiazide and, thus, to improve its detection. Detection limits between 0.5 and 1.5 $\mu$g/ml were reported using LC analysis and, in spite of the low recoveries for some compounds, all studied diuretics were detected in urine until 24 h after administration of a therapeutic dose, except etacrynic acid [54].

When the detection of basic, acidic and neutral diuretics is aimed, two separated liquid–liquid extraction procedures in neutral or basic medium, and in acidic medium have been proposed. Extraction with diethyl ether at pH 7.4, for weakly acidic, basic and neutral diuretics, and extraction at pH 2 with diethyl ether or ethyl acetate, for strongly acidic compounds, have been described by Schänzer [55] and Ueki [56].

Cooper et al. [57] and Tsai et al. [58] described similar procedures consisting of two simultaneous extractions of the urine: at pH 5 with ethyl acetate and clean-up of the organic phase with lead acetate solution; and at pH 9–9.5 with ethyl acetate. Organic extracts were analyzed by LC. Basic compounds were best recovered in the basic extract, although even under these optimal conditions, amiloride had a poor extraction recovery (23–25%). For neutral and weakly acidic compounds, both extraction procedures gave similar yields, except for acetazolamide not recovered in the basic extract. Extraction at acidic pH was better for strongly acidic compounds. For some of the compounds poor detection limits were reported [57,58] which would make difficult their detection after a normal ingestion of the drug.

Park et al. [59] compared liquid–liquid extractions at different pH values (pH 5, 7, 9 and 11) using diethyl ether as organic solvent and anhydrous sodium sulphate to promote salting-out effect. Amiloride was not recovered at any pH, and the extraction of triamterene was improved at high pH values. The extraction of weakly acidic diuretics was dependent of the compound, and strongly acidic compounds were best recovered at the lowest pH value. The best compromise was achieved at pH 7. Under these extraction conditions and using LC analysis, detection limits of 0.2 $\mu$g/ml were reported for most of the compounds.

After studying different liquid–liquid extraction conditions (pH, organic solvent, salting-out effect), alkaline extraction (pH 9.5) with ethyl acetate and using sodium chloride to promote salting-out effect was chosen as the best compromise to screen for diuretics in urine [60]. Extraction of basic and weakly acidic diuretics was favoured by the pH and/or the salting-out effect, depending on the compound. The recovery of strongly acidic compounds in the alkaline extract was clearly due to the salting-out effect. Detection limits in the range of 0.01 to 0.2 $\mu$g/ml were obtained for most of the compounds using LC analysis. An example of the analysis of a urine spiked with different diuretics is presented in Fig. 2.

Solid-phase extraction has also been evaluated to screen for diuretics in urine. Sep-Pak $C_{18}$ columns have been studied by Park et al. [59] using a mixture of diethyl ether and methanol as elution solvent. After LC analysis, detection limits of 0.2 $\mu$g/ml were obtained. Using Sep-Pak $C_{18}$ columns, cleaner organic extracts were obtained compared to the liquid–liquid extraction at pH 7 described by the same authors.

Campins et al. [61] compared the extraction recoveries of neutral, acidic and basic diuretics using different Bond-Elut columns: octadecyl, octyl, ethyl, cyclohexyl, phenyl and cyanopropyl. In all cases, the sample was loaded in the pretreated column and, after a wash step with water, the elution of the analytes was performed using pure methanol; the chromatographic analysis was carried out by LC. The results showed the difficulty to obtain single extraction conditions to extract all the compounds of the group due to its different polarity. The best compromise was achieved using the most apolar packing materials (octadecyl or octyl), except for the most polar compounds of the family, acetazolamide and hydrochlorothiazide, that were lost in the wash step. In order to increase the recovery of such compounds, the volume of water used in the washing step was reduced (from 2 ml to 0.5 ml), leading to a concomitant increase in the number of interfering peaks in the final extract. Coelution of urinary endogenous peaks with diuretics was observed in spite of the high concentrations of diuretics tested compared to the levels of the drugs found in urine after ingestion of clinical doses.

The use of Bond-Elut octadecyl cartridges for screening purposes, and cyclohexyl or phenyl col-

