Rapid determination of diuretics in human urine by gas chromatography–mass spectrometry following microwave assisted derivatization

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Abstract

This work presents a complete method for the screening and confirmation analysis of diuretics in human urine by gas chromatography–mass spectrometry (GC–MS). The method comprises a pretreatment stage (extraction, preconcentration and derivatization to form the corresponding methyl derivatives) and the subsequent analysis of the derivatized extracts by GC–MS. Particularly, the derivatization stage, necessary to form the methyl derivatives of the compounds detectable by the GC–MS technique, is carried out under microwave irradiation rather than with direct thermal heating, thus reducing the incubation time from 3 h to 10 min. Microwave assisted derivatization also allowed an improvement of the limits of detection (LODs) for all the compounds here considered. The technique is particularly suitable for the rapid analysis of huge population of samples, as it is the case of urine analysis by the antidoping laboratories, and especially in all those occasions where rapid response times are requested.
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1. Introduction

Diuretics are a wide class of drugs eliciting an increased production of urine. Apart from the prophylaxis of renal failure, their main indication in the clinical practice is for the mobilization of edemas and for the therapy of hypertension and of congestive heart failure [1].

In the field of sport medicine, the use of diuretics has been first banned by the International Olympic Committee (IOC) in 1988, on the occasion of the winter Olympic Games of Calgary, and they are still included in the list of doping substances and methods [2]. Diuretics may be misused in sport for two main reasons: (i) to achieve acute weight loss before competition, in sports where weight categories are involved, and (ii) to mask the use of other doping agents by altering their mechanism of excretion, mainly reducing their concentration in urine. The latter effect may be accomplished either directly, by increasing the urine volume, and/or indirectly, for instance by altering the urinary pH, thus reducing the excretion in urine of acid/basic doping agents. In addition to this, a massive, as well as an unnecessary, use of diuretics can lead to dangerous side effects, including the reduction of the total blood volume, with the risk

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of hypotension and collapse, and an increase of blood viscosity, with the risk of thrombosis.

The family of diuretics includes compounds with widely different molecular structures and physico-chemical properties. From a pharmacological point of view, apart from the osmotic diuretics, like mannitol and sorbitol, four different groups of drugs acting on the nephron can be considered: carbonic anhydrase inhibitors (such as acetazolamide and diclofenamide), blocking HCO_3^- reabsorption in the proximal tubule; thiazides (bendroflumethiazide, chlorothiazide and hydrochlorothiazide) and long-acting thiazide type drugs (chlorthalidone), inhibiting Na^+/Cl^- cotransport in the distal tubule; diuretics of the loop (bumetanide, furosemide), characterized by a very rapid onset of the effect caused by the inhibition of Na^+–K^+/2Cl^- cotransport in the Henle's loop; and potassium-sparing diuretics (triamterene, amiloride and the aldosterone antagonists spironolactone and its active metabolite canrenone), acting in the distal portion of the distal tubule and in the proximal part of the collecting duct.

The simultaneous detection of all these groups of drugs in biological fluids is a quite complicated task, mainly because the variety of chemical structures makes very problematic their recovery from the biological matrix. At present, the analysis of diuretics is generally carried out by the antidoping laboratories accredited by the IOC by gas chromatography–mass spectrometry (GC–MS) of the methyl derivatives (reviewed in [3]). However, since the complete derivatization of all diuretics requires an incubation period of 3 h at 70 °C, this method becomes less competitive whenever a rapid response time is requested; this is the case, for instance, of major international sport events, including Olympic Games, when the test report has to be transmitted to the sport authority within 24 h from the reception of the urine samples. In these cases specifically developed methods, in which the extraction and derivatization processes are carried out simultaneously, are generally used [4].

This work describes an alternative, rapid method to detect and confirm the presence of diuretics in human urine by GC–MS of the corresponding methyl derivatives. The performance of the method was verified for 18 representative diuretics (Table 1), following the same operative conditions of the anti-

doping laboratory of Rome; the novel aspect of the method is represented by the technique used for the derivatization reaction, that is carried out by incubation of the purified urine extracts by methyl-iodide and potassium carbonate in acetone, supplying the energy transfer by microwave irradiation instead of by direct thermal heating. The proposed approach allowed to set up a robust analytical procedure for the screening and confirmation analysis of diuretic agents searched by the antidoping laboratories, requiring 10 min instead 3 h for the derivatization step.

2. Experimental

2.1. Materials and reagents

All reagents (analytical grade) were supplied by Carlo Erba (Milano, Italy). Ammonium iodide was supplied by Fluka Sigma Aldrich (St. Louis, MO, USA). Indomethacine and mefruside (used as internal standards, ISTD) and the 18 compounds considered in the present study (acetazolamide, althiazide, bendroflumethiazide, bumetanide, canrenone, chlorothiazide, chlorthalidone, clopamide, diclofenamide, ethacrynic acid, furosemide, hydrochlorothiazide, hydroflumethiazide, indapamide, probenecid, spironolactone, triamterene, trichlormethiazide) were supplied by Sigma (St. Louis, MO, USA). Stock standard solutions were prepared by dissolving the reference standard in methanol ($1 g l^{-1}$); all stock solutions were stored in screwed cap vials at $T = -20 °C$ in the dark. Retention times, full scan spectra, and diagnostic ions for the selected ion monitoring (SIM) assays of all compounds here considered were obtained in standard solution ($10–100 mg l^{-1}$) of reference standard in methanol. Working standard solutions were daily prepared, at the appropriate dilution, from the correspondent stock solution. Limits of detection (LODs) in urine were obtained on spiked reference urines. Twice distilled/deionized water was used for the preparation of all reagents and solutions. Spiked urine samples were prepared by diluting the corresponding methanol working standard solution with blank reference urine to the final desired concentration. C18 cartridges (Sep-Pak) were supplied by Waters (Waters SpA, Milano, Italy).

