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Gas chromatographic–mass spectrometric analysis of the loop diuretic torasemide in human urine

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Abstract

A fast and reliable gas chromatographic–mass spectrometric (GC–MS) method for the identification and determination of the loop diuretic torasemide in human urine is described. The usefulness of different derivatization procedures and reagents was studied. Flash methylation using trimethylanilinium hydroxide was the most convenient and appropriate procedure. The optimal urine isolation method comprised alkaline liquid–liquid extraction with ethyl acetate. After evaporation of the organic layer to dryness, the solid residue was reconstituted in the derivatizing reagent and was directly injected into the GC–MS system. Samples were analysed in the multiple ion detection mode using electron impact ionization. No interferences from other urinary compounds were found. Torasemide gave rise to a derivative that was identified by GC with Fourier transform infrared detection. There was a 70±5% recovery of torasemide. The coefficient of variation was 5% at a concentration of 0.05 μg/ml. The method was used for the determination of torasemide in urine samples obtained from a healthy volunteer that had received a single, 10 mg dose of torasemide.

Keywords: Torasemide

1. Introduction

1-Isopropyl-3-(4-*m*-toluidinopyridine-3-sulphonyl)urea, torasemide, is the leader in the sulphonylurea class of high ceiling loop diuretics.

Diuretics are drugs that are used mainly in the treatment of hypertension and for some kinds of oedema, where the aim is to eliminate extracellular fluid. One adverse effect of loop diuretics is that they cause kaliuresis because of increased potassium excretion rates. Torasemide is more potent, natriuretic and more potassium-sparing than the most often used loop diuretic, furosemide [1,2]. Therefore, its use is favoured compared with the other loop diuretics.

Some evidence indicates that in low, once daily dosage, torasemide is effective in the treatment of hypertension. Its effects compare favourably with those of the thiazide group and with indapamide. In low dosage, the drug does not cause significant hypokalemia, elevation of blood sugar or lipid disorders [3].

Torasemide differs from other loop diuretics (i.e. bumetanide and furosemide) in its metabolic profile.

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Only 20% of the drug is excreted unchanged in the urine, with the remaining 80% being eliminated by metabolism in the liver [4]. This property accounts for some of the differences that occur with torasemide pharmacokinetics and response in disease states, when it is compared with other high ceiling diuretics.

The present trend is to use low doses of diuretics, in terms of the renal excretory effects of these substances, in the treatment of uncomplicated essential hypertension [5].

Diuretics belong to the class of drugs that are banned by the International Olympic Committee for use by athletes in Olympic events, due to their diluting and masking properties [6]. Hence, sensitive and reliable methods for their determination must be developed and made available.

The general approach to address illegal use of drugs includes two stages: screening analysis followed by confirmation of positive results. The International Olympic Committee has recommended two gas chromatographic–mass spectrometric analyses, using aliquots from two sample containers. Gas chromatography–mass spectrometry (GC–MS) is accepted as the most powerful technique for confirmation analyses [7].

GC–MS has emerged as a very useful technique for drug analysis. The coupling of GC with MS combines the high separation power of GC with the sensitivity and specificity of MS, making it the most suitable method for confirming the results obtained with other techniques. It can provide evidence for the presence of suspected drugs and it is useful for sensitive and selective quantitation. It is being used increasingly for screening itself, without any preliminary testing procedure [8].

Few methods were found in the literature for the determination of torasemide. These include electrochemical methods by differential pulse [9] and by adsorptive stripping voltammetry [10] as well as a few liquid chromatographic separations followed by photometric [11–14] or amperometric [15] detection. In all of the chromatographic methods reported, identification of the compound is based solely on the retention time, while GC–MS provides additional spectrometric information for the identification of the diuretic of interest.

The aim of this work was to develop a GC–MS method for the identification and quantitative determination of the loop diuretic torasemide in human urine samples.

