Journal of Chromatography, 564 (1991) 567–578
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5712

Rapid high-performance liquid chromatographic determination with fluorescence detection of furosemide in human body fluids and its confirmation by gas chromatography–mass spectrometry

MARTIAL SAUGY* and PHILIPPE MEUWLY

Unité d'Analyse du Dopage, Institut de Médecine Légale, Université de Lausanne, Rue du Bugnon 21, 1005 Lausanne (Switzerland)

A. MUNAFO

Laboratoire de Pharmacologie Clinique, Centre Hospitalier Universitaire Vaudois, 1005 Lausanne (Switzerland)

and

LAURENT RIVIER

Unité d'Analyse du Dopage, Institut de Médecine Légale, Université de Lausanne, Rue du Bugnon 21, 1005 Lausanne (Switzerland)

ABSTRACT

Furosemide (FD; Lasix®) is a loop diuretic which strongly increases both urine flow and electrolyte urinary excretion. Healthy volunteers were administered 40 mg orally (dissolved in water) and concentrations of FD were determined in serum and urine for up to 6 h for eight subjects, who absorbed water at a rate of 400 ml/h. Quantification was performed by HPLC with fluorescence detection (excitation at 233 nm, emission at 389 nm) with a limit of detection of 5 ng/ml for a 300-μl sample. The elution of FD was completed within 4 min using a gradient of acetonitrile concentration rising from 30 to 50% in 0.08 *M* phosphoric acid. The delay to the peak serum concentration ranged from 60 to 120 min. FD was still easily measurable in the sera from all subjects 6 h after administration. In urine, the excretion rates reached their maximum between 1 and 3 h. The total amount of FD excreted in the urine averaged 11.2 mg (range 7.6–14.0 mg), with a mean urine volume of 3024 ml (range 2620–3596 ml). Moreover, the urine density was lower than 1.010 (recommended as an upper limit in doping analyses to screen diuretics) only for 2 h. An additional volunteer was administered 40 mg of FD and his urine was collected over a longer period. FD was still detectable 48 h after intake. Gas chromatography–mass spectrometry with different types of ionization was used to confirm the occurrence of FD after permethylation of the extract. Negative-ion chemical ionization, with ammonia as reactant gas, was found to be the most sensitive method of detection.

INTRODUCTION

Furosemide (FD; Lasix®) is the most widely used loop diuretic for the treatment of oedema and hypertension, enhancing renal excretion of salt and water

0378-4347/91/$03.50 © 1991 Elsevier Science Publishers B.V.

and lowering the blood pressure. The pharmacokinetics and pharmacodynamics of FD have recently been extensively reviewed [1]. The inter- and intra-individual variability of the bioavailability of FD is also known [1,2].

Recently, diuretics have been misused and abused in sports where weight categories are involved, such as weightlifting, wrestling and boxing, in order to reduce body weight rapidly. Diuretics could be also used to decrease the urine concentration of doping agents in order to avoid their detection. It is known that the administration of high-ceiling diuretics such as furosemide reduces by four- of five-fold the range of the urinary concentration of doping agents [3–5]. Diuretics have also been used to control water retention, one of the most frequent adverse effect of anabolic steroids [6]. Therefore, the Medical Commission of the International Olympic Committee has banned diuretics since the 1988 Olympic Games.

Several methods have been reported for the separation, detection and quantification of FD in body fluids, including thin-layer chromatography (TLC) [7], high-performance liquid chromatography (HPLC) [8–16] and gas chromatography–mass spectrometry (GC–MS) [17–20].

In doping analyses, an abused drug has to be identified by its mass spectrum after a GC separation, and for this purpose FD must be derivatized. Because of its carboxylic function and sulphonamide group, furosemide can be trimethylated with methyl iodide [17].

The aim of this work was to develop a reliable and reproducible HPLC method for the quantification of furosemide that is rapid and sensitive enough for pharmacokinetic studies in serum and urine. Different ionization modes were tested to achieve the most sensitive GC–MS detection allowing the confirmation of the occurrence of FD in urine more then 6 h after intake.