Table 1
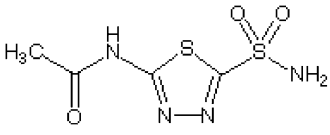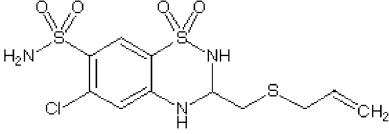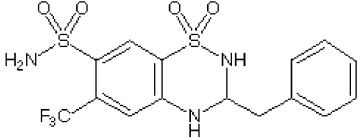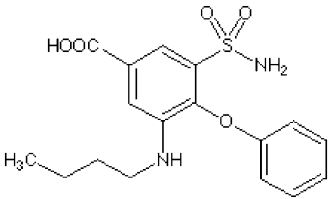Chemical properties of the drugs under investigation
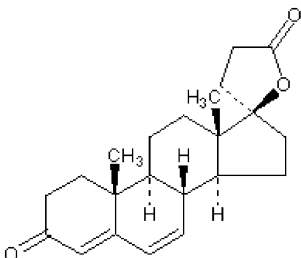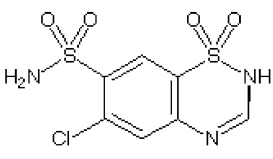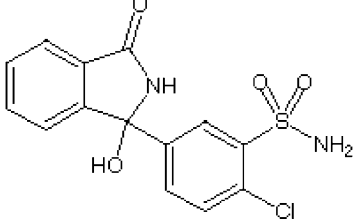
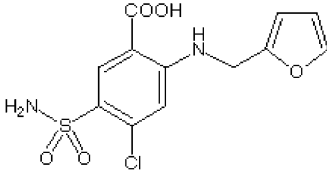| Drug	Molecular structure	Formula (molecular weight)	International names
Acetazolamide		$C_4H_6N_4O_3S_2$ (222.25)	*N*-[5-(Aminosulfonyl)-1,3,4-thiadiazol-2-yl]acetamide
Althiazide		$C_{11}H_{14}ClN_3O_4S_3$ (383.90)	6-Chloro-3,4-dihydro-3-[(2-propenylthio)methyl]-2H-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide; 3-[(allylthio)methyl]-6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide
Bendroflumethiazide		$C_{15}H_{14}F_3N_3O_4S_2$ (421.42)	3,4-Dihydro-3-(phenylmethyl)-6-(trifluoromethyl)-2H-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide
Bumetanide		$C_{17}H_{20}N_2O_5S$ (364.42)	3-(Aminosulfonyl)-5-(butylamino)-4-phenoxybenzoic acid
Canrenone		$C_{22}H_{28}O_3$ (340.46)	17α-17-Hydroxy-3-oxopregna-4,6-diene-21-carboxylic acid γ-lactone
Chlorothiazide		$C_7H_6ClN_3O_4S_2$ (295.73)	6-Chloro-2H-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide
Chlorthalidone		$C_{14}H_{11}ClN_2O_4S$ (338.77)	2-Chloro-5-(2,3-dihydro-1-hydroxy-3-oxo-1H-isoindol-1-yl)benzenesulfonamide