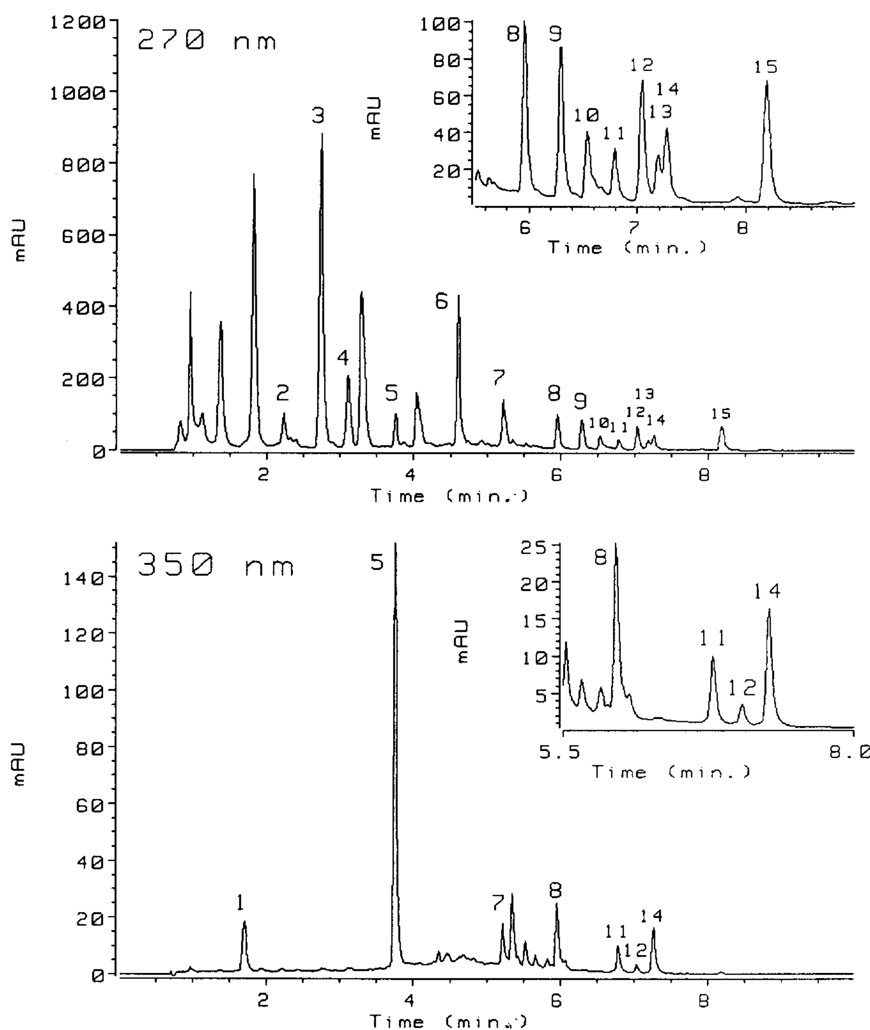


Fig. 2. Results obtained after analysis by liquid chromatography of a urine spiked with: (1) amiloride (0.1 μg/ml); (2) acetazolamide (5 μg/ml); (3) caffeine (3 μg/ml); (4) hydrochlorothiazide (0.5 μg/ml); (5) triamterene (0.5 μg/ml); (6) 7-propyltheophylline, ISTD (1 μg/ml); (7) torasemide (0.5 μg/ml); (8) furosemide (0.2 μg/ml); (9) buthiazide (0.2 μg/ml); (10) benzthiazide (0.2 μg/ml); (11) piretanide (0.2 μg/ml); (12) bendroflumethiazide (0.2 μg/ml); (13) xipamide (0.2 μg/ml); (14) bumetanide (0.2 μg/ml) and (15) canrenone (0.2 μg/ml). For extraction and chromatographic conditions see Ref. [60].

umns for quantitation and confirmation of amiloride, triamterene, chlorthalidone and furosemide was recommended for the same authors in a subsequent study [62]. For confirmation of acetazolamide, liquid–liquid extraction at acidic pH with ethyl acetate was suggested by these authors. Detection limits from 3 to 200 ng/ml were reported using methanolic solutions.

The isolation of diuretics from urine by adsorption on a polystyrene resin (XAD-2) before derivatization and GC–MS analysis has been used by some authors [55,63]. Another purification procedure used before GC analysis which combines extraction and derivatization, is extractive methylation described in Section 4.1.

A simplified screening procedure using column switching techniques coupled to LC has been recently described [64]. A 50-μl volume of urine were

directly injected onto a precolumn with Hypersil ODS material. The precolumn was flushed for 1 min with water to eliminate polar matrix components and afterwards the retained analytes were back-flushed by means a six-port switching valve onto a Hypersyl ODS analytical column where they were separated using gradient elution. A back-flush mode was preferred to minimize the dispersion of the sample in the chromatographic system. Extraction recoveries higher than 90% were obtained for most of the compounds. Acetazolamide and hydrochlorothiazide were only partially recovered due to their high polarity. Detection limits in the range of 2 to 200 ng/ml for methanolic solutions were reported.

Direct injection of the urine sample into the chromatographic system using micellar LC has been studied for some authors [65–68]. The main drawback of such approach to screen for diuretics is the difficulty to obtain single chromatographic conditions for the detection of all the compounds of the family in an acceptable run time, due to the differences in the capacity factors and also to coelutions with sample matrix components.

