Fast gaschromatographic/mass spectrometric determination of diuretics and masking agents in human urine. Development and validation of a productive screening protocol for antidoping analysis

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Abstract

An analytical procedure was developed for the fast screening of 16 diuretics (acetazolamide, althiazide, amiloride, bendroflumethiazide, bumetanide, canrenoic acid, chlorthalidone, chlorthiazide, clopamide, ethacrynic acid, furosemide, hydrochlorthiazide, hydroflumethiazide, indapamide, triamterene, trichlormethiazide) and a masking agent (probenecid) in human urine. The whole method involves three analytical steps, including (1) liquid/liquid extraction of the analytes from the matrix, (2) their reaction with methyl iodide at 70 °C for 2 h to form methyl derivatives, (3) analysis of the resulting mixture by fast gas chromatography/electron impact mass spectrometry (fast GC/EI-MS). The analytical method was validated by determining selectivity, linearity, accuracy, intra and inter assay precision, extraction efficiencies and signal to noise ratio (S/N) at the lowest calibration level (LCL) for all candidate analytes. The analytical performances of three extraction procedures and five combination of derivatization parameters were compared in order to probe the conditions for speeding up the sample preparation step. Limits of detection (LOD) were evaluated in both EI-MS and ECNI-MS (electron capture negative ionization mass spectrometry) modes, indicating better sensitivity for most of the analytes using the latter ionization technique.

The use of short columns and high carrier gas velocity in fast GC/MS produced efficient separation of the analytes in less than 4 min, resulting in a drastic reduction of the analysis time, while a resolution comparable to that obtained from classic GC conditions is maintained. Fast quadrupole MS electronics allows high scan rates and effective data acquisition both in scan and selected ion monitoring modes.

Keywords

- Fast GC/MS;
- Diuretics;
- Masking agents;
- Doping control;
- Urine screening;
- ECNI

1. Introduction

Diuretic drugs increase the rate of urine production by improving the excretion of electrolytes (especially sodium and chloride ions) and water from the body [1]. For these pharmacological properties, diuretics are used in the treatment of edematous conditions resulting from a variety of diseases and in the management of hypertension [2].

The diuretics family includes compounds with widely different molecular structures, physical and chemicals 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 are classified [3]:

1. carbonic anhydrase inhibitors, blocking HCO_3^- reabsorption in the proximal tubule;
2. thiazides and long acting thiazide type drugs, inhibiting Na^+/Cl^- cotransport in the distal tubule;
3. diuretics of the loop, characterized by rapid onset of the inhibition of $Na^+/K^+/2Cl^-$ cotransport in the Henle's loop;
4. potassium-sparing diuretics, acting in the distal portion of the distal tubule and in the proximal part of the collecting duct.

In sport medicine, diuretics are included in the prohibited list of substances compiled by the World Antidoping Agency (WADA) [4], because they may be misused for three main reasons:

a. to achieve quick weight loss before competition, in sports involving weight categories;
b. to relieve the water retention induced by assumption of anabolic androgenic steroids (e.g., bodybuilders) [5];
c. to mask the use of other doping agents by altering their excretion mechanism, mainly reducing their concentration in urine. The latter effect may be accomplished either directly, by increasing the urine volume, or indirectly by altering the urinary pH, thus reducing the excretion in urine of acid/basic doping agents.

In order to ensure that all doping control laboratories can report the presence of prohibited substances uniformly, WADA establishes a minimum detection capability for testing methods called “minimum required performance limits” (MRPL). The limit for each analyte in the class of diuretics is 250 ng/ml [6].

Other drugs with masking action, like probenecid (a lipid-soluble benzoic acid derivative), are active mostly on the renal tubule, where the transport of organic acids across epithelial barriers is inhibited [1]. As a doping agent, probenecid reduces the urinary excretion of anabolic steroids.