EXPERIMENTAL

Reagents

Acetonitrile was of HPLC grade (Merck, Zurich, Switzerland). Ethyl acetate, phosphoric acid, anhydrous potassium carbonate and iodomethane were of analytical-reagent grade (Merck). Water was doubly distilled. Furosemide and warfarin (internal standard) were purchased from Sigma (Chemie Brunschwig, Basle, Switzerland). The structures of furosemide and its trimethyl derivative are shown in Fig. 1.

Clinical studies

Eight healthy male volunteers received in the morning 40 mg of FD orally (dissolved in water, 10 ml/kg) and drank 400 ml of water per hour throughout the experiment. Blood (10 ml) and urine were collected (blood every 30 min up to 2 h, then every hour; urine every hour) for up to 6 h. Blood was centrifuged and serum and urine were stored at −20°C until extraction.

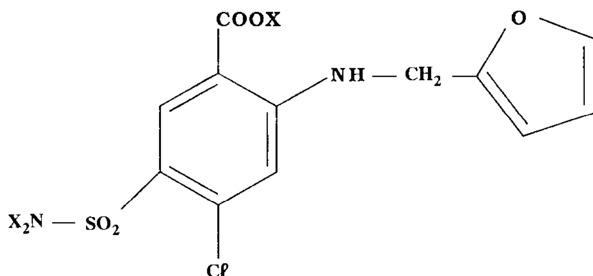

Fig. 1. Structures of furosemide (X = H) and its trimethylated derivative (X = CH_3).

In a second experiment, an additional male volunteer was administered 40 mg of FD dissolved in 100 ml of water and his urine was collected during the following 96 h. During the first 5 h, this volunteer drank only 100 ml of water in order to mimic a rapid loss of weight.

Urine density was measured with a table clinical refractometer (Atago 2, Müller & Krempel, Bülach, Switzerland).

Sample preparation

This extraction procedure was adapted from Straughn *et al.* [13] and Rapaka *et al.* [9]. To 300 μl of serum or urine, 50 μg of warfarin [WA; 4-hydroxy-3-(3-oxo-1-phenylbutyl)coumarin] in methanol as internal standard, 500 μl of acetonitrile and 500 μl of ethyl acetate were added to the samples. For the calibration graphs, different amounts of FD (as a methanolic solution) were added at the beginning of the procedure to blank serum or blank urine. The sample mixture was vortexed for 10 s after each addition and then centrifuged (10 min, 2370 *g*). After freezing, the supernatant was collected and evaporated to dryness at 45°C under vacuum. The residue was resuspended in 150 μl of acetonitrile–0.08 *M* phosphoric acid (90:10) and 20 μl were injected automatically into the HPLC system.

HPLC conditions

The extract was eluted through a 150 mm × 3.9 mm I.D. C_{18} reversed-phase column, particle size 4 μm (Nova-Pak; Waters Assoc., Milford, MA, U.S.A.) and the effluent was detected with a fluorescence monitor (HP 1046A; Hewlett-Packard, Palo Alto, CA, U.S.A.) with excitation at 233 nm and emission at 389 nm. Gradient elution was performed with a Perkin-Elmer Series 4 HPLC system and using acetonitrile and 0.08 *M* phosphoric acid as shown in Table 1.

GC–MS identification

Furosemide, with a carboxylic function and sulphonamide group, has to be derivatized in order to show good GC properties. This was achieved by the following procedure, adapted from Dünges and Bergheim-Irps [21] and Donike [22].

TABLE I

SOLVENT GRADIENTS USED FOR ELUTION OF FUROSEMIDE AND WARFARIN

Sector (No.)	Time (min)	Flow-rate (ml/min)	Acetonitrile (%)	0.08*M* H_3PO_4 (%)
Equil.	1	1	30	70
1	1	1	30	70
2	0.5	2	35	65
3	1.5	2	50	50
4	1	2	50	50
5	2	1	30	70
6	1	1	30	70

The volume was reduced by evaporation (45°C, nitrogen) to 1 ml, and 1 ml of acetronitrile plus 1 ml of ethyl acetate were added to the residual phase. The mixture was vortex-mixed for 10 s and the organic layer was removed and evaporated to dryness (45°C, nitrogen). Anhydrous potassium carbonate was added to the residue, which was dissolved in 100 μl of a 10% iodomethane solution in acetonitrile. The mixture was heated at 60°C for at least 3 h, and after cooling 1 μl of the supernatant was injected into the GC–MS system.