2. Experimental

2.1. Chemicals, reagents and stock solutions

Torasemide was provided by Boehringer Mannheim and piretanide (used as the internal standard) was provided by Hoechst Ibérica. MethElute (trimethylanilinium hydroxide; TMAH; 0.2 mol/l in methanol) as well as all other derivatizing reagents, i.e. bis(trimethylsilyl)trifluoroacetamide (BSTFA), pentafluorobenzoyl chloride (PFBOCl), pentafluorobenzyl bromide (PFB-Br), 3,5-bis(trifluoromethyl)benzyl bromide (BTFMB-Br), heptafluorobutyric anhydride (HFBA), trifluoroacetic anhydride (TFAA), triethylamine (TEA) and iodomethane, were obtained from Pierce (Rockford, IL, USA). Methanol, acetone, sodium chloride, potassium carbonate and potassium hydroxide were from Merck (Darmstadt, Germany) and ethyl acetate was from Rathburn (Walkerburn, UK). Water was obtained from a MilliQ system (Millipore, Bedford, MA, USA).

Stock solutions of torasemide and piretanide were prepared in methanol.

2.2. Apparatus

Incubation of spiked samples as well as evaporation of solutions was carried out on a Pierce Reacti-Vap III (Rockford, IL, USA). Shaking was performed with an Edmund Bühler S.M. mechanical shaker (Netherlands). Samples were centrifuged using a Hettich Universal centrifuge (Dépex, Bilthoven, Netherlands).

2.3. Gas chromatography–mass spectrometry

All GC–MS analyses were carried out on a Hewlett-Packard HP5890 series II gas chromatograph (Palo Alto, CA, USA) directly coupled to a Finnigan MAT SSQ710 mass spectrometer (San Jose, CA, USA) equipped with a Digital 5000/25

workstation. Sample separation was performed on a DB-1701 capillary column (30 m×0.25 mm I.D., 0.25 μm film thickness) (J&W Scientific, Folsom, CA, USA). The column temperature was programmed from 40°C (held for 1 min) to 300°C at 20°C/min. Samples were injected in the splitless mode (45 s sampling time) at an injector temperature of 275°C. Helium was used as the carrier gas at a constant linear velocity of 40 cm/s. The mass spectrometer was operated in the electron impact (EI) mode. For structure elucidation, full-scan spectra were acquired from M_r 50 to 650 at a rate of 1 scan/s. Molecular mass information of the torasemide derivative was obtained from positive chemical ionization (PCI) mass spectra, with methane as the reagent gas. Quantitation was performed using EI with multiple ion detection (MID) at m/z 197 and 305 for torasemide and at m/z 295 for piretanide.

2.4. Gas chromatography–Fourier transform infrared spectroscopy (GC–FTIR)

GC–FTIR was used for identification of the torasemide derivatives. Separations were carried out on a Carlo-Erba MEGA 5160 gas chromatograph. The GC was equipped with a HP-5MS capillary column (Hewlett-Packard; 25 m×0.25 mm I.D., 0.25 μm film thickness). The injection volume was 1 μl. Splitless helium was used as the carrier gas. The temperature program was 40°C (held for 1 min), followed by a temperature increase of 20°C/min to 290°C, where it was maintained isothermally for 10 min. The column was connected to a fused-silica transfer line with an internal diameter of 150 μm. The transfer line was guided into the FTIR spectrometer by means of a stainless steel tube, heated at 250°C. The infrared spectrometer was a Digilab FTS-40 Fourier Transform instrument equipped with a Digilab Tracer cryotrapping GC interface and an SPC 3200 computer for data processing. Chromatograms were processed as the Gram–Schmidt plot and as six functional group chromatograms of pre-selected wave-number intervals. Spectra were recorded on-the-fly at a rate of 2 scans/s (4 scans co-added).

All spectra were recorded at an optical resolution of 8 cm^{-1}.