Table 1 (*Continued*)
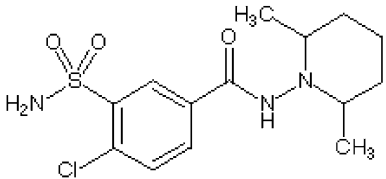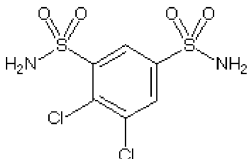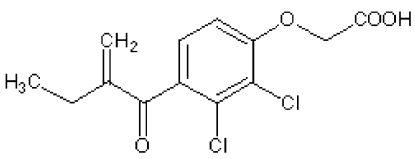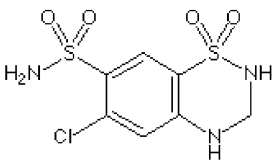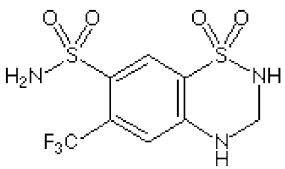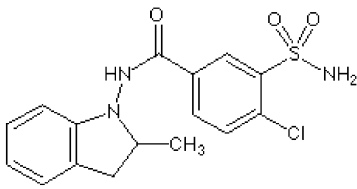
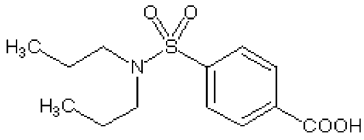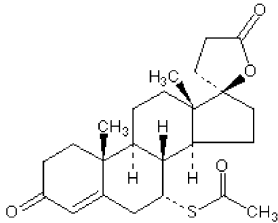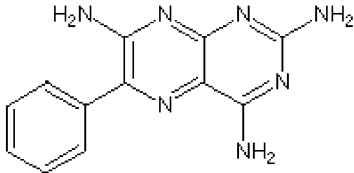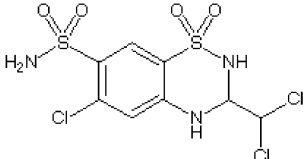| Drug	Molecular structure	Formula (molecular weight)	International names
Clopamide		$C_{14}H_{20}ClN_3O_3S$ (345.85)	*cis*-3-(Aminosulfonyl)-4-chloro-*N*-(2,6-dimethyl-1-piperidinyl)benzamide
Diclofenamide		$C_6H_6Cl_2N_2O_4S_2$ (305.16)	4,5-Dichloro-1,3-benzenedisulfonamide
Ethacrynic acid		$C_{13}H_{12}Cl_2O_4$ (303.14)	[2,3-Dichloro-4-(2-methylene-1-oxobutyl)phenoxy]acetic acid
Furosemide		$C_{12}H_{11}ClN_2O_5S$ (330.75)	5-(Aminosulfonyl)-4-chloro-2-[(2-furanylmethyl)amino]benzoic acid
Hydrochlorothiazide		$C_7H_8ClN_3O_4S_2$ (297.74)	6-Chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide
Hydroflumethiazide		$C_8H_8F_3N_3O_4S_2$ (331.30)	3,4-Dihydro-6-(trifluoromethyl)-2H-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide
Indapamide		$C_{16}H_{16}ClN_3O_3S$ (365.84)	3-(Aminosulfonyl)-4-chloro-*N*-(2,3-dihydro-2-methyl-1H-indol-1-yl)benzamide
Probenecid		$C_{13}H_{19}NO_4S$ (285.36)	4-[(Dipropylamino)sulfonyl] benzoic acid

Table 1 (*Continued*)

Drug	Molecular structure	Formula (molecular weight)	International names
Spironolactone		$C_{24}H_{32}O_4S$ (416.58)	7α,17α-7-(Acetylthio)-17-hydroxy-3-oxopregn-4-ene-21-carboxylic acid γ-lactone
Triamterene		$C_{12}H_{11}N_7$ (253.27)	6-Phenyl-2,4,7-pteridinetriamine
Trichlormethiazide		$C_8H_8Cl_3N_3O_4S_2$ (380.66)	6-Chloro-3-(dichloromethyl)-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide

2.2. Urine pretreatment

The pretreatment of urine samples, apart from the final derivatization stage, was the same followed by the antidoping laboratory of Rome for the screening analysis by GC–MS of the methyl derivatives of diuretics and masking agents: 5 ml of urine, added with 50 µl of ISTD (indomethacine and/or mefruside 200 µg l^{-1} in methanol) were passed across a C18 Sep-Pak (previously activated by 3 ml of MeOH and 3 ml of H$_2$O) and then eluted, after washing with 3 ml of H$_2$O, with 3 ml of methanol. The eluate was evaporated to dryness under a stream of N$_2$ at $T = 40\,^\circ$C.

2.2.1. Derivatization with thermal incubation

In the traditional procedure the residue was taken up in 200 µl of acetone/methyl-iodide 1:10. Fifty milligrams of potassium carbonate were added and the tubes were warmed up to 70 $^\circ$C for 3 h.

2.2.2. Derivatization under microwave irradiation

In the alternative procedure the energy transfer necessary to support the derivatization reaction was supplied by a microwave oven. All materials were preliminarily tested to verify their resistance to the operating conditions. The reaction tubes were stoppered by silicon septa and placed in a water bath to maintain the temperature ≤100 $^\circ$C during the MW irradiation. More in details, after addition of the derivatizing mixture (200 µl of acetone/methyliodide 1:10 + 50 mg of potassium carbonate) the glass tubes were placed in a 1000 ml glass beaker filled with 250 ml of distilled water. The beaker was in turn placed in the microwave oven and irradiated at 900 W (emitted nominal power). The reproducibility of the amount of energy supplied by the microwave oven was performed by a daily verification of the temperature increase of a known volume of distilled water recorded following incubation for a known period of time at a fixed value of the emitted power: in practice, 1000 ml of distilled water were irradiated daily for 5 min at 900 W (emitted nominal power) with an increase of temperature of 38±1 $^\circ$C, thus corresponding to an effective absorbed power of 530 ± 14 W.

2.3. Instrumentation and GC–MS parameters

The microwave oven used for the MW assisted derivatization of the compounds here considered was a Whirlpool MWO105 (Whirlpool Italia). The

Table 2
Gas chromatographic conditions

Carrier gas	He
Injector T (°C)	260
Transfer line T (°C)	280
Source T (°C)	200
Constant pressure (psi)	30
Injection mode	Split
Split ratio	1:20
Injection volume	1 μl
Oven temperature program	
Initial T (°C)	190
Initial time (min)	0
Rate 1 (°C/min)	15
Final T_1 (°C)	280
Hold time 1 (min)	6
Rate 2 (°C/min)	5
Final T_2 (°C)	290
Hold time 2 (min)	6

GC–MS system was a HP6890-5973 (Agilent Technologies Italia, Milano, Italy), equipped with a phenyl-methylsilicone column, length $18\,\text{m} \times 0.2\,\text{mm}$ i.d., $0.33\,\mu\text{m}$ film (HP5, Agilent Technologies Italia, Milano, Italy). Details on the GC method are reported in Table 2.