The GC–MS system consisted of a Hewlett-Packard Model 5988 A mass spectrometer, with a combined electron impact–chemical ionization (EI–CI) source coupled with a Hewlett-Packard Model 5890 gas chromatograph. Splitless injections of 1 μl were made onto an HP-1 bonded fused-silica capillary column (12 m × 0.2 mm I.D.), film thicknes 0.33 μm. Typical GC conditions were injector temperature 260°C and oven temperature 150°C for 1 min, then programmed at 20°C/min to 300°C and maintained at that temperature for 3 min. Helium was used as the carrier gas at a flow-rate of *ca.* 1 ml/min. The mass spectrometer ion source temperature was 200°C and the ionization potential was 70 eV for EI and 150 eV for CI. The pressures of gases for CI were adapted in order to obtain the highest signal from the calibrator (benzophenone).

RESULTS AND DISCUSSION

Chromatography

Fig. 2 shows typical HPLC chromatograms of serum and urine samples with the internal standard (I.S.) warfarin. The total run time for each sample was 7 min (+ 1 min equilibration; see Table I) in which FD and I.S. were resolved with retention times of 3.9 and 6.3 min, respectively.

The FD concentration in a sample was determined by comparing the peak-area ratio of furosemide to warfarin (I.S.) with a calibration graph of peak-area ratio *versus* FD concentration. These curves were established with blank serum and urine samples spiked with increasing amounts of FD and analysed by the

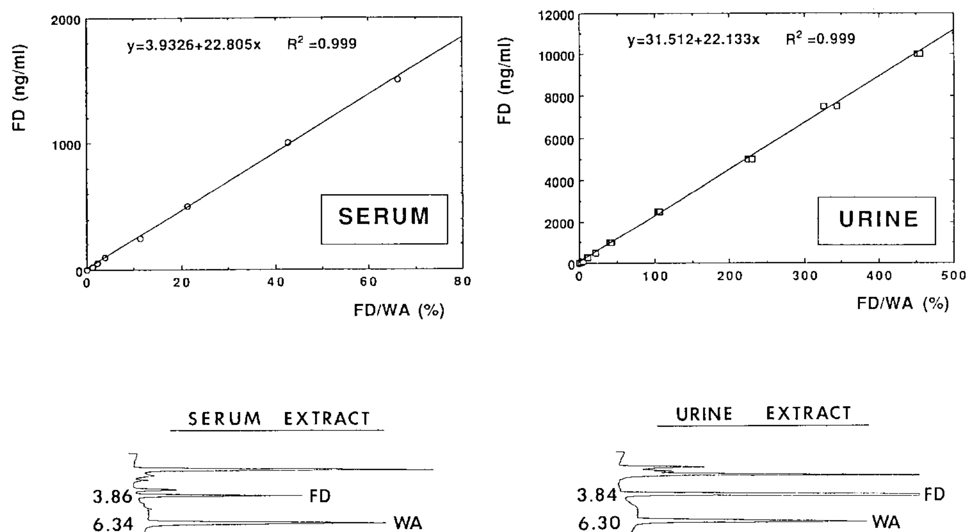

Fig. 2. Correlation graphs and typical HPLC traces for serum and urine. The range of linearity for serum was 10–1500 and for urine 50–10 000 ng/ml FD with a starting sample volume of 300 μl. FD = Furosemide; WA = warfarin.

same procedure. A good linear relationship was found for FD concentration ranges of 10–1500 and 50–10 000 ng/ml for serum and urine, respectively (Fig. 2).

The recovery with the extraction procedure was between 85 and 90% for both serum and urine. The limit of detection (five times the baseline noise) of the method was 10 ng/ml for both types of sample (3 ng in 0.3 ml). The intra-assay relative standard deviation varied between 1.0 and 1.1% for urine and between 3.8 and 4.0% for serum over an FD concentration range of 0.071–10.8 μg/ml.

FD in serum

Data obtained from only four significant subjects are presented in some instances in order to interpret the results more easily. These subjects were chosen because they were representative of the variability found in the eight-volunteer population.

The peak serum FD concentration was reached 90–120 min after intake, as can be seen in Fig. 3. Maximum FD concentration ranged from 604 ng/ml for the lowest after 120 min (subject 3) to 1506 ng/ml for the highest after 90 min (subject 4), which represents a variation of 1:2.5.