2.5. Derivatization procedure

Several chemical derivatization procedures were tested on a standard sample of torasemide (20 μl of 500 mg/l in methanol) evaporated to dryness under a stream of nitrogen. Residues were submitted to: (1) silylation with BSTFA (100 μl) in pyridine (100 μl) for 1 h at 60 °C; (2) acylation with PFBOCl (10 μl) in acetonitrile (50 μl) and TEA (10 μl) for a few seconds at room temperature followed by a wash step and extraction of the reaction products into ethyl acetate; (3) alkylation with PFB-Br (10 μl) in acetonitrile (1 ml), using solid anhydrous potassium carbonate (50 mg) as the catalyst, for 1 h at 90°C; (4) alkylation with PFB-Br (10 μl) in acetonitrile (50 μl) and TEA (10 μl) for a few seconds at room temperature. After that, the reaction mixture was washed with water (250 μl) and the reaction products were extracted into ethyl acetate (2 ml); (5) extractive alkylation with PFB-Br (10 μl) in phosphate buffer (2 ml, pH 7.4) and dichloromethane after shaking for 50 min in the presence of tetrahexylammonium hydrogensulphate (10 mM); (6) alkylation with BTFMB-Br (40 μl) in acetonitrile (100 μl), using solid anhydrous potassium carbonate (50 mg) as the catalyst, for 1 h at 90°C; (7) acylation with neat HFBA (100 μl) for 1 h at 60°C followed by washing of the reaction mixture with water (250 μl) and extraction of the reaction products in ethyl acetate; (8) acetylation with neat TFAA (100 μl) at 50°C for 1 h followed by evaporation to dryness under a stream of nitrogen and addition of diazomethane (0.5 ml) in diethylether. After 5 min at room temperature, the ether was removed and the solid residue was reconstituted in ethyl acetate; (9) methylation with iodomethane (20 μl) in acetone using anhydrous potassium carbonate (100 mg) for 4 h at 60°C; (10) flash methylation with TMAH (100 μl solution 0.2 M in methanol).

2.6. Extraction procedure for urine samples

The extraction of torasemide from urine is based on the alkaline liquid–liquid extraction described by Barroso et al. [15]. A 2-ml volume of urine was alkalinized to pH 10 with potassium hydroxide. To this solution, 20 μl of the internal standard (100 μg/ml piretanide) were added as well as 1.5 g of

NaCl and 4 ml of ethyl acetate. After the mixture had been shaken mechanically in a horizontal position at a frequency of 200 strokes min^{-1} for 10 min, the mixture was centrifuged at 1700 *g* and the organic layer was evaporated to dryness under a gentle stream of nitrogen at 40°C. The solid residue was dissolved in 100 μl of TMAH and 1 μl of this was injected into the GC–MS system.

3. Results

3.1. Optimization of the derivatization procedure

The polar nature of torasemide makes it impossible to determine this drug by direct GC without derivatization. Silylation, alkylation as well as acylation with different derivatizing reagents were investigated. In all cases, the derivatives were analyzed in full scan mode and the results were compared with a procedural blank analysis.

Since negative chemical ionization can provide high sensitivity for electrophilic compounds, some fluoride reagents, such as pentafluorobenzyl bromide, pentafluorobenzoyl chloride, trifluoroacetic anhydride and heptafluorobutyric anhydride, were tried first. In all cases, however, no derivatives of torasemide were detected. Finally, methylation procedures were used.

For this purpose two different reagents were used, i.e., iodomethane and TMAH. The procedure used for methylation with iodomethane was performed according to the official method for doping control analysis of diuretics [16]. With both reagents, two peaks that resulted from torasemide were observed in the chromatogram (Fig. 1). The yield with flash methylation was approximately three-times better than with iodomethane. In addition, the flash methylation method was less laborious. Hence, it was