3. Results

Table 3 reports the GC–MS–EI data for all compounds under investigation; the diagnostic ion fragments (≥ 2) used for the screening analysis in SIM are indicated in boldface. Relative retention times (RRt) instead of absolute retention times are used, to take into account the variability consequent to minor perturbations of the chromatographic system.

The effectiveness of the derivatization technique by microwave irradiation, was evaluated on blank urine samples spiked with all compounds considered in the present study. All urine samples were analyzed in duplicate, varying the conditions of the derivatization step. LOD (SIM mode) in urine, corresponding to a value of the signal to noise ratio ≥ 3 for at least three diagnostic ions, were obtained by progressively diluting the above mentioned spiked urine with blank urine. All data regarding the LOD and the comparison among the derivatization yields obtained following the traditional derivatization procedure (incubation at $T = 70\,°\text{C}$ for 3 h) and microwave irradiation (10 min at 900 W emitted power) are reported in Table 4.

Figs. 1 and 2 show the GC–MS chromatograms respectively of the screening and of the confirmation

Table 3
GC–MS–EI data of the compounds under investigation (ion fragments used for the screening analysis in SIM are indicated in boldface)

Compound	Me	Molecular weight	RRt 1	RRt 2	Ions (*m/z*)	
					Base	Others
Indomethacin (ISTD1)	1	371	1.00	0.98	139	373, **371**
Mefruside (ISTD2)	2	411	1.02	1.00	**325**	218, 282, 367
Acetazolamide	3	264	0.39	0.38	**249**	**310**, **264**, 251, 108
Althiazide	4	439	1.78	1.74	**352**	**354**, 246, 244
Bendroflumethiazide	4	477	1.68	1.64	**386**	388, 387, **278**, 91
Bumetanide	3	406	1.08	1.06	**406**	**363**, 318, 298, 254
Canrenone/spironolactone	–	340/416	1.61	1.57	**267**	**340**, 325, 227
Chlorothiazide	3	339	0.88	0.86	**248**	**275**, 250, 169
Chlorthalidone	4	394	1.04	1.01	**363**	289, **287**, 255, 176
Clopamide	2	373	0.93	0.91	**111**	139, **127**, 112, 55
Diclofenamide	4	360	0.68	0.67	**253**	**360**, 255, 108
Ethacrynic acid	1	316	0.49	0.48	**261**	**316**, 263, 243
Furosemide	3	372	0.94	0.92	81	374, **372**, 96
Hydrochlorothiazide	4	353	1.18	1.15	**353**	**310**, 288, 218, 202
Hydroflumethiazide	4	387	0.84	0.82	**387**	**344**, 322, 252, 236, 215
Indapamide	3	407	1.25	1.22	**161**	**407**, 132
Probenecid	4	299	0.46	0.45	**270**	271, 104, **199**, 135
Triamterene	6	337	1.24	1.21	**336**	**337**, 322, 294, 279
Trichlormethiazide	4	435	1.46	1.43	**352**	**354**, 246, 244, 108

Me: number of methyl residues in the derivatized molecule; RRt: relative retention time (vs. ISTD1 and ISTD2).

Table 4

Limit of detection (LODs) by GC–MS SIM (3 ions), of all diuretics considered in this study following derivatization by microwave (MW) irradiation, and comparison with data obtained following derivatization with thermal incubation

Drug	Limit of detection (μg l^{-1}) after MW derivatization	LOD ratio thermal/MW
Acetazolamide	50	1.1
Althiazide	140	1.5
Bendroflumethiazide	60	3.5
Bumetanide	50	2.4
Canrenone/spironolactone	250	1.0
Chlorothiazide	70	3.3
Chlorthalidone	50	3.7
Clopamide	50	2.4
Diclofenamide	80	1.3
Ethacrynic acid	20	1.1
Furosemide	40	2.6
Hydrochlorothiazide	100	2.0
Hydroflumethiazide	50	2.1
Indapamide	40	2.5
Probenecid	10	1.1
Triamterene	130	3.3
Trichlormethiazide	100	3.0

analysis, compared with the chromatogram of a positive reference sample, of a real urine sample found positive for diclofenamide.

4. Discussion

A particular field of analytical chemistry applied to forensic toxicology is represented by the antidoping analysis, where the urines of athletes ruled for national/international sport federations are analyzed to detect the putative use of forbidden drugs/methods. At present, the classes of banned drugs include: (1) stimulants, (2) narcotics, (3) anabolic agents (both androgenic steroids and beta-2-agonists), (4) diuretics and (5) peptide hormones, while additional classes (beta blockers, local anaesthetics, cannabinoids and glucocorticoids) are subject to certain restrictions [2].