FD in urine

The FD excretion rate in urine exhibited the same kind of variability (Fig. 4). It ranged from 2.7 mg/h after 3 h (volunteer 3) to 5.6 mg/h after 2 h (volunteer 4). Cumulative data for FD in urine (Fig. 5) show that after 6 h subject 4 had

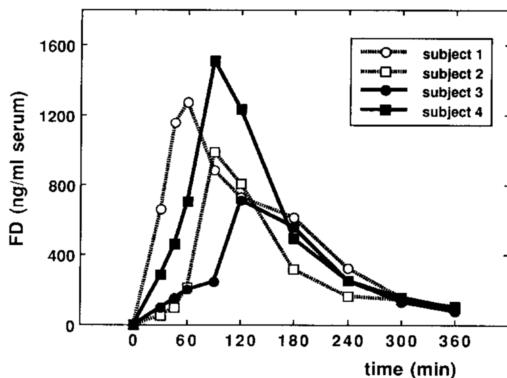

Fig. 3. Serum concentration of FD (ng/ml) expressed as a function of time for four volunteers. Measurements of FD were made by HPLC with fluorescence detection.

excreted 14 mg of unchanged FD (35% of the intake), whereas subject 2, after the same time had only eliminated 7.6 mg (19% of the intake).

The volume of excreted urine also showed some varations between volunteers (Fig. 6). In that case also, subject 4 excreted the maximum volume of urine after 2 h with the highest value of the group (1030 ml), whereas subject 3 excreted 746 ml after 3 and 4 h. Cumulative urine volumes after 6 h ranged from 2620 to 3596 ml.

These kinetic data show that, for almost all the subjects, there is a shift of 30–60 min between the FD serum peak and the maximum rate of FD excretion in urine. The maximum FD excretion rate virtually corresponds to the maximum urine excretion. However, there is a non-negligible variability between individuals for the time of peak serum concentration, the value of this peak concentration and the FD excreted in urine. This variability between subjects has been studied by Granhén *et al.* [2], who showed that the range of bioavailability between subjects was 20–84% and also the maximum difference within one subject was 20

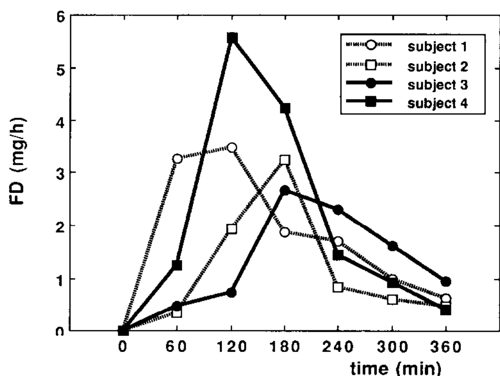

Fig. 4. Urine excretion rate of FD (in mg/h) as a function of time. Each point corresponds to the total amount of FD found in each collection of urine. Same volunteers as in Fig. 3.

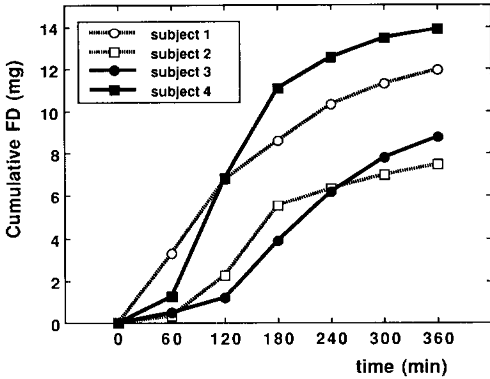

Fig. 5. Cumulative FD (mg) in urine as a function of time. Same volunteers as in Fig. 3.

to 61% (four assays), *i.e.*, three-fold, whereas the maximum difference in the amount of furosemide excreted in urine was two-fold. This intra-subject variability was mainly attributed to the absorption process.

Density: good criterion for suspect urine?