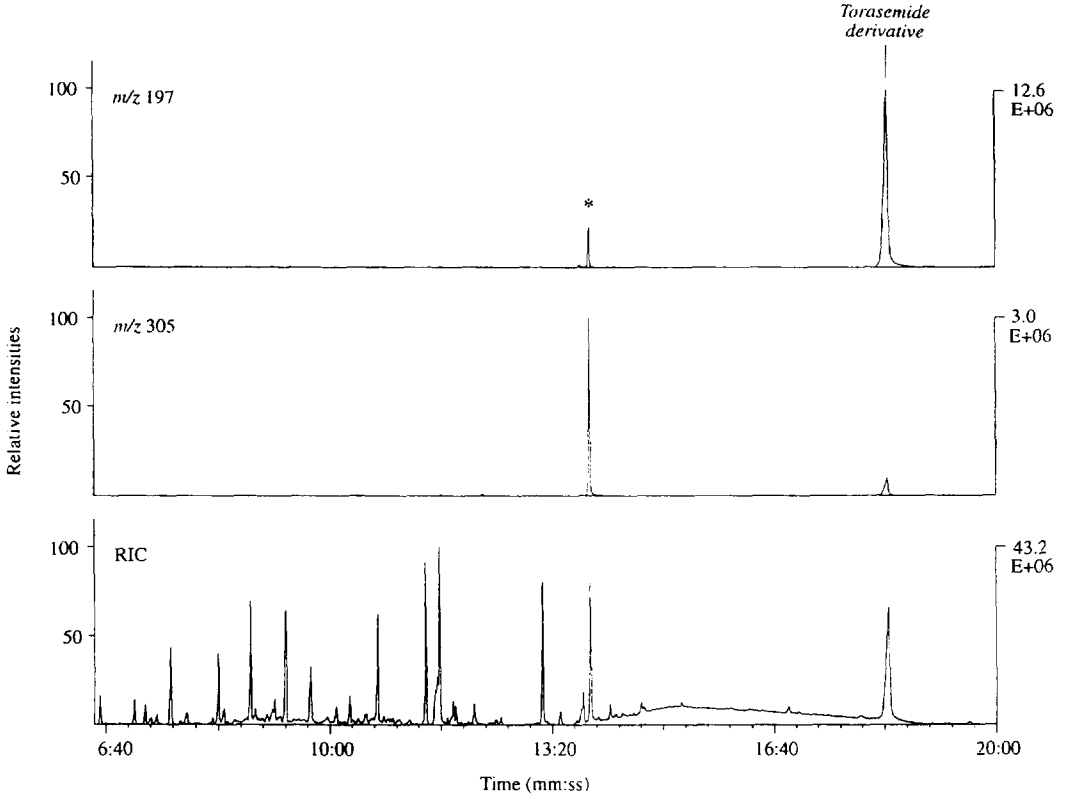

Fig. 1. Chromatograms corresponding to standard torasemide after flash methylation.

considered to be the best choice. When a solution of TMAH is heated in the injector port of a gas chromatograph in the presence of the drug containing extracts of urine, reactive amino and hydroxyl groups will be converted to the corresponding methyl derivatives. These volatile methylated compounds then pass through the chromatograph for separation and quantitation. The derivatization is based on the methylation of the groups containing N-bonded hydrogen atoms (Fig. 3).

Taking into account that chemical ionization often produces protonated molecular ions with high intensity and that less fragmentation occurs compared to that found with EI, this ionization method was used to elucidate the molecular mass of both products. Chemical ionization spectra were very similar for both derivatives, indicating that they have the same molecular mass.

Flash methylation of torasemide yielded two derivatives. EI mass spectra of both were quite similar, with only small differences in the intensity ratio for *m/z* 195 and 197, indicating the possible formation of two isomeric products. However, GC–FTIR analysis revealed that only one product (retention time 18:25 min) contained the secondary amide moiety (IR absorption at 1657 and 1569 cm^{-1}). This derivative has a molecular mass of 305 a.m.u., which corresponds to the loss of the $-NHCH(CH_3)_2$ group from torasemide during flash heating, accompanied by the simultaneous addition of a methyl group (Fig. 3, A_1).

The EI mass spectrum (Fig. 2A) shows an intense fragment ion at *m/z* 197, corresponding to the loss of the SO_2NHCHO moiety of the torasemide derivative. Furthermore, low intensity fragment ions are present at *m/z* 305 (M^{+}*) and at *m/z* 262 (i.e. loss of the secondary amide functionality, HN=C=O). This ion was used for quantitation of torasemide.