At present, diuretics are generally determined by chromatographic-spectrometric techniques (mainly LC/MS and GC/MS) [3], [7], [8], [9] and [10]; in GC/MS they are generally screened for as methyl derivatives, in the selective ion monitoring (SIM) mode.

For most GC applications using conventional capillary columns, the separation requires from 15 to 60 min, depending on the complexity of the matrix and number of analytes to be determined. Obviously, reducing the analysis time speeds up sample processing and decreases costs.

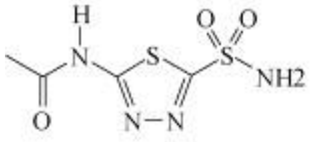
Moreover, antidoping analyses require wide screening and rapid response time, especially in the case of major international sport events, including the Olympic Games, when test reports have to be transmitted to the sport authority within 24 h from the reception of urine samples.

Therefore, the use of methods that reduce the analysis time without sacrificing the analytical information, such as fast gas chromatography (fast GC), is likely to meet an important need of antidoping control. Fast GC achieves efficient analytical separation basically by using a shorter column (i.e. 5–10 m; i.d. 0.05–0.1 mm) and a higher carrier gas velocity with respect to classic GC conditions.

The experimental parameters that affect the speed of analysis are: (1) the carrier gas flow rate, (2) the oven temperature heating rate, (3) the column length, (4) the column internal diameter, (5) the thickness of the stationary phase, (6) the outlet pressure at which the detector operates.

The development of fast electronics to control mass analyzers allows fast scans and high data acquisition rates, that are necessary to support fast GC with an appropriate mass detector [11]. Fast GC/MS has been recently proposed for the determination of drugs of abuse [12].

The present work describes the development and validation of an analytical method for the simultaneous determination of sixteen diuretics (Fig. 1) and one masking agent (probenecid) in human urine [13],[14] and [15] based on fast GC/MS using a benchtop quadrupole instrument. Besides the optimization of GC parameters, a comparison of extraction procedures and derivatization conditions was undertaken, in the perspective of further reducing the overall analysis time.



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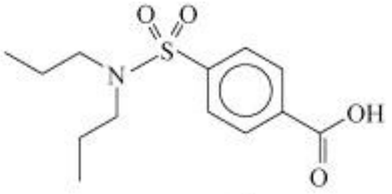

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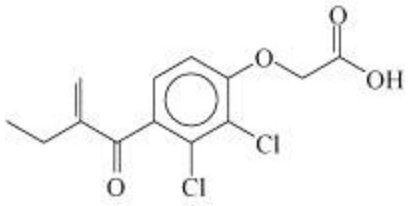

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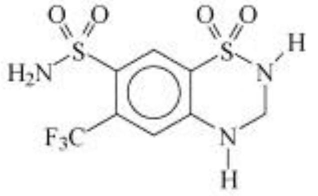

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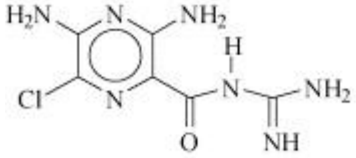

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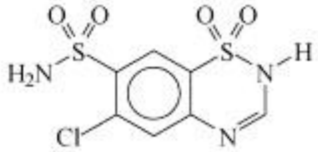

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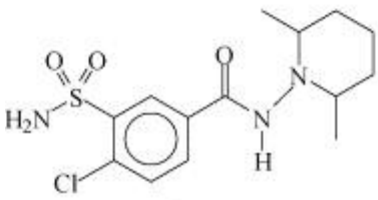

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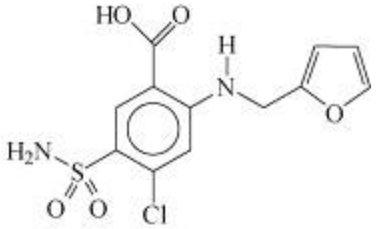

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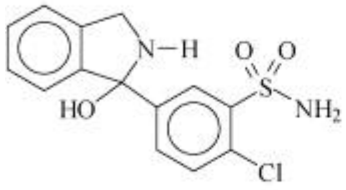