A urine density of 1.010 is often taken as the upper limit for suspecting an athlete to have been doped with diuretics. The data in Fig. 7 show that this was the case only for a short period of time in our experiment. Before FD intake (time 0), the density of urine from two volunteers was already $\leqslant 1.010$; after 1 and 2 h, this was the case for all eight subjects; after 3 and 4 h, two urines were still suspect and later all urine densities were > 1.010. These results demonstrate that this density criterion is not usable in doping controls; it makes no sense to use diuretics 2 h before a competition or weighing.

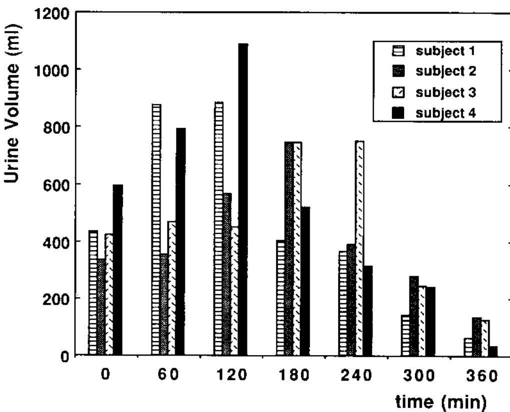

Fig. 6. Urine volume (ml) as a function of time.

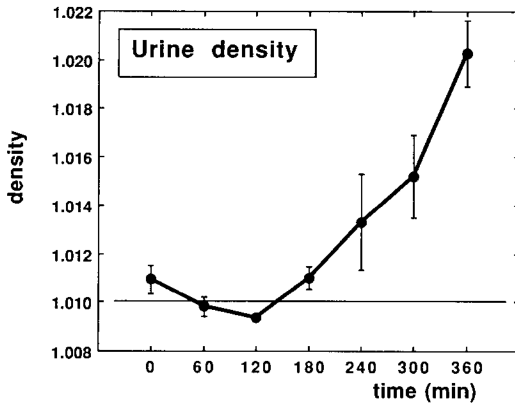

Fig. 7. Mean urine density (± standard error) expressed as a function of time. The horizontal line corresponds to the limit 1.010.

Long-term detection

It was considered of interest to detect FD in urine over periods longer than 6 h. An additional volunteer was administered 40 mg of FD dissolved in water and during the first 5-h period he did not receive any water. The data showed (Fig. 8) that when using the same HPLC–fluorescence detection method of quantification with 300 μl of sample the maximum FD concentration in urine occurred after 5 h and reached 160 ng/ml after 48 h. Traces of FD were still present 52 h after the intake, without any possibility of quantification, and later, even after increasing the volume of the urine sample to be extracted, FD was not detectable. The maximum rate of FD excretion and the maximum volume of urine (data not shown) occurred between 2 and 3 h. The urine density was < 1.010 at 1.5, 2 and 3 h and finally, after 6 h, the cumulative urine volume was 1950 ml.

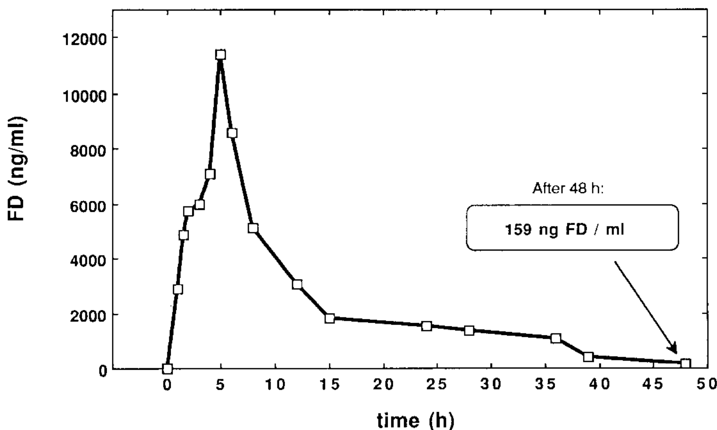

Fig. 8. Urine FD concentration (ng/ml) as a function of time (h). A single volunteer took 40 mg of FD at time 0 and urine was collected for 96 h. After 52 h, no quantification was possible.

These results lead to the conclusion that FD could be easily detectable when using this HPLC method for a much longer period than when the urine density is $\leqslant 1.010$.