The other peak (with a retention time of 13:54 min) could be another derivative formed during methylation, the structure of which is proposed in Fig. 3, A_2. The molecular mass also would be 305 a.m.u., with main fragments at *m/z* 262 and 197.

After establishing the method for torasemide, a suitable internal standard had to be found. In general, it is recommended to use internal standards with chemical and physiological properties that are similar to the drug, forming analogous derivatives [17]. The method for torasemide was assayed with other loop diuretics and piretanide was found to be the most suitable. The EI mass spectrum of the trimethyl derivative of piretanide, formed after flash methylation (Fig. 2B), shows the molecular ion *m/z* 404 and an intense fragment ion at *m/z* 295 ($[M-109]^{+}$*), corresponding to the loss of $[SO_2N(CH_3)_2^{+}]$*. The gas chromatographic retention time for piretanide (18:05 min) is close to that for torasemide (18:24 min) but both compounds were well resolved.

3.2. Analytical results

Flash methylation was applied to human urine samples spiked with torasemide. A calibration curve determined for urine samples was linear over a wide concentration range, from 50 ppb to 5 ppm (Table 1).

Recovery was evaluated in fortified urine at two different concentration levels corresponding to those usually found in urine, i.e., 50 ppb and 1 ppm. Recoveries were (70±3)% and (72±3)% at 50 ppb and 1 ppm, respectively.

The reproducibility, evaluated at 50 and 500 ppb levels was good, with coefficients of variation ranging from 1.3 to 4.8% (Table 1).

Accuracy was calculated from urine samples spiked with a known concentration of torasemide and using the standard additions method. Results in Table 1 show that relative errors are lower than 7%.

The limit of quantitation of the method (defined as the amount of torasemide giving rise to a signal-to-noise ratio of 10) was 25 ppb in urine samples.

3.3. Urinary excretion studies

In order to demonstrate the applicability of the method developed, urine samples from a healthy volunteer that had received a single oral dose of torasemide (10 mg) were collected over a 24-h period at several time intervals and were analyzed.

The urine samples were treated as described in Section 2.6 and 1 μl of each sample was injected in the GC–MS system. In Fig. 4, chromatograms of a real urine sample and a blank urine sample are shown. The concentrations were calculated using the standard addition method. Results corresponding to