The procedure for the preliminary screening and the subsequent confirmation (if necessary) of these drugs is generally constituted by the preliminary pretreatment of the urine samples and by the subsequent

analysis by chromatographic-spectrometric methods. Specifically, the analysis of diuretics represents a stimulating analytical challenge for the antidoping laboratory, mainly because of the marked physico-chemical properties (pK_a, partition coefficient, water and lipid solubility) of the single components of the class. In addition to this, diuretics have to be searched for in all urine samples, since their use is forbidden in all sport disciplines and on both "in competition" and "out of competition" tests, since, unlike stimulants and narcotics, the pharmacologic effect of diuretics can be effective also in the training period, and not only at the moment of the competition.

Analysis and quantitative determination of diuretics in routine screening of biological fluids is usually being accomplished by a variety of chromatographic-spectrometric methods, including HPLC-UV, HPLC-MS, GC–MS. Screening procedures by HPLC with UV detection and with MS detection have been diffusely reported [3–7]. In the last years new screening procedures, based on the use of relatively new separation techniques, like micellar liquid chromatography and capillary electrophoresis, were also developed [8,9]. Analysis by direct GC–MS screening is made problematic by the polar nature of most diuretics, so that GC is not suitable for the determination of the native drugs, and preliminary derivatization is therefore needed for GC analysis. For these reasons the analysis of diuretics in human urine for doping control is routinely performed by LC-DAD (screening analyses) and/or by GC–MS (both screening and confirmation analyses) after derivatization (usually methylation) of the reactive residues (mainly –OH and/or –NH groups). The advantage of the latter approach consists in the versatility of GC–MS detection, combined with high sensitivity and selectivity. However, the derivatization step that represents the final stage of the urine pretreatment is time consuming: an incubation time of at least 3 h at $T = 70\,^\circ$C is indeed necessary to achieve sufficient yields for the methylation reaction of most compounds [3,5].