GC–MS confirmation

The identification of an abused drug has to be confirmed by a mass spectrum, after a GC separation. In this instance, the carboxylic and sulphonamine groups of FD were methylated with methyl iodide in acetonitrile. The EI mass spectrum (Fig. 9A) of the trimethyl derivative has already been published [12]. It shows a base peak (m/z = 81) which represents the furan ring after scission of the nitrogen–carbon bond. The molecular ion (m/z = 372) intensity is less than 15% of the base peak and an isotope peak (m/z = 374) can also be detected with 38–40% intensity of the molecular ion, typical of a chloride atom in a molecule.

EI ionization did not allow the confirmation of the presence of FD found in

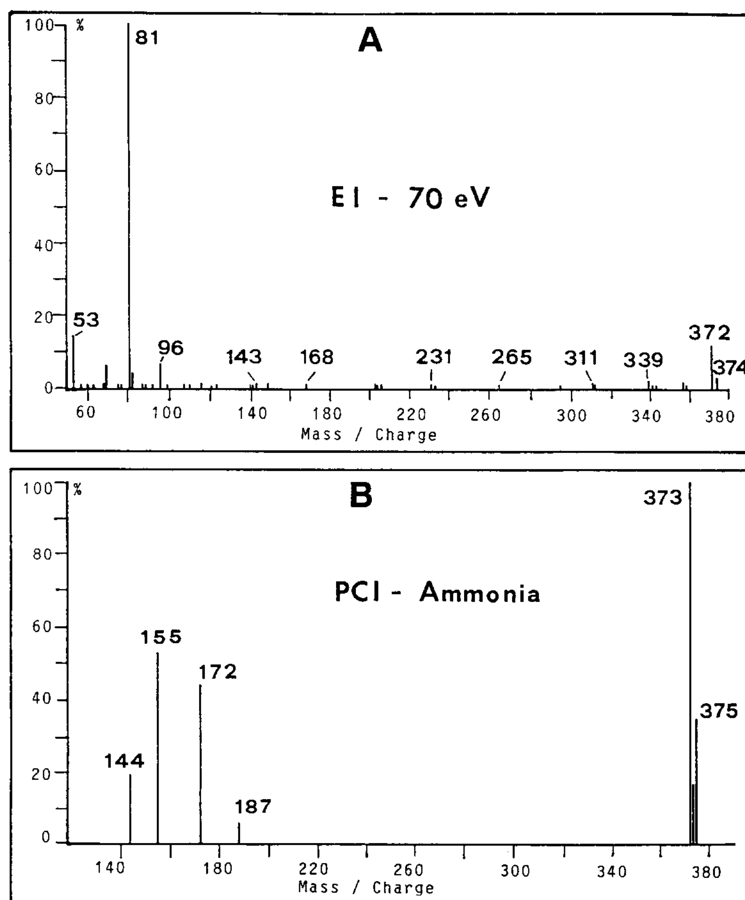

Fig. 9.

(Continued on p. 576)