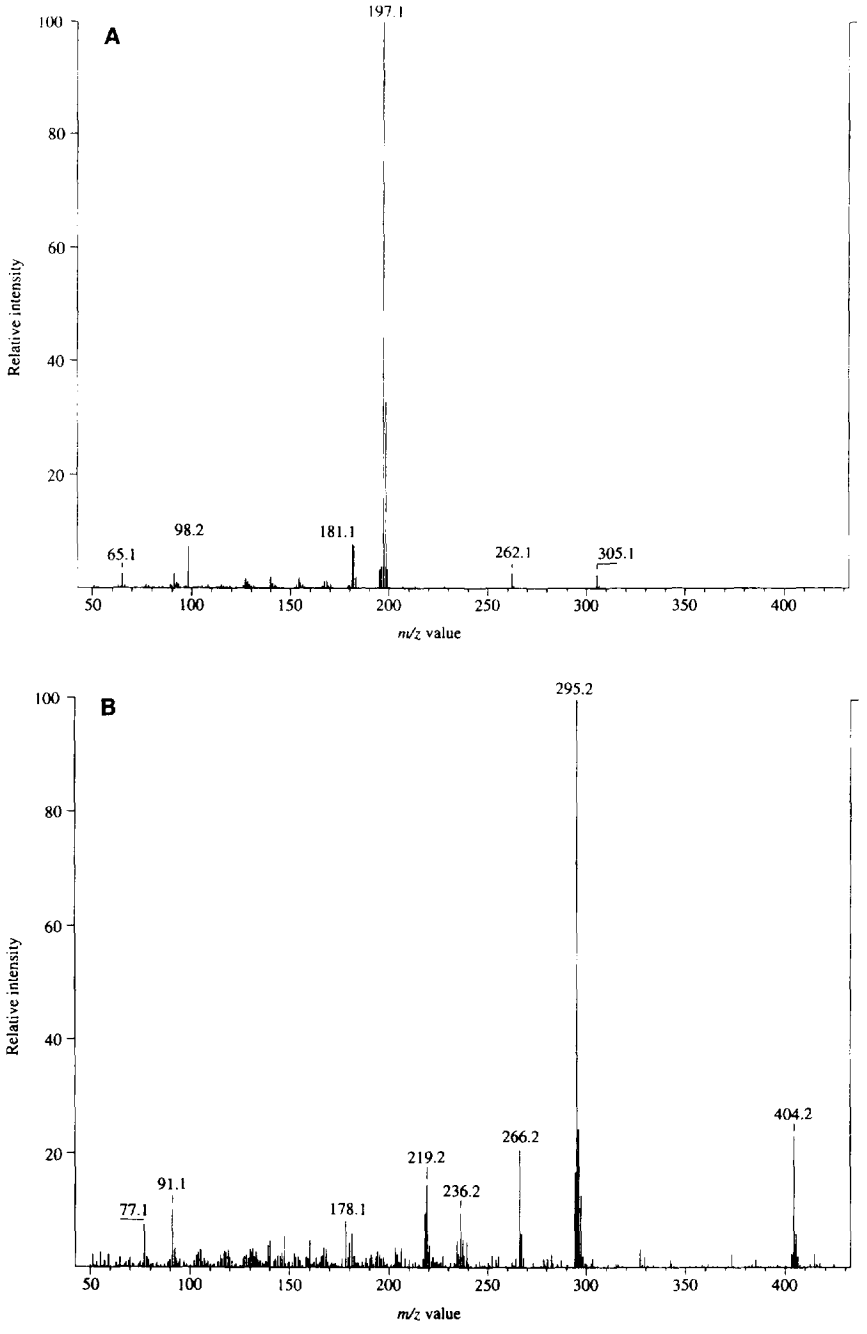

Fig. 2. EI mass spectrum of torasemide (A) and piretanide (B) after flash methylation.

the different time intervals are summarized in Table 2. As can be seen, the percentage of unchanged drug found in urine after 24 h (20.6%) is in accordance with the 20% value reported in the literature [4].

4. Discussion

GC coupled with MS using flash methylation has resulted in a fast, reliable and sensitive method for