## 3. Liquid chromatography

LC procedures optimized to the analysis of single diuretics have been recently reviewed by Herraez et al. [69]. LC methods described to screen for a group of diuretics are presented in Table 3. The separation of the compounds has been usually achieved using octadecylsilane columns with 5 $\mu$m particles. The use of octadecylsilane columns with particles of 3 $\mu$m allowed the reduction of the column length to obtain the same chromatographic efficacy and, thus, a substantial reduction in the analysis time was also achieved [60]. A typical chromatogram obtained using octadecylsilane columns with particles of 3 $\mu$m [60] is shown in Fig. 2. The influence of the length of the alkyl chain in the stationary phase on the chromatographic behaviour of diuretics has been studied by De Croo et al. [71].

Gradient elution is preferred by most of the authors owing to the different polarity of the compounds belonging to the group of diuretics (Table 3). Mobile phases consisting of an acidic aqueous buffer and acetonitrile as organic modifier, have been used. The effect of the mobile phase composition on the LC behaviour of diuretics has been studied by different authors [70,72–74]. Thiazide diuretics can be analyzed using mixtures of water and organic modifier (methanol or acetonitrile); in the pH range between 3 and 7, the retention of such compounds was not influenced by the pH of the aqueous phase, and better resolution was obtained using acetonitrile as organic modifier [70,72].

Reversed-phase LC analysis of strong acidic diuretics required mobile phases of acidic pH in order to increase the lipophilic interactions of the compounds with the stationary phase [70]. Basic compounds were highly retained using mobile phases containing water and organic modifier at pH 3 [70,73]; this high retention can be explained by interactions with the residual silanol groups of the stationary phase. In order to analyze basic diuretics together with acidic compounds, a basic modifier, such as propylamine [70], or an ammonium salt [73] were added to the acidic aqueous phase to minimize the interactions of the basic compounds with residual silanol groups. Under these conditions, a reduction of the retention times and an improvement in the peak shapes was observed for basic compounds.

The retention time of etacrynic acid was found to be very sensitive to small variations of the mobile phase pH in the range between 2.95 and 3.25, owing to the proximity of the $pK_a$ value [74].

For compounds that may form intramolecular hydrogen bonding, such as bendroflumethiazide and hydrochlorothiazide, the effect of the injection solvent on the peak shape and peak response was important [75]. For reversed-phase systems, higher responses and better peak shapes were obtained when the polarity of the solvent was increased.

Ultraviolet absorbance (UV) detection is described in all LC screening procedures reported in the literature (Table 3). In most cases, diode array detection is used due to the possibility to monitorize different wavelengths (Fig. 2) and to obtain UV spectra which facilitates the identification of the peaks detected. Special software developments have been made to comprehensively analyze diuretics by LC using diode array detection in a large number of samples [76]. For some of the compounds, as furosemide, bumetanide, amiloride and triamterene, fluorescence detectors have also been used allowing

Table 3
Reversed-phase LC conditions described to screen for diuretics

| Reference | Compounds | Column | Mobile phase | Detection |
|---|---|---|---|---|
| [53] | 9 acidic diuretics (thiazides) | μBondapak $C_{18}$<br>10 μm, 30×0.39 cm | Acetic acid 1%<br>Acetonitrile<br>Isocratic elution | UV[a]: 271 nm |
| [70] | 20 diuretics (acidic, basic and neutral) | LiChrosorb RP $C_{18}$<br>5 μm, 15×0.46 cm | Different mobile phases<br>Isocratic elution | UV: 238 or 275 nm |
| [71] | 20 diuretics (acidic, basic and neutral) | LiChrosorb RP $C_{18}$, $C_8$ or $C_2$<br>5 μm, 15×0.46 cm | Water or 0.05 *M* phosphate buffer pH 3<br>Acetonitrile<br>Isocratic elution | UV: 238 or 275 nm |
| [72] | 16 diuretics (thiazides) | Different columns | Acetic acid 1%<br>Acetonitrile<br>Isocratic elution | UV: 271 nm |
| [54] | 12 acidic diuretics<br>(thiazide and loop diuretics) | LiChrosorb RP-18<br>5 μm, 12.5×0.44 cm | 0.05 *M* phosphate buffer pH 3<br>Acetonitrile<br>Gradient elution | DAD[b]: 271 nm |
| [56] | 12 diuretics (acidic, basic and neutral) | Nucleosil $C_{18}$ or $C_8$<br>25×0.46 cm | 0.1% Phosphoric acid<br>Acetonitrile<br>Gradient elution | UV: 216, 254, 275 nm |
| [57] | 22 diuretics (acidic, basic and neutral) | Hypersil ODS 5μm, 20×0.46 cm<br>Lichrosorb RP-18 5μm, 20×0.46 cm | 0.05 *M* sodium phosphate buffer,<br>0.016 *M* propylamine, pH3<br>Acetonitrile<br>Gradient elution | DAD: 230, 275 nm |
| [59] | 13 diuretics (acidic, basic and neutral) | Hypersil ODS<br>5 μm, 10×0.46 cm | Potassium phosphate buffer pH 6.8<br>Acetonitrile<br>Gradient elution | DAD: 220, 273, 328 nm |
| [73] | 9 diuretics (acidic, basic and neutral) | Ultrasphere ODS<br>5 μm, 25×0.46 cm | 0.05 *M* ammonium acetate or<br>phosphate buffer, pH 3<br>Acetonitrile<br>Gradient elution | DAD |
| [58] | 13 diuretics (acidic, basic and neutral) | Bondclone ODS<br>10 μm, 30×0.39 cm | 0.05 *M* sodium phosphate buffer,<br>0.016 *M* propylamine, pH 3<br>Acetonitrile<br>Gradient elution | DAD 230, 275 nm |
| [60] | 20 diuretics (acidic, basic and neutral) | Ultrasphere ODS<br>3 μm, 7.5×0.46 cm | 0.1 *M* ammonium acetate buffer, pH 3<br>Acetonitrile<br>Gradient elution | DAD: 240, 270, 290,<br>300, 318, 350 nm |
| [61,62,64,74] | 11 diuretics (acidic, basic and neutral) | Hypersil ODS<br>5 μm, 25×0.4 cm | 0.05 *M* sodium phosphate or acetate buffer,<br>0.016 *M* propylamine, pH 3<br>Acetonitrile<br>Gradient elution | DAD: 230, 254, 275 nm |