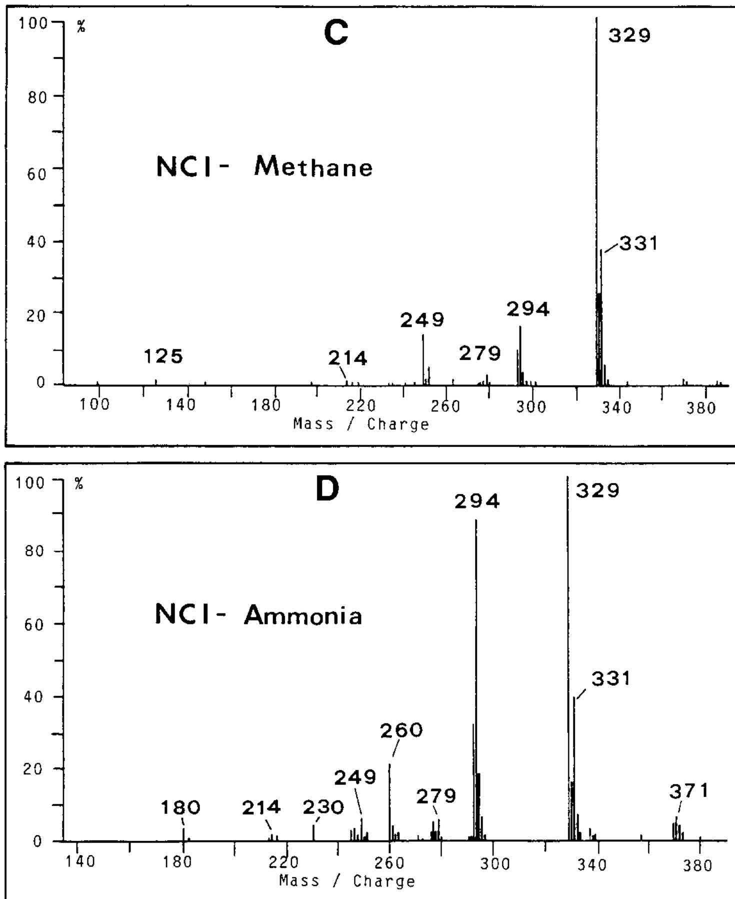

Fig. 9. Mass spectra of the trimethyl derivative of FD (5-ng injection in each instance. (A) Electron-impact mode; (B) positive-ion chemical ionization with ammonia as reactant gas; (C) negative-ion chemical ionization with methane as reactant gas; (D) negative-ion chemical ionization with ammonia as reactant gas. For GC–MS conditions, see Experimental.

urine after 24 h and later, because the signal-to-noise ratio was too low. To establish better conditions of sensitivity, other types of ionization were tried. Mass spectra of the same derivative obtained with different types of chemical ionization are shown in Fig. 9B, C and D.

Positive-ion CI (PICI) with ammonia as reactant gas gave a spectrum with good fragmentation properties. The base peak is at *m/z* 373, $[M+1]^+$, *m/z* 375 being its isotope ion.

Negative-ion CI (NICI) was performed with methane (Fig. 9C) and ammonia (Fig. 9D). Methane produced less fragmentation of the molecule with a lower signal at *m/z* 294 and no signal at *m/z* 260. The intensity of the base peak (*m/z* 329) with methane was 72% (data not shown) of that of the same fragment with ammonia.

TABLE II

EFFECT OF IONIZATION MODE ON THE FRAGMENTATION AND INTENSITY OF THE RESPONSE WHEN 5 ng OF FUROSEMIDE TRIMETHYL DERIVATIVE WERE INJECTED

Ionization mode	Peak (m/z)	Area response	Ratio to EI base peak
EI (70 eV)	81 (base peak)	4546	1.00
	372	166	0.09
	374	65	0.02
NICI (ammonia)	329 (base peak)	395 637	87.03
	331	154 365	33.96
	371	2428	0.53
PICI (ammonia)	373 (base peak)	417	0.09
	375	160	0.03

The sensitivity of the different types of ionization are evaluated in Table II. A 5-ng amount of FD derivative was injected into the GC–MS system operated in the scanning mode with the electron multiplier at the autotune value of the voltage. Ions of interest were then selected to measure their signal intensity at the retention time of FD derivative.

NICI gave the best results as the area response of the base peak was 87 times higher than the EI base peak. PICI, although producing a good spectrum, was not as sensitive as the other two types of ionization. PICI with methane (data not shown) also was not as sensitive.

It was easily possible with the sensitive NICI mode to detect FD in different types of samples. Chromatograms of two extracted main ions in a urine sample (5 h after intake of 40 mg of FD) in this mode are shown in Fig. 10. The detection

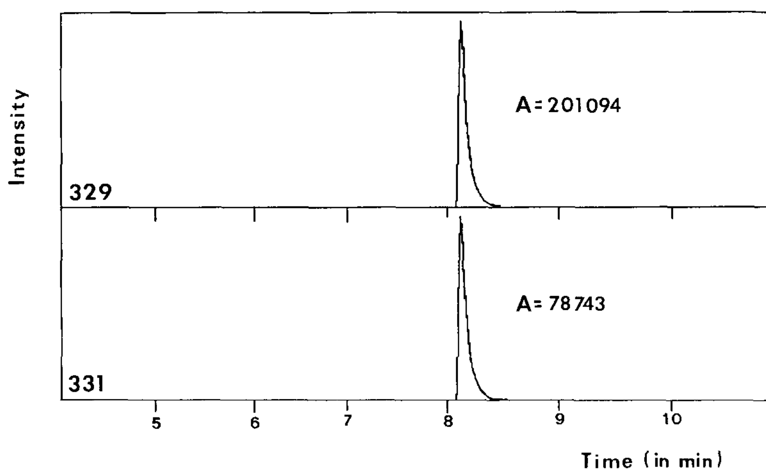

Fig. 10. GC–MS of two extracted ions (base peak m/z = 329 and its isotope ion m/z=331) of a 1-μl splitless injection of a urine extract (5 h after intake) in the negative-ion chemical ionization mode with ammonia as reactant gas. A is the area response of the peak (see Experimental).

limit of this method is similar to that obtained with HPLC with fluorescence detection (10 ng/ml).