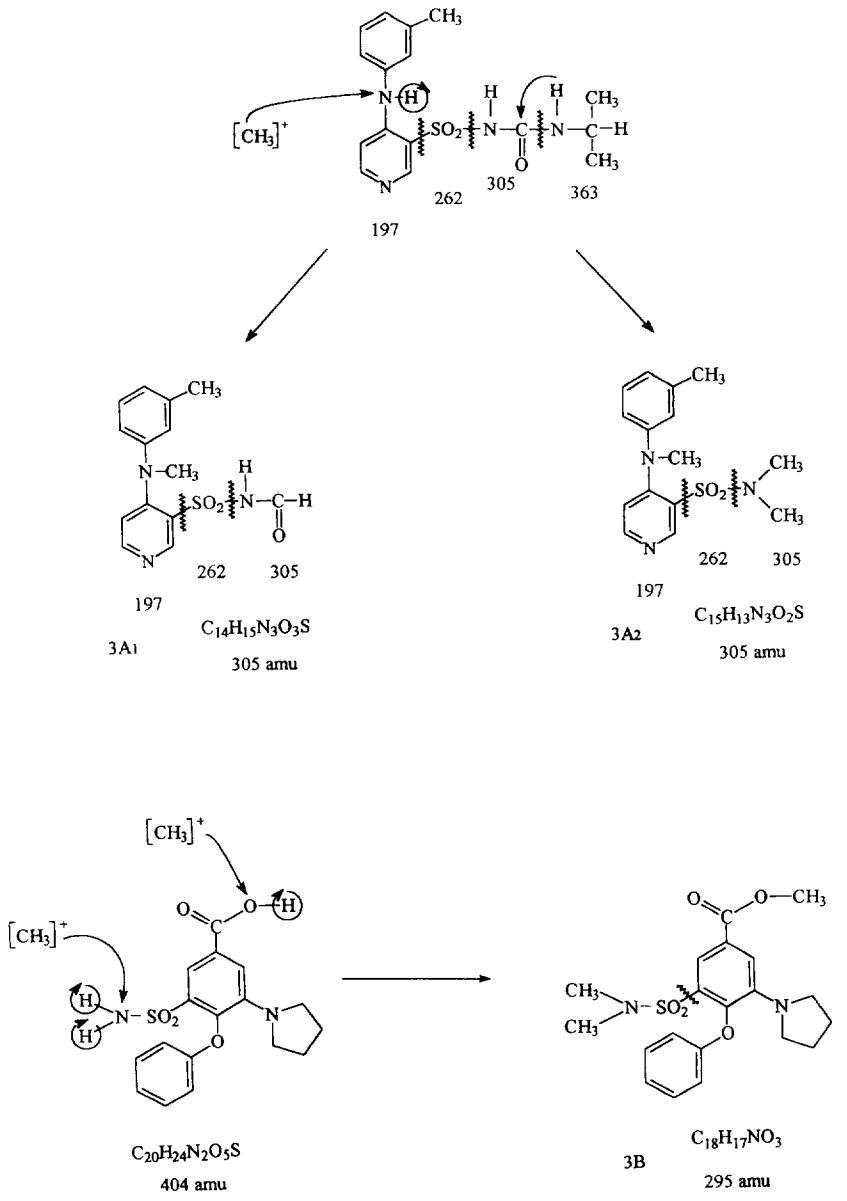

Fig. 3. Schemes for the proposed formation of torasemide (A1 and A2) and piretanide (B) derivatives at flash methylation in a hot GC injector.

quantitation and identification of torasemide in human urine.

The diuretic is readily identifiable, based on the characteristic mass fragment ion under EI ionization and the signal obtained was free from any interferences from other urinary compounds.

Although more than one derivative is formed, this is not a problem, since both are linear with torasemide concentration and can be useful for confirmation analyses.

The limit of quantitation was slightly higher than reported in a previous high-performance liquid chromatographic method [15], but it was sufficient for measuring the levels of torasemide usually found in

Table 1
Analytical characteristics of the GC–MS method used for torasemide determination

Linear concentration range in urine	Limit of quantitation (ng)[a]	Repeatability as R.S.D.		Accuracy	
		at 50 ppb	at 0.5 ppm	at 50 ppb	at 0.5 ppm
50 ng/ml–5 μg/ml	0.50	2.5%[b] and 4.9%[c]	1.3%[b] and 3.2%[c]	49.5±2.1	0.49±0.03

[a] as an absolute injection amount.
[b] for $n=10$ injections of one urine sample.
[c] for $n=10$ aliquots of the same urine sample.