[a] UV: ultraviolet detector.
[b] DAD: diode array detector; UV spectrum of the peaks detected were stored in all cases.

a more selective and sensitive detection than UV detectors [69,77,78].

## 4. Gas chromatography

The polar nature of most of diuretics makes it difficult the direct analysis by GC and a derivatization step is needed to improve gas chromatographic behaviour of most of diuretics. Only spironolactone and canrenone can be directly analyzed by GC [59]. Silylation is the derivatization procedure mainly used in analytical methodologies of doping control [79–83], but its application to the analysis of diuretics is limited by the instability of the trimethylsilyl derivatives of sulphonamide functions [55]. Methylation is the procedure chosen for most authors to derivatize diuretics (Table 4). Three main methylation procedures have been described: extractive methylation, on column methylation or flash methylation, and methylation with methyl iodide in acetone.

### *4.1. Extractive methylation*

Extractive methylation involves the extraction of the organic acid as an ion pair into an organic solvent where the methylation reaction occurs. A quaternary ammonium salt is used as phase-transfer reagent to extract the organic acid from the alkaline aqueous phase into an aprotic reagent with low solvation power for anions containing the methylation reagent (methyl iodide).

Extractive methylation procedures optimized for the detection of chlorthalidone [84,88], furosemide [85], hydrochlorothiazide [86,89], bendroflumethiazide [87], diclofenamide [91], and bumetanide [92] have been described. Fagerlund et al. [90] proposed a methylation procedure to analyze eleven sulphonamidic diuretics, but its application to biological samples was only evaluated with acetazolamide. The application of extractive methylation to the analysis of fifteen diuretics in urine has been studied by Lisi et al. [93,94].

In the firsts procedures, extractive methylation was applied to an aqueous extract of the biological sample. Sample was extracted at appropriate pH with an organic solvent, in most cases, methyl-isobutylketone [84–89]; the organic phase was reextrated with an alkaline aqueous solution (0.1 *M* or 0.2 *M* sodium hydroxide solution). The extractive methylation was applied to this aqueous extract: the phase-transfer reagent (tetrahexylammonium hydroxide or hydrogensulphate solutions) and a solution of the methylation reagent (normally, methyl iodide in dichloromethane) were added to the aqueous phase and, in all cases, the phases were mixed at 50°C to achieve complete reaction.

Extractive methylation procedures applied to the dry residue obtained after complex extractions have also been described for diclofenamide [91] and bumetanide [92].

Fagerlund et al. [90] and Lisi et al. [93,94] performed extractive methylation directly to the urine sample. Fagerlund et al. [90] compared different phase-transfer reagents and best results were obtained using tetrahexylammonium hydroxide with an increase in the reaction temperature. Using methyl iodide in toluene, as methylation reagent, and reaction at room temperature, Lisi et al. [93] showed an increase of the derivatization efficacy when the hydrophillic character of the phase-transfer reagent was decreased.

The phase-transfer reagent leads to interferences during GC analysis and can cause a loss in the efficacy of the capillary column due to its pyrolysis to the corresponding amines in the injector [90,93,94]. Additionally, the presence of the phase-transfer reagent can lead to secondary derivatization reactions of the compound in the injector [90]. The elimination of this reagent is, thus, necessary. Evaporation of the organic solvent used for extractive methylation and redisolution of the methyl derivatives in an apolar solvent with low solubility for quaternary ammonium salts, as cyclohexane [89], hexane [85,88] or mixtures of toluene and hexane [86,87], have been used for this purpose. The elimination of the quaternary ammonium salts was also attained by washing the organic phase with saturated silver sulphate solution [93]. Lisi et al. [94] described an efficient clean-up procedure based on the solid-phase extraction of the organic extract obtained after extractive methylation using a macro reticular acrylic copolymer. Recoveries between 80 and 90% and detection limits of 10–50 ng were obtained using the later procedure.