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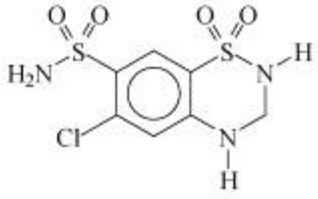

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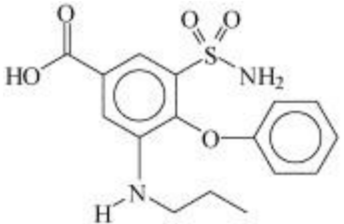

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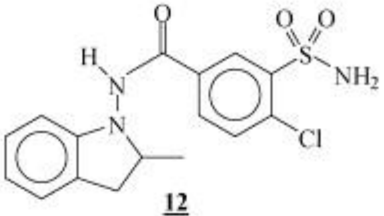

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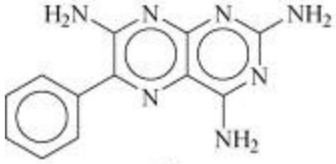

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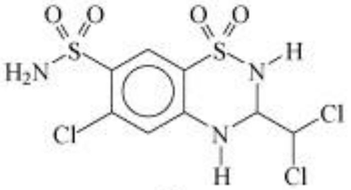

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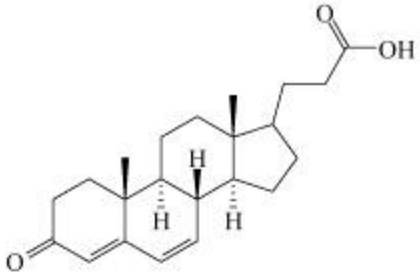

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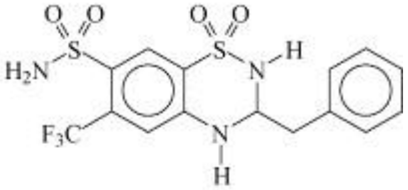

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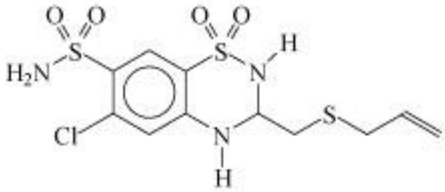

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Fig. 1. Chemical structure of investigated compounds: acetazolamide (**1**), probenecid (**2**), ethacrynic acid (**3**), hydroflumethiazide (**4**), amiloride (**5**), chlorthiazide (**6**), clopamide (**7**), furosemide (**8**), chlorthalidone (**9**), hydrochlorthiazide (**10**), bumetanide (**11**), indapamide (**12**), triamterene (**13**), trichlormethiazide (**14**), canrenoic acid (**15**), bendroflumethiazide (**16**), althiazide (**17**).

2. Experimental

2.1. Materials, reagents and standard solutions

Methanol was supplied by Riedel de Haën (Seelze, Germany). Sodium hydroxide, hydrochloric acid and potassium carbonate were supplied by Carlo Erba Reagenti (Milan, Italy). Acetone, 2-propanol, methylene chloride, methyl iodide and the 17 compounds studied in this work (acetazolamide, althiazide, amiloride, bendroflumethiazide, bumetanide, canrenoic acid, chlorthalidone, chlorthiazide, clopamide, ethacrynic acid, furosemide, hydrochlorthiazide, hydroflumethiazide, indapamide, probenecid, triamterene, trichlormethiazide, see Fig. 1) were supplied by Sigma–Aldrich (St. Louis, MO, USA). Mefruside (Internal Standard, IS) was kindly supplied by the FMSI Antidoping Laboratory (Rome, Italy).