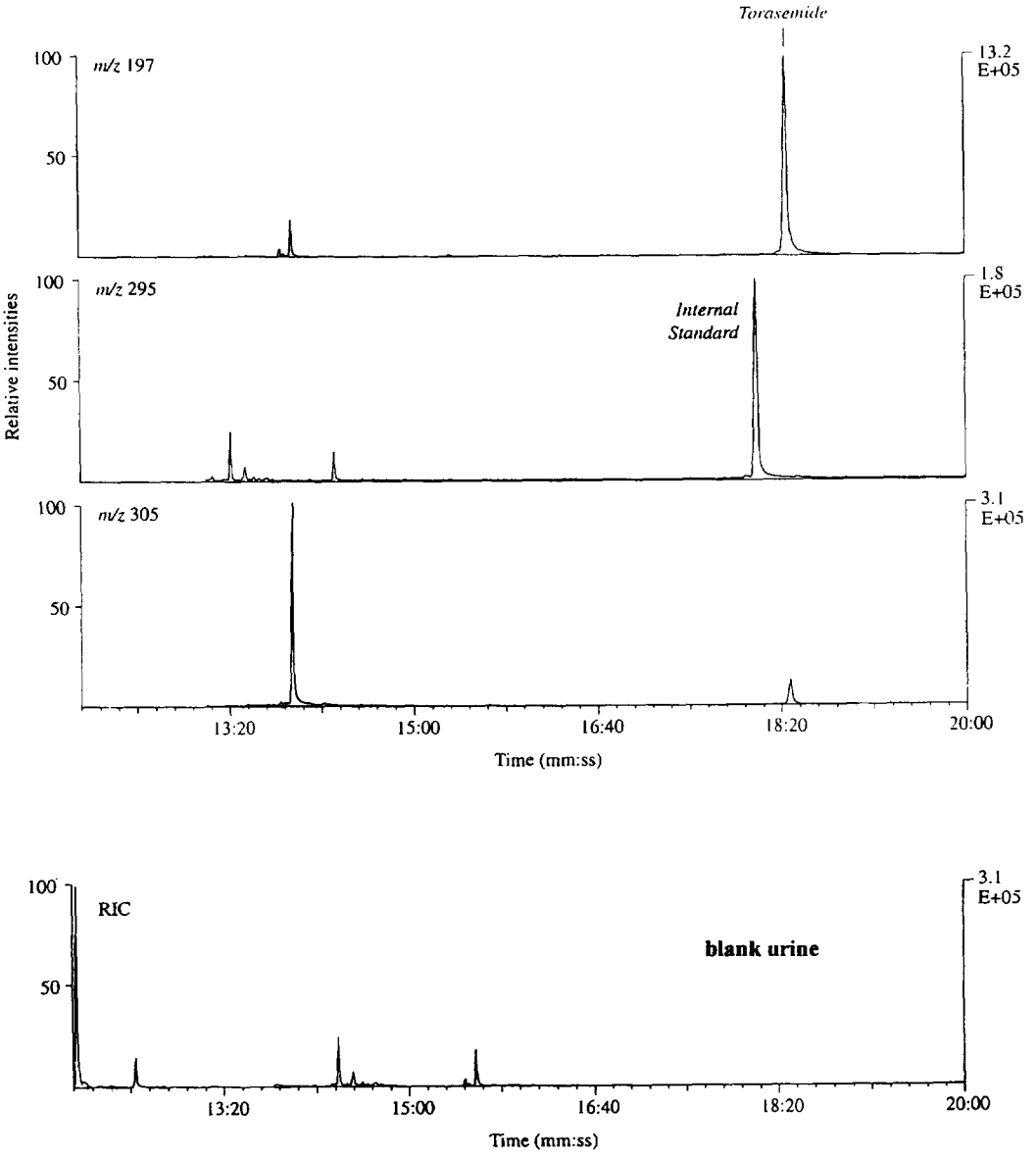

Fig. 4. Ion chromatograms of a real urine sample obtained from a healthy volunteer 2–8 h after ingestion of a single dose of 10 mg of torasemide, compared with a chromatogram obtained with a blank urine sample.

Table 2
Excretion data of torasemide after administration of a single oral dose (10 mg)

Interval	Δt	Urine volume (ml)	Concentration (μg/ml)	Amount (μg)
0–2	2	250	1.82	455
2–8	6	335	1.65	553
8–20	12	960	0.89	854
20–24	4	705	0.28	197
0–24	24	2250		2059

urine samples. In addition, the high specificity makes this technique adequate for confirmation analyses in urine samples for doping control purposes.

The good selectivity allows for the use of relatively large sample volumes or for the final sample extract to be dissolved in a very small volume of reagent, both resulting in enhanced sensitivity.

5. Acknowledgements

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