These results show that GC–NICI-MS is a very sensitive and specific method for FD detection in urine samples. This technique allows the confirmation of the presence of FD at low levels in urine and thus could be very helpful in some doping analyses when the urine was collected more than 24 h after intake. Experiments are in progress to apply the same method to the detection of other diuretics of interest in doping analyses.

ACKNOWLEDGEMENT

We thank Dr. V. Raverdino of Hewlett-Packard in Geneva for skillful technical assistance.

REFERENCES

1 M. Hammarlund-Udenaes and L. Z. Benet, *J. Pharmacokin. Biopharm.*, 17 (1989) 1.
2 A. Grahnén, M. Hammarlund and T. Lundqvist, *Eur. J. Clin. Pharmacol.*, 27 (1984) 595.
3 F. T. Delbecke and M. Debackere, *J. Pharm. Biomed. Anal.*, 3 (1985) 141.
4 F. T. Delbecke and M. Debackere, *Arzneim.-Forsch.*, 36 (1986) 134.
5 F. T. Delbecke and M. Debackere, *Arzneim.-Forsch.*, 36 (1986) 1413.
6 S. M. Cooper, R. Massé and R. Dugal, *J. Chromatogr.*, 489 (1989) 65.
7 B. W. Hadzija and A. M. Mattocks, *J. Chromatogr.*, 229 (1982) 435.
8 W. Snedden, J. N. Sharma, P. G. Fernandez, *Ther. Drug Monit.*, 4 (1982) 381.
9 R. S. Rapaka, J. Roth, C. Viswanathan, T. J. Goehl, V. K. Prasad and B. E. Caban, *J. Chromatogr.*, 227 (1982) 463.
10 K. Uschino, S. Isozaki, Y. Saitoh, F. Nakagawa and Z. Tamura, *J. Chromatogr.*, 308 (1984) 241.
11 L. J. Lovett, G. Nygard, P. Dura and S. K. W. Khalil, *J. Liq. Chromatogr.*, 8 (1985) 1611.
12 T. C. Pinkerton, J. A. Perry and J. D. Rateike, *J. Chromatogr.*, 367 (1986) 412.
13 A. B. Straughn, G. C. Wood, G. Raghow and M. C. Meyer, *Biopharm. Drug Dispos.*, 7 (1986) 113.
14 F. G. M. Russel, Y. Tan, J. J. M. Van Meijel, F. W. J. Gribnau and C. A. M. Van Ginneken, *J. Chromatogr.*, 496 (1989) 234.
15 A. K. Singh, C. McArdle, B. Gordon, M. Ashraf and K. Grandley, *Biomed. Chromatogr.*, 3 (1989) 262.
16 W. Radeek and M. Heller, *J. Chromatogr.*, 497 (1989) 367.
17 W. Schänzer, in P. Bellotti, G. Benzi and A. Ljungqvist (Editors), *Official Proceedings of International Athletic Foundation World Symposium on Doping in Sports, Florence, May 10–12, 1987*, Fidal, Florence, 1988, p. 89.
18 Chang-No Yoon, Tae-Huyn Lee and Jongsei Park, *J. Anal. Toxicol.*, 14 (1990) 96.
19 Song-Ja Park, Hee-Soo Pyo, Yung-Je Kim, Mi-Sook Kim and Jongsei Park, *J. Anal. Toxicol.* 14 (1990) 84.
20 A. F. Casy, *J. Pharm. Biomed. Anal.*, 5 (1987) 247.
21 W. Dünges and E. Bergheim-Irps, *Anal. Lett.*, 5 (1973) 185.
22 M. Donike, *8th Cologne Workshop in Dope Analysis, March 26–30, 1990*, personal communication.