Table 4
Derivatization procedures and detection systems used to analyze diuretics by gas chromatography

| Reference | Compounds | Sample | Derivatization | Detection[a] |
|---|---|---|---|---|
| [84] | Chlortalidone | Plasma, Serum, Urine | Extractive methylation | ECD |
| [85] | Furosemide | Plasma | Extractive methylation | ECD |
| [86] | Hydrochlorothiazide | Plasma, Erythrocyte, Urine | Extractive methylation | ECD, FID |
| [87] | Bendroflumethiazide | Plasma | Extractive methylation | ECD |
| [88] | Chlorthalidone | Plasma, Erythrocyte, Urine | Extractive methylation | NPD |
| [89] | Hydrochlorothiazide | Plasma | Extractive methylation | ECD |
| [90] | 11 sulphonamide diuretics | Reference solutions | Extractive methylation | FID, ECD, MS (EI) |
| [91] | Diclofenamide | Serum | Extractive methylation | MS (EI) |
| [92] | Bumetanide | Urine | Extractive methylation | MS |
| [93] | 15 diuretics | Urine | Extractive methylation | MS (EI) |
| [94] | 15 diuretics | Urine | Extractive methylation | MS (EI) |
| [95] | 16 diuretics | Urine | Extractive methylation | MS (CI) |
| [96] | Clopamide, indapamide | Reference solutions | Extractive methylation | MS (EI, CI) |
| [6] | Bumetanide | Urine | Flash methylation | FID |
| [97] | Hydrochlorothiazide | Blood, Plasma | Flash methylation | ECD |
| [98] | Acetazolamide | Blood, Plasma, Saliva | Flash methylation | ECD |
| [99] | Chlorthalidone | Urine | Flash methylation | FID |
| [100] | Acetazolamide | Blood, Saliva | Flash methylation | MS (EI) |
| [101] | Bumetanide, furosemide, etacrynic acid | Urine | Flash methylation | MS (EI) |
| [55] | Furosemide, clopamide, other diuretics | Urine | Methylation with $ICH_3$ in acetone | MS (EI) |
| [59] | 9 diuretics | Urine | Methylation with $ICH_3$ in acetone | MS (EI) |
| [102] | 10 diuretics | Reference solutions | Methylation with $ICH_3$ in acetone | MS (EI) |
| [78] | Furosemide | Urine, Serum | Methylation with $ICH_3$ in acetone | MS (EI, CI) |
| [58] | Hydrochlorothiazide | Urine | Methylation with $ICH_3$ in acetone | MS (EI) |
| [103] | Etacrynic acid | Plasma | Methylation (HCl/Methanol) | ECD |
| [104] | Etacrynic acid | Plasma | Acylation (pentafluorobenzyl bromide) | MS (CI) |
| [105] | Etacrynic acid | Plasma, Urine | Silylation (BSTFA[b]) | MS (EI) |
| [106] | Amiloride | Urine | Methanolysis and Silylation (MSTFA[c]) | MS (EI) |
| [107] | Amiloride | Urine | Silylation (MSTFA) | MS (EI) |

[a] ECD, electron capture detector; FID, flame ionization detector; NPD, nitrogen–phosphorus detector; MS, mass spectrometric detector; EI, electron impact ionization; CI, chemical ionization.
[b] BSTFA: N,O-*bis*(trimethylsilyl)trifluoroacetamide.
[c] MSTFA: N-methyl-N-trimethylsilyltrifluoroacetamide.

### 4.2. Flash methylation

Flash methylation has also been used to analyze diuretics in biological samples (Table 4). Procedures optimized for the detection of bumetanide [6], hydrochlorothiazide [97], acetazolamide [98,100], and chlorthalidone [99] have been described. Hagedorn and Schulz [101] described a single procedure for the simultaneous analysis of bumetanide, furosemide and etacrynic acid in horse urine. In all cases, the compounds were extracted from the biological sample using liquid–liquid procedures of different complexity depending on the compound and the biological matrix. The dry residue was redissolved in the methylation reagent, normally a quaternary ammonium hydroxide solution. Trimethylaniline hydroxide [97–100], tetramethylammonium hydroxide [98], or a mixture of both [6] have been described for this purpose. The reaction occurs in the injector of the gas chromatograph which is kept at high temperatures. Methyl derivatives similar to those described for other methylation procedures have been, in general, obtained. Feit et al. [6] and Hagedorn and Schulz [101] described the formation of the tetramethylderivatives of bumetanide, and bumetanide and furosemide, respectively, in contrast to the trimethylderivatives obtained using other methylation procedures.