The method we are proposing combines the versatility of the GC–MS analysis with the rapidity of microwave assisted organic synthesis [10,11] applied to derivatization reactions [12,13]. Apart from the net reduction of the overall time of analysis, mi-
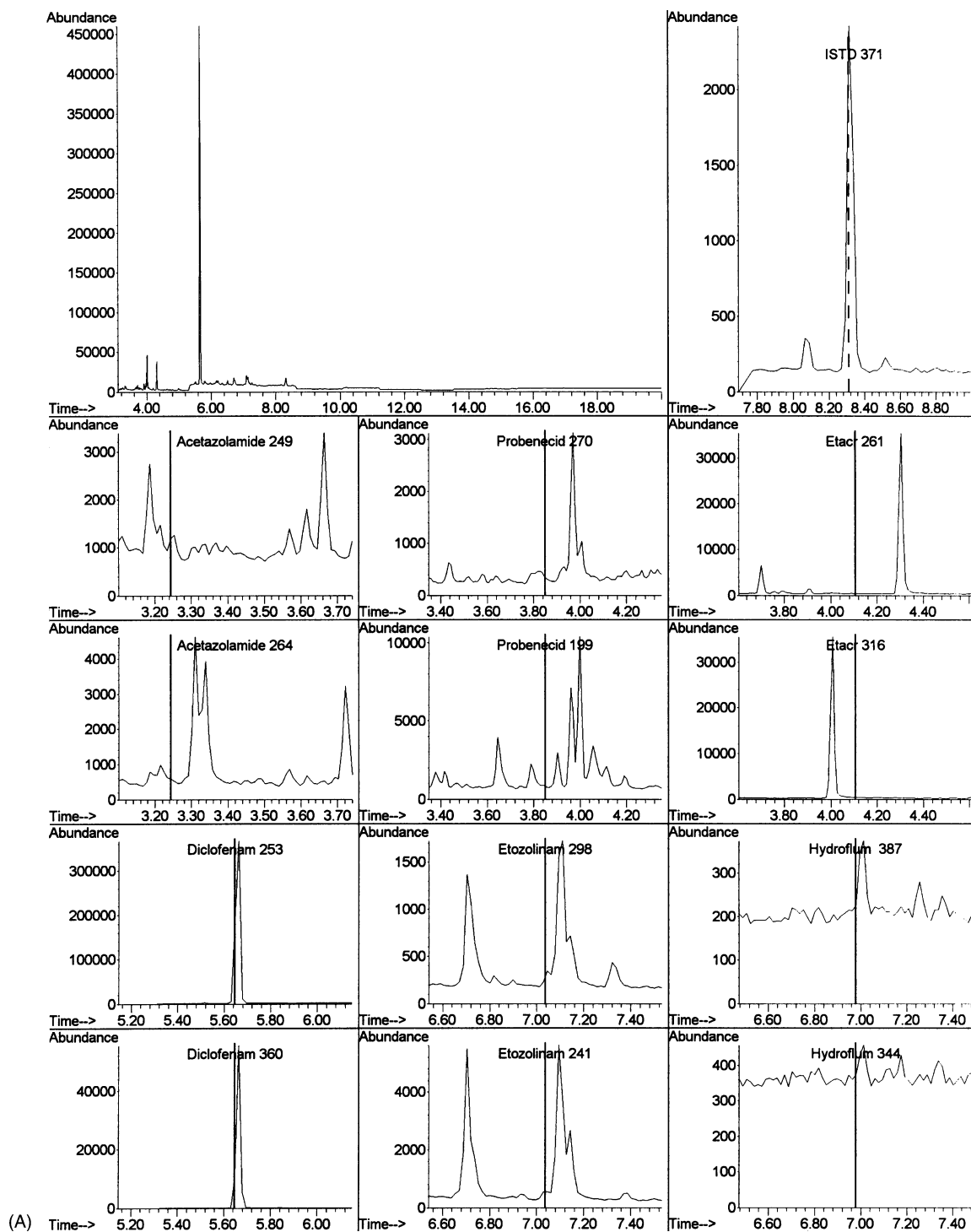
Fig. 1. Screening analysis, by GC–MS (in SIM mode) of a real