Stock standard solutions were prepared in methanol at a concentration of 5000 mg/l for all substances, except chlorthiazide, triamterene (400 mg/l) and bumetanide (2000 mg/l) and were stored at +4 °C until used. Calibration curves were obtained from urine spiked with standard solutions at five to seven different concentration levels for each analyte, except for amiloride which was not detected at the concentration levels used for the other compounds. In this case a single concentration level (10,000 ng/ml) was used. Derivatization assays were performed on methanolic solutions of the 17 analytes at the MRPL concentration fixed by the WADA for the class of diuretics (250 ng/ml).

Spiked urine samples for validation assays were prepared by adding the adequate volume of the corresponding methanolic standard solutions to 3 ml of negative reference urine, yielding a final concentration equal to the MRPL value. Hundred microliters of IS (30 μg/ml in methanol) was added to all spiked urine samples after the extraction stage and before solvent evaporation.

2.2. Extraction

Three different procedures were followed:

Method 1.

Spiked samples (3 ml) were mixed with 80 μl of NaOH 1 M ($10 < pH < 10.3$) and extracted with 5 ml of 85:15 v/v methylene chloride/2-propanol mixture using a vortex mixer for 5 min. The resulting biphasic solution was centrifuged at 5000 rpm for 5 min and the organic fraction was collected. The surnatant urine fraction was acidified with 130 μl of HCl 1 M to $3.5 < pH < 3.8$. Three milliliters of the same 85:15 v/v methylene chloride/2-propanol mixture was added and the resulting solution was centrifuged again at 5000 rpm for 5 min. The two organic fractions were collected together and evaporated to dryness by a gentle stream of nitrogen.

Method 2.

Spiked urine samples (3 ml), acified to pH 5 (HCl 1 M), were passed through a SPE cartridge (Varian ABS ELUT Nexus, 60 mg/3 ml). The SPE column was then washed with 1 ml of water and 1 ml of 20% methanol in water by gravity or using a gentle negative pressure. Afterwards, the analytes were recovered from the cartridge by flowing 2 ml of methanol at a rate of 1–2 ml/min. The resulting solution was lastly evaporated to dryness.

Method 3.

Spiked urine samples (2 ml) were added with 80 µl of NaOH 1 M (10 < pH < 10.3) and 1 ml of 85:15 v/v methylene chloride/2-propanol mixture. The resulting solutions were mixed for 1 min with a customized ultrasonic bath [16] and the organic fraction was collected. The surnatant urine fraction was acidified with 130 µl of HCl 1 M to 3.5 < pH < 3.8. 1 ml of the same 85:15 v/v methylene chloride/2-propanol mixture was added and the resulting solution was sonicated again for 1 min. The two organic fractions were collected together and evaporated to dryness by a gentle stream of nitrogen.

The dry residue arising from each method was derivatized under the conditions described below.

2.3. Derivatization

The reaction forms methyl derivatives on the sulfonamide groups and other active hydrogen atoms (carboxylic, amine and hydroxyl groups, Fig. 1) [17].

Fifty milligrams of potassium carbonate and 400 µl of acetone/methyl iodide 10:1 (v/v) were added to the dry residue of methanolic standard solution at the MRPL concentration. The resulting solution was subjected to thermal incubation under various conditions. In previous works [3], [17] and [18], incubation was generally reported to occur in 3 h at 70 °C, in 3 h at 60 °C and in 10 min at 900 W (microwaves). In the present work, experiments were carried out with incubation periods of (i) 2 h at 70 °C, (ii) 1 h at 70 °C, (iii) 10 min at 100 °C, (iv) 20 min at 100 °C and (v) 10 min at 900 W. The resulting solutions were evaporated to dryness; the residue was redissolved with 100 µl of acetone and 1 µl of the final solution was injected into the GC.

2.4. Instrumentation and GC/MS parameters

Sonication was performed in a customized SONIFIER II W-450 sonication bath (BRANSON; Danbury, CT, USA) [16].

Liquid–liquid extraction was performed with a Zx^3 vortex mixer (VELP Scientifica, Milan, Italy).