### 4.3. Methylation with methyl iodide in acetone

This procedure consists of refluxing a dry acetone solution of the compound and the alkylating reagent (methyl iodide) with a mildly basic condensation reagent (dry potassium carbonate). This procedure, described for the analysis of barbituric acids [108], was first applied to the analysis of diuretics by Schänzer [55]. In addition to carboxylic acid and sulphonamide functions, this procedure allows the methylation of primary amine functions (as those of triamterene). Its main drawback is the long incubation time needed for some compounds: incubation at 60°C for 2–8 h has been described [55,58,59,78, 102,109]. Nevertheless, compounds with only carboxylic acid functions, such as etacrynic acid, can be methylated without incubation [107]. Detection limits of 20 ng in scan mode and 0.05 ng in selective ion monitoring mode have been reported [59]. Typical results obtained after GC–MS analysis of urines spiked with different diuretics subjected to extraction at alkaline pH with ethyl acetate and salting-out effect [60] and derivatized with methyl iodide in acetone are shown in Fig. 3.

Extractive methylation, flash methylation and methylation with methyl iodide in acetone have been recently compared by Carreras et al. [63]. These authors suggest methylation with methyl iodide in acetone as the best choice for screening purposes due to the derivatization of a great number of compounds. Extractive methylation and flash methylation are faster and more effective procedures for some of the compounds and can be of interest for confirmation purposes.

### 4.4. Other derivatization procedures

Derivatization of compounds with only carboxylic functions has been achieved using other procedures (Table 4). Methylation with hydrochloric acid in methanol [103], acylation with pentafluorobenzyl bromide [104] or silylation with N,O-*bis*-(trimethylsilyl)trifluoroacetamide [105] have been described for etacrynic acid.

Finally, the analysis by GC–MS of the potassium-sparing diuretic amiloride has been accomplished using a complex procedure based on a methanolysis followed by a silylation with N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) [106]. Amiloride can also be analyzed by GC–MS using an extraction with ethyl acetate at alkaline pH [60], followed by derivatization with MSTFA to form its tris-trimethylsilyl derivative [107].

### 4.5. Chromatographic separation and detection

Separation of diuretic compounds by GC has been performed with different stationary phases, methylsilicone and phenylmethylsilicone being the most widely used. Packed columns, initially used, have been replaced by cross-linked fused-silica capillary columns in the latest published methods. Electron capture detection was the most described in the initial studies; in the last years, due to the great diffusion of bench-top GC–MS instruments, mass spectrometric detection using electron impact ioniza-