sample, found positive for diclofenamide. GC–MS windows of the analysis carried out following derivatization under microwave irradiation (10 min, emitted power = 900 W) of the sample under investigation (A) and of a positive reference urine (B).
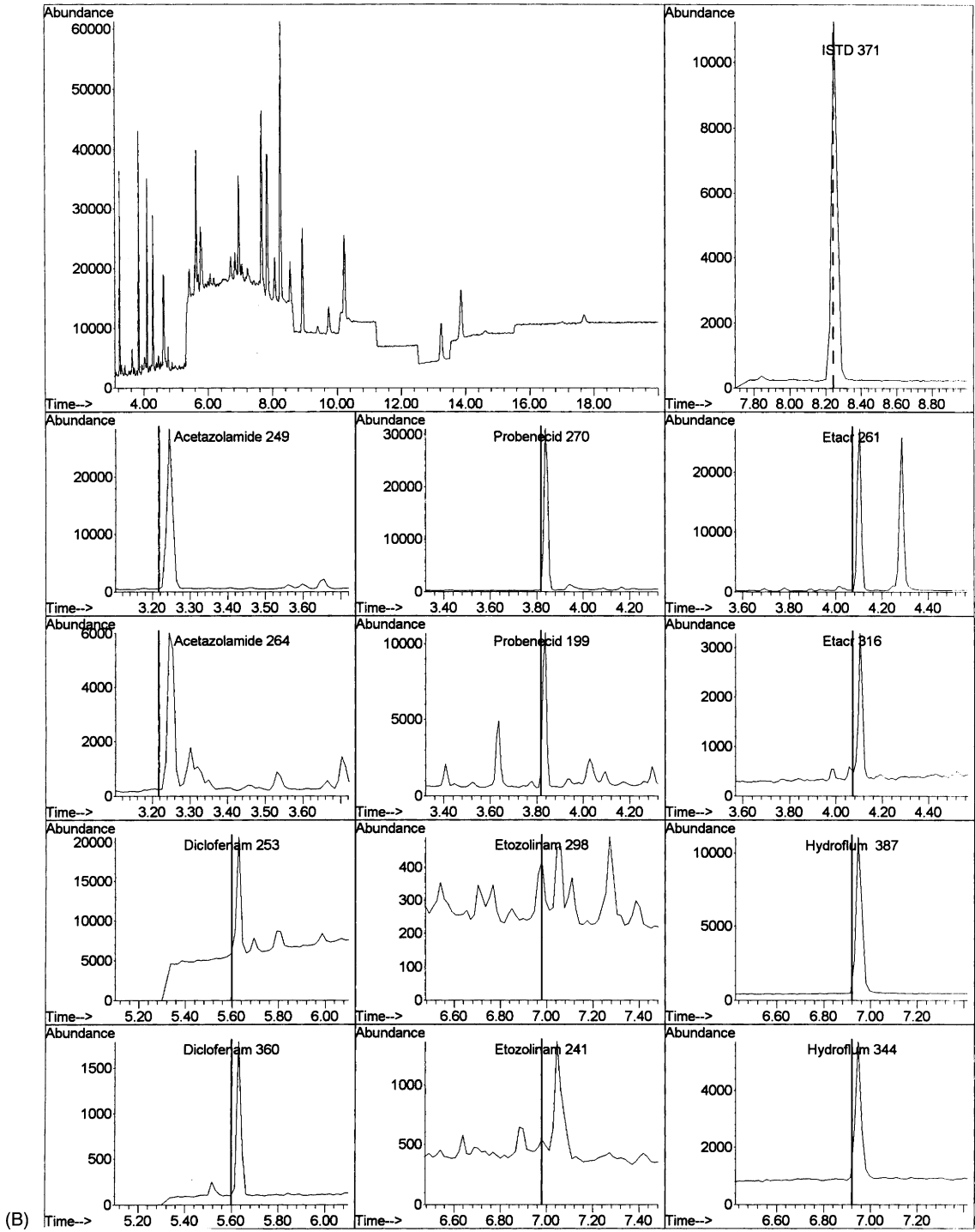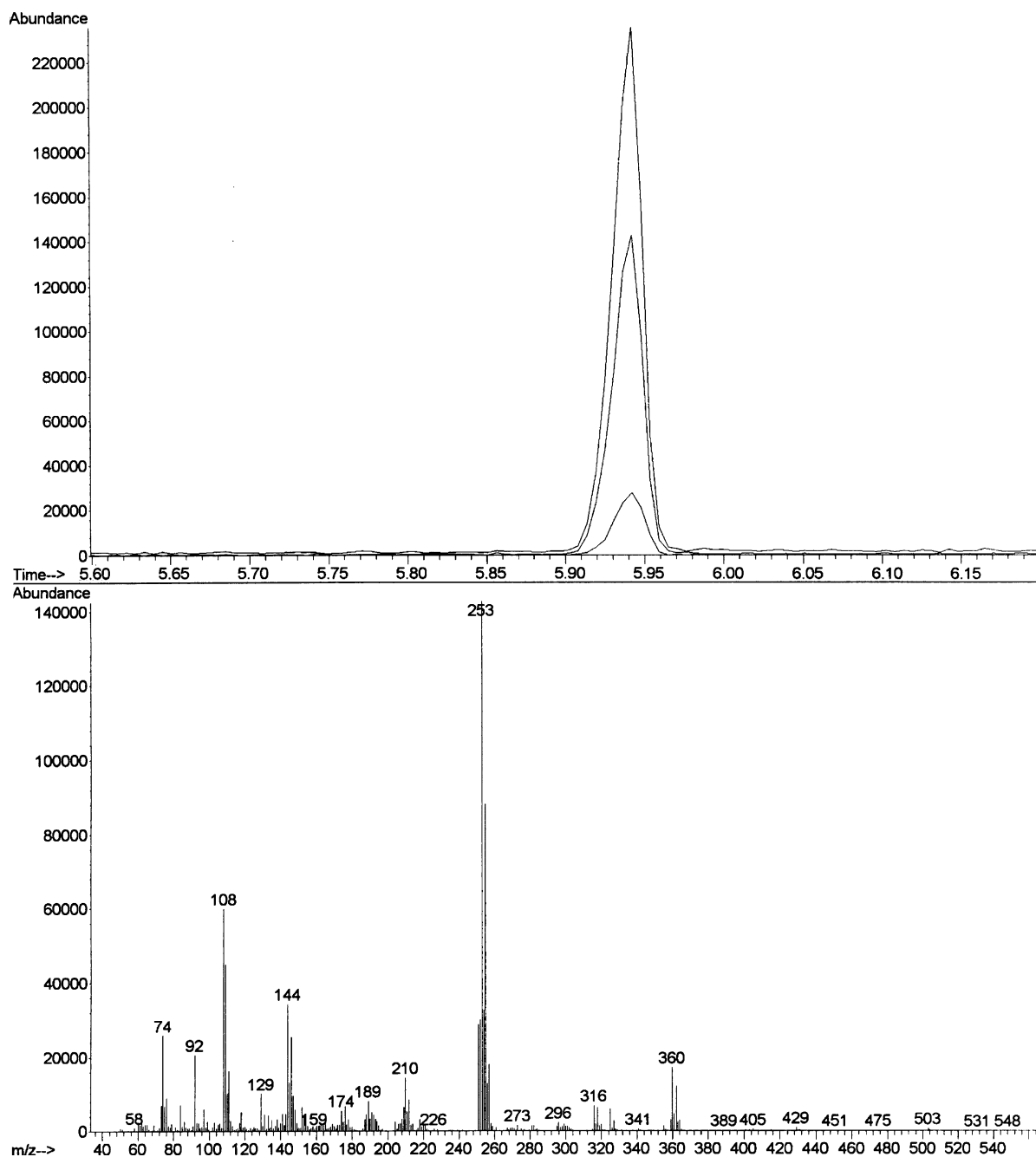
Fig. 1. (*Continued*).