Microwave-assisted derivatization was performed with a MARS microwave oven (CEM Corporation, Matthews, North Carolina, USA).

GC/MS analyses were performed in electron impact (EI) and electron capture negative ionization (ECNI), using Agilent 6890 N GC instruments operating in fast GC mode, coupled with either a 5973 *inert* or a 5975*inert* Mass Selective Detector (Agilent Technologies, Milan, Italy). A DB1MS column, of 5 m length × 0.10 mm I.D., 0.10 µm film thickness (Agilent Technologies, Milan, Italy) was used.

The GC injector operated at 250 °C in the split mode, with a split ratio of 1:20. Helium was used as the carrier gas at a constant flow of 0.8 ml/min (average velocity 83 cm/s). During the chromatographic run, the GC oven temperature was initially kept at 150 °C for 0.5 min, then increased at a rate of 50 °C/min to 300 °C and maintained at 300 °C for 1 min. The transfer line was kept at 280 °C.

The MS quadrupole temperature was maintained at 150 °C, while the MS ion volume was kept at 300 °C in the EI mode and 150 °C in the ECNI mode. In the ECNI mode, methane was used as the moderating gas at 40% flow. The full scan MS spectrum for each analyte (recorded in the m/z 75–450 range) was obtained from the corresponding diluted standard solution. From MS spectra, diagnostic ions for SIM experiments were extracted. Quantitative determinations were performed in the SIM mode. In order to collect sufficient data points along the GC peaks, the cycle time for SIM ion groups was reduced by applying short dwell times for each ion (25 ms).

2.5. Validation

2.5.1. Linearity

The linear calibration model was checked using urine samples spiked with standard solutions at five to seven concentration levels for each analyte. The calibration curves were obtained using the least squares regression method while the squared correlation coefficients (R^2) were utilized to estimate linearity.

2.5.2. Limits

Limit of detection (LOD) values were calculated on the target ion as the analyte concentrations providing a S/N value equal to 3, as determined by the Agilent MSD proprietary software ("Chemstation"). Sensitivity tests performed on spiked urine samples at concentration levels proximate to LOD confirmed the calculated values. The S/N value was also calculated at the lowest calibration level (LCL), defined as the lowest concentration providing a useful data point on the calibration curve for each analyte. The software determines the S/N for each analyte from the corrected signal (ratio between peak height and average noise) divided by the RMS noise (SQRT($\sum$(noise − average noise)^2)/points)). The noise was measured from −0.05 min before the peak onset till the beginning of the GC peak for each analyte.

2.5.3. Selectivity

Nine blank urine samples, obtained from different sources and pretreated with the most effective extraction and derivatization conditions, were analyzed to check for possible chemical and chromatographic interferences.

2.5.4. Extraction efficiency

Extraction efficiency was determined from negative urine samples spiked with standard working solutions, giving a final concentration of each analyte equal to the MRPL value (250 ng/ml). In addition three blank urine aliquots were prepared as extraction controls, using the procedure previously described. For quantitation, the peak areas of the analytes were corrected by the IS coefficient and then compared with the calibration straight lines. The results were expressed as the mean values of the three spiked samples subtracted by the corresponding mean values of the three blanks and extraction efficiencies were calculated in percentage.

2.5.5. Precision and accuracy

Intra assay precision (%) and accuracy (expressed as bias %) were evaluated by extracting and analyzing three replicates of urine samples spiked at the MRPL concentration, performed by three different operators. Inter assay precision (%) was determined on the mean value of nine replicates (three replicates for each operator). Calibration straight lines were obtained from spiked urine samples, as mentioned above.

2.6. GC/ECNI-MS experiments

In order to compare the relative sensitivities of EI-MS and ECNI-MS as pure instrumental responses for the various analytes, LOD values were obtained from experiments performed on aqueous standard solution mixtures at progressive dilution, after extraction and derivatization.