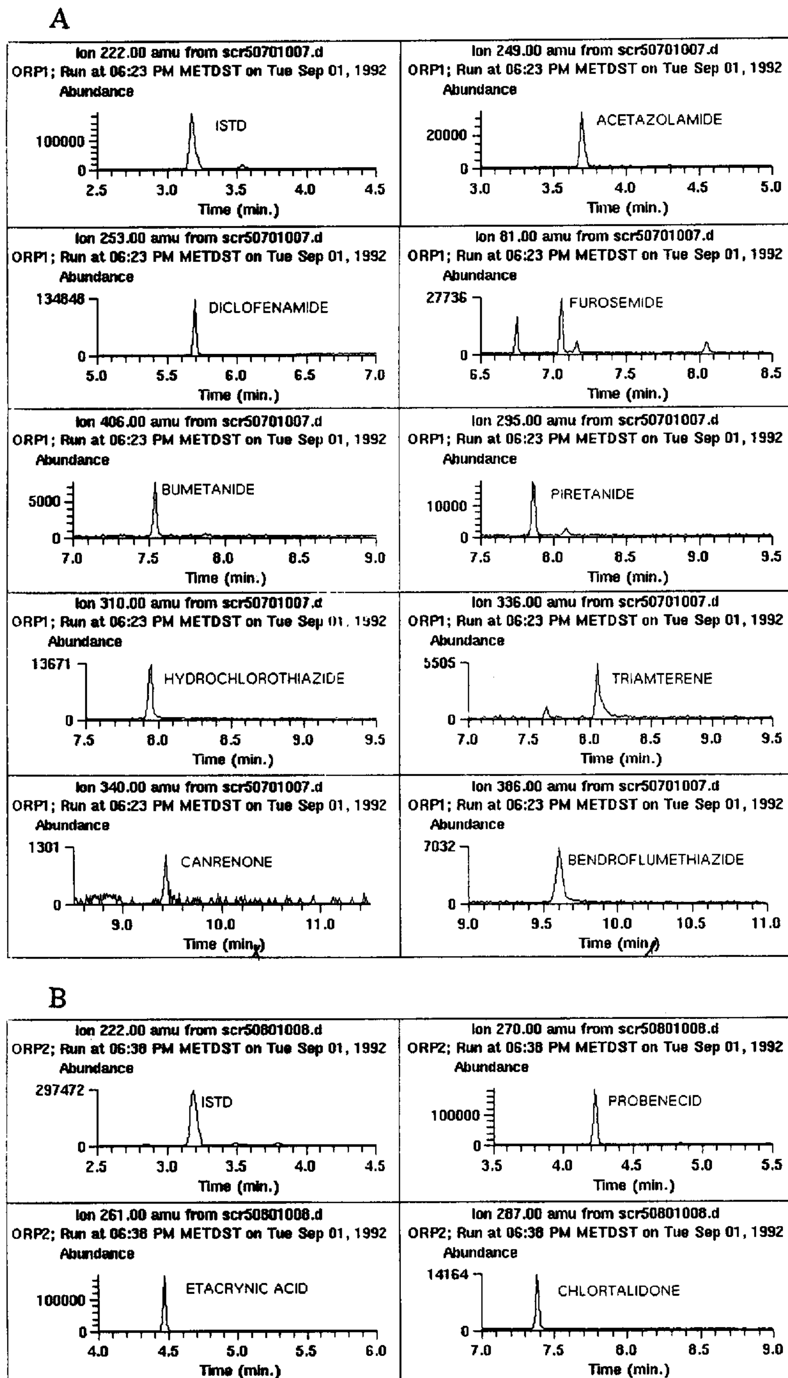


Fig. 3. Results obtained after analysis by gas chromatography–mass spectrometry: (A) Urine spiked with: 7-propyltheophylline (7-PT), ISTD (1 μg/ml); acetazolamide (5 μg/ml); diclofenamide and hydrochlorothiazide (0.5 μg/ml); furosemide, bumetanide, piretanide, triamterene, canrenone and bendroflumethiazide (0.2 μg/ml). (B) Urine spiked with: 7-propyltheophylline (7-PT), ISTD (1 μg/ml); etacrynic acid and chlortalidone (0.5 μg/ml) and probenecid (0.2 μg/ml). For chromatographic and sample preparation conditions see Refs. [60,107].

tion is the method of choice for most authors (Table 4).

## 5. Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) coupled to UV detection has also been studied for screening of diuretics [110,111]. Jumpannen et al. [110] developed a method to analyze diuretics in urine and blood serum, after solid-phase extraction. Owing to the heterogeneity of the family of diuretics, two consecutive runs at different pH values were required. Compounds containing sulphonamide and/or carboxylic groups were separated as anions using 3-(cyclohexylamino)-1-propanesulphonic acid buffer at pH 10.6. Diuretics with amino functions were separated at acidic pH as cations using an acetate–betaine buffer at pH 4.5. Under these conditions, diuretics can be screened in less than 30 min. The procedure was assayed with blank serum and urine samples spiked with diuretics.

González and Laserna [111] reported the electrophoretic migration parameters of diuretics and other drugs banned in sport using a borate buffer at pH 8. The procedure was only tested with urine samples of atenolol.

## 6. Mass spectrometry

Electron impact (EI) mass spectra of most diuretics obtained by direct introduction of the sample into the mass spectrometer have been reported in different mass spectral libraries [112]. Fragmentation profiles in EI conditions (70 eV) have been studied by Casy [113] using high resolution MS.

However, MS is normally used as detection system after gas or liquid chromatographic separations. As discussed before, GC–MS under EI conditions is widely used to analyze diuretics as their methyl derivatives and, in general, it has shown to be suitable for doping purposes in terms of sensitivity and selectivity of the mass spectra. The mass spectra of the methyl derivatives of diuretics have been described by different authors [55,63,90,93,94,102] and their fragmentation profiles have been proposed by Yoon et al. [102] by comparison with the deuterated methyl derivatives. GC–MS with EI ionization has also proved to be suitable to analyze the trimethylsilyl derivatives of amiloride in urine samples [106,107].

Using direct introduction into the mass spectrometer, negative ion mass spectra of several methylated diuretics have been studied [95,96]. In general, mass spectra with less fragment ions than in EI conditions have been obtained. For most of the compounds, no data are available regarding the sensitivity of GC–MS with negative ion chemical ionization (NICI) compared to EI ionization. However, for the methyl derivative of furosemide, NICI with ammonia has been found to be more sensitive than EI allowing a substantial improvement of the detection limit [78]. GC–MS CI with isobutane has been used to quantitate etacrynic acid in plasma as its pentafluorobenzyl derivative [104]; a mass spectrum with the molecular ion and no fragmentation was obtained.