Fig. 2. Confirmation analysis of a real sample, recorded as suspected positive for diclofenamide after the screening analysis (see also Fig. 1). Plots show the extracted ion (*m/z* 253, 360, 255) GC–MS chromatograms (above) and the full scan MS spectrum (below) of the sample to be confirmed (A) and of a positive reference urine containing diclofenamide in a concentration $0.4 \, \text{mg} \, \text{l}^{-1}$ (B), both obtained following derivatization under microwave irradiation (10 min, emitted power = 900 W).
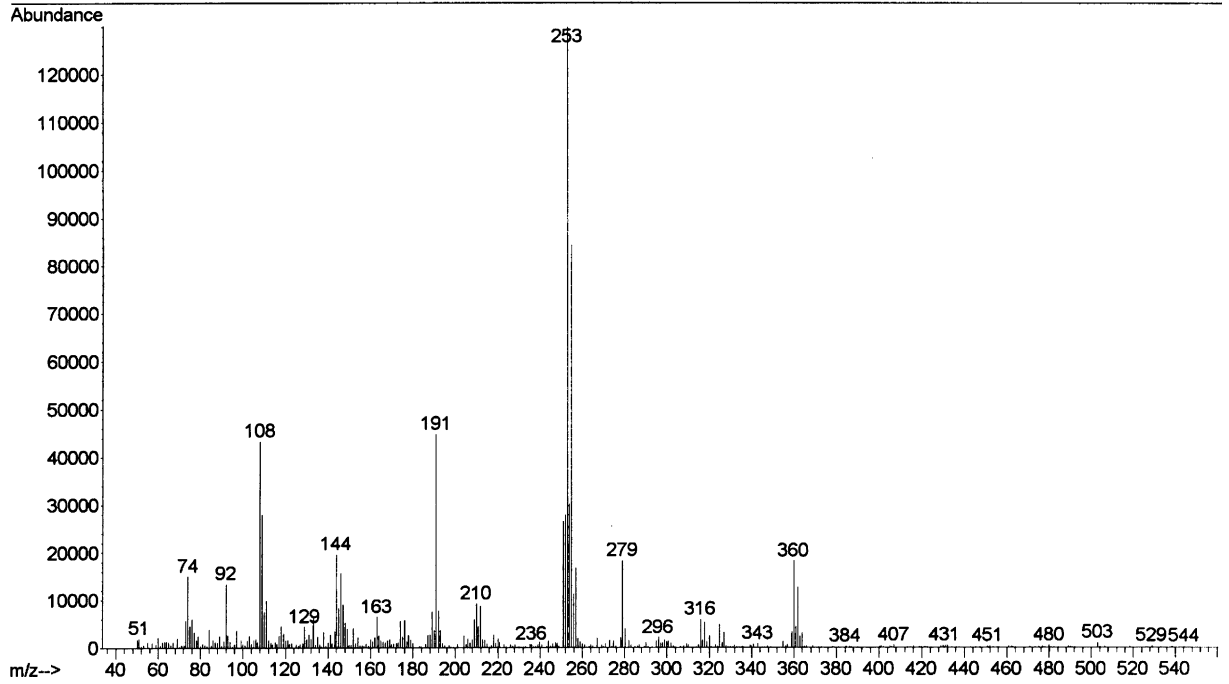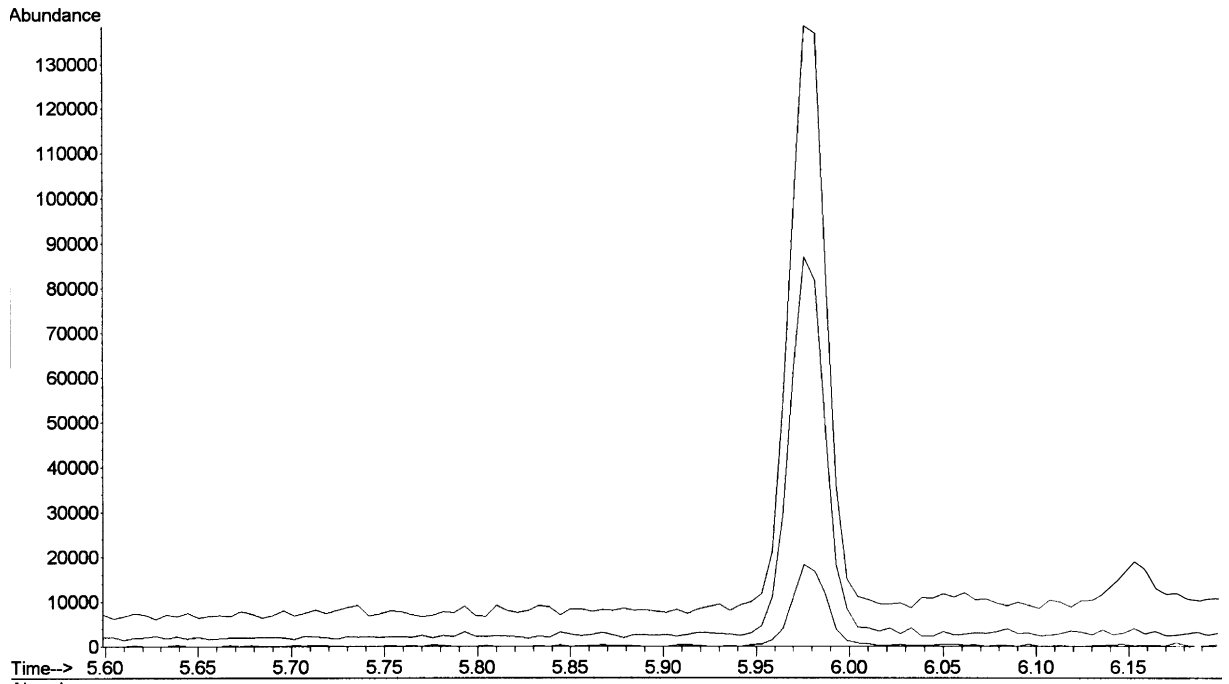
Fig. 2. (*Continued*).

crowave assisted derivatization led to an increase of the derivatization yields for all compounds here considered, with a consequent improvement of the LOD (Table 4). This effect is particularly evident on those compounds for which the formation of poly-methyl derivatives is more problematic.

Evaluation of the performance of the method was verified by comparing, for all samples analyzed by the antidoping laboratory of Rome in the period January–June 2002, the results obtained following microwave irradiation with those obtained following thermal incubation. In the case of samples in which the presence of diuretics was suspected after the screening analysis, the confirmation analysis was carried out also after microwave assisted derivatization. Data of Figs. 1 and 2 show that both the screening and the confirmation analysis allow the unambiguous identification of the diuretic present in the sample (in this case diclofenamide).

The method is presently under validation according to ISO 17025 to be applied as a certified routine analytical procedure in the antidoping laboratory.

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