3. Results and discussions

3.1. Derivatization products

Alkylation is the chemical process in which an active hydrogen is replaced by an alkyl group. Carboxylic acids, alcohols, thiols, phenols, primary and secondary amines, amides and sulfonamides are the main functional groups amenable to alkylation reactions. For GC/MS analysis, alkylation (in particular methylation) makes the analytes more volatile and produces a molecular weight increase of 14 u, for each active hydrogen that is replaced.

GC analysis of diuretics requires a preliminary alkylation reaction, due to the polar character of the functional groups present in their structures. Reaction with methyl iodide and dry potassium carbonate allows the methylation of amines, sulfonamides, carboxylic and hydroxylic groups. Previous studies showed that protracted incubation of the reaction mixture at 70 °C is requested in order to achieve derivatization of the diuretics containing sulfonamide or amino groups [16]. Alternatively, an increase of the derivatization reaction rate can be achieved under microwave irradiation [3].

3.2. Fast GC/MS characterization

The chromatographic separation of the diuretic derivatives mixture was initially optimized using two solutions of nine and eight analytes, respectively. In the final conditions, two couples of derivatives (hydrochlorthiazide/bumetanide and indapamide/triamterene) exhibited coincident retention times. From full scan mass spectra, three ions for each derivative (two in the case of bendroflumethiazide) were selected for the subsequent SIM experiments. The relative abundances of these characteristic ions (qualifier ions vs. target ion) along the GC peak, together with the coincidence of their retention times, were used for the positive identification of the 17 derivatives. The mass spectra of coeluting compounds did not show common fragment ions, enabling the creation of a single GC/MS-SIM method for the separation and quantitation of all 17 analytes. Table 1 reports retention times and selected ions, used for the identification of diuretics methyl derivatives in the EI mode. Fig. 2 shows typical GC/EI-MS chromatograms, obtained in SIM mode for the two mixtures of analytes at the highest calibration level concentration. It is noteworthy that the entire GC/MS run is completed in less than 4 min. Two more minutes are needed for the oven cool down, temperature equilibration and injection.

Table 1. Molecular weights of the 17 analytes and their methyl derivatives, GC retention times, characteristic ions (EI mode) used in SIM experiments and the corresponding retention time windows

	Analytes	Analyte molecular weight	Number of active hydrogens	Methyl derivative molecular weight	t_R(min)	Ions (m/z) EI		RT time window
						Base	Others	
1	Acetazolamide	222	3	264	1.41	249	108, 264	1.00–1.52
2	Probenecid	285	1	299	1.66	270	135, 199	1.52–1.69
3	Ethacrynic acid	303	1	316	1.74	261	243, 281	1.69–2.04
4	Hydroflumethiazide	331	4	387	2.48	387	322, 344	2.04–2.54
5	Amiloride	229	8	341	2.50	225	239, 268	2.04–2.54
6	Chlorthiazide	295	3	339	2.56	275	220, 248	2.54–2.60
7	Clopamide	345	2	373	2.66	111	127, 358	2.60–2.72
8	Furosemide	330	3	372	2.70	372	339, 357	2.60–2.72
I.S.	Mefruside	411	2	439	2.75	325	282, 218	2.72–2.84
9	Chlorthalidone	338	4	394	2.80	363	176, 287	2.72–2.84
10	Hydrochlorthiazide	297	4	353	2.88	310	138, 353	2.84–2.94
11	Bumetanide	364	3	406	2.89	363	254, 406	2.84–2.94
12	Indapamide	365	3	407	3.00	161	132, 407	2.94–3.06
13	Triamterene	253	6	337	3.01	336	279, 322	2.94–3.06
14	Trichlormethiazide	380	4	435	3.12	352	354, 399	3.06–3.19
15	Canrenoic acid	340	–	–	3.25	267	325, 340	3.19–3.40
16	Bendroflumethiazide	421	4	477	3.30	386	278	3.19–3.40
17	Althiazide	383	4	439	3.50	352	244, 354	3.40–4.00