The potential of LC–MS with a thermospray (TSP) interface for confirmation analysis of diuretics has been investigated by Ventura et al. [114]. Better ionization efficiency was obtained using discharge ionization mode than filament-off TSP ionization. The response in discharge ionization mode was found to be dependent on the mobile phase composition: higher signals were obtained with mobile phases containing water (acidified with trifluoroacetic acid) and methanol as main organic modifier, than with eluents containing an ammonium acetate solution (pH 2.1 with trifluoroacetic acid) and acetonitrile. Under these conditions, LC–MS was found to be sensitive enough to confirm most of the compounds in urine extracts, including benzthiazide which, as far as the authors know, can not be analyzed by GC–MS owing to the inability to form suitable derivatives. The analysis of benzthiazide using LC–TSP–MS in filament-on mode has been studied by Kim et al. [115]; an improvement in the response was observed when different organic acids were added to the LC eluent.

TSP positive ion mass spectra of diuretics using discharge ionization were characterized by a protonated molecular ion with little fragmentation [114]. The coupling of this technique with tandem MS could offer more structural information suitable for confirmation purposes in doping control.

The application of LC–MS using a particle-beam

(PB) interface to the analysis of diuretics has also been studied [107]. PB interface is compatible with conventional ion sources and, thus, conventional ionization modes can be employed. Although selective EI mass spectra similar to those reported using direct introduction have been obtained, sensitivity in EI conditions was not suitable to analyze diuretics in urine. NICI with methane was found to be more sensitive due to the electrophilic nature of diuretics. The sensitivity achieved using NICI allowed the detection of most of the compounds in actual urine extracts. NICI mass spectra showed a molecular ion resulting from an electron capture and more fragment ions than in TSP-discharge ionization.

Fast atom bombardment (FAB) has been applied to the analysis of diuretics after thin layer chromatographic separation [116]. Only amiloride yielded a useful positive ion FAB mass spectrum; for the other compounds the negative ion mode had to be used. Mass spectra with the molecular ion and little fragmentation were described. The method was used to analyze reference solutions and results obtained after direct analysis of a urine sample positive to amiloride and hydrochlorothiazide were also reported.

## 7. Conclusions

The pharmacological group of diuretics includes compounds with a wide variety of chemical structures and important differences in physico-chemical properties, making the development of common screening procedures for all of the substances difficult. Moreover, the presence of polar functional groups in most of the compounds makes their analysis by GC not feasible without prior derivatization.

Liquid–liquid procedures have been mainly described to extract unchanged diuretics from the urine matrix. The extraction of all the compounds from the urine has been achieved using two simultaneous extractions at acidic and basic pH, or only one extraction at neutral or alkaline pH using salting-out effect.

Solid-phase extraction procedures have been also evaluated. Promising results have been obtained using Sep-Pak $C_{18}$ cartridges or octadecyl Bond-Elut cartridges, in spite of the wide differences in the acid-basic behaviour and in the polarity of different diuretics. Adsorption of the compounds on polystyrene resins has been successfully used for some authors before derivatization and GC–MS analysis.

Solid-phase extraction has proved to be a powerful tool for the analysis of diuretics in view of the possibilities of direct coupling with LC using column-switching techniques. Using this technique the sample preparation step is eliminated and, consequently, the sample throughput can be substantially increased. In the same sense, attempts to use direct introduction of the urine into the LC–UV system have been made using micellar LC but, at present, interferences due the sample matrix components and the long run times make this approach not suitable for screening purposes.

LC–UV using diode array detectors and GC–MS after methylation are the methods of choice to screen for diuretics in urine. LC has the advantage of circumventing the need for derivatization which is in most instances a time-consuming step, and offers a more comprehensive screening than GC–MS due to the unability to form suitable methylated derivatives for some of the compounds. Diode array detection improves the selectivity for identification of the compounds after LC separation. Reversed-phase separations and gradient elution using mobile phases containing an acidic aqueous buffer and acetonitrile have been usually described. Propylamine or an ammonium salt have been added to the acidic aqueous phase in order to improve the chromatographic behaviour of basic diuretics.

GC–MS under EI conditions is a more selective approach than LC–UV and, furthermore, the high degree of the standardization of this technique achieved in the last years, makes GC–MS one of the most widely used tools to screen for diuretics in urine. The need for derivatization is the main limiting factor. Methylation using different reagents is the method of choice for screening purposes. Derivatization with methyl iodide in acetone seems to be the most comprehensive methylation method, although extractive methylation or flash methylation are less time-consuming procedures.

The separation of diuretics using CZE has also been studied. Although this technique offers a high separation power, further research is needed to

evaluate the relevance of this approach when dealing with actual urine samples.

GC–MS using EI mode is also the most used technique for confirmation purposes. GC–MS with NICI has demonstrated to be a useful alternative for some compounds to improve sensitivity and selectivity. LC–MS analysis using PB and TSP interfaces have also proved to be suitable for confirmation of some diuretics, in terms of sensitivity, and is the only technique available to analyze by MS some of them. PB interface using NICI offers better possibilities than TSP discharge ionization taking into account the selectivity of the mass spectra. The potential of tandem MS coupled to these interfaces or the use of other LC–MS interfaces as electrospray for the analysis of diuretics has still to be investigated.

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