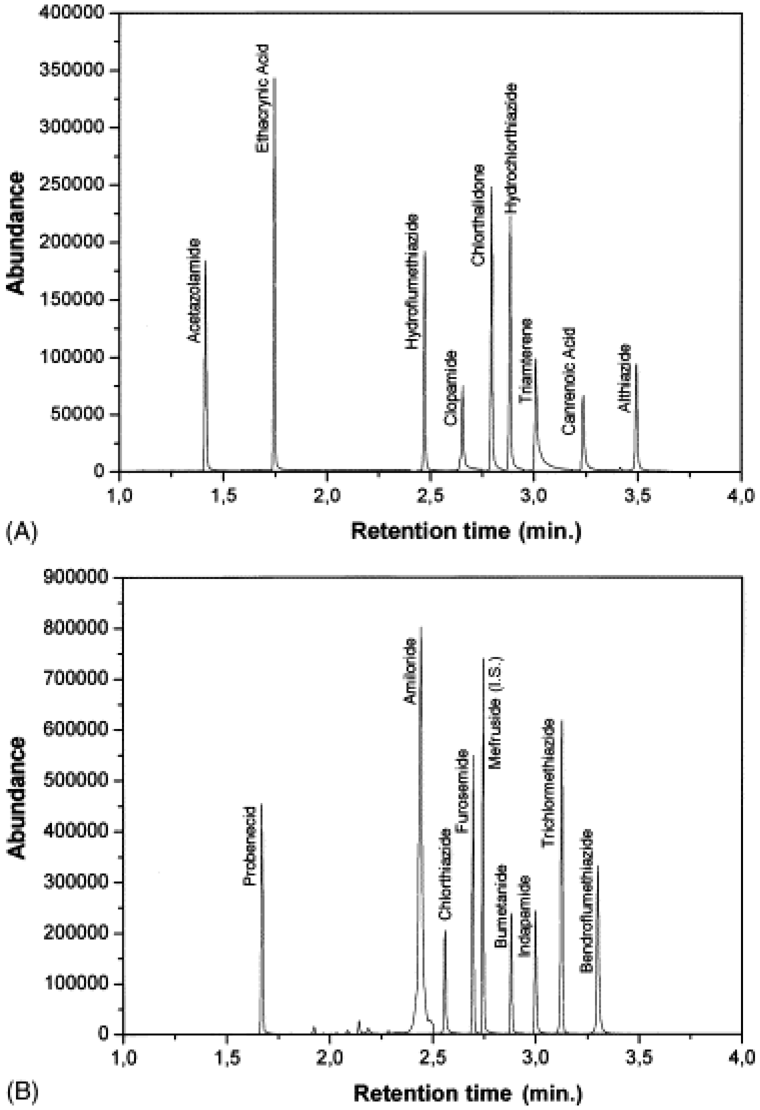

Fig. 2. Fast GC/EI-MS SIM chromatograms of mixture 1 (a) and mixture 2 (b) at the highest calibration level concentration.

The SIM protocol described in Table 1 was used to build the calibration graphs for the 17 analytes. Peak areas from the chromatographic profiles of target and qualifier ions were plotted against five to seven concentration levels, followed by linear regression of data points (see Table 2). The range of concentrations studied was planned according to the response factors obtained from the analytes. The concentration levels used for calibration included the MRPL value for diuretics fixed by the WADA (250 ng/ml), with the notable exception of amiloride, which proved undetectable at this concentration level.

Table 2. Calibration levels concentrations; gradients, intercepts and R^2 values for calibration curves obtained using a linear model; LOD and S/N at LCL values for the methyl derivatives of the 17 analytes

	Analytes	Calibration level concentration (ng/ml)	Gradient (area counts)	Intercept (area counts ng^{-1} ml)	R^2	LOD (ng/ml)	S/N at LCL
1	Acetazolamide	2000 1000 500 200 100	2.03	1100	0.998	11.0	27.3
2	Probenecid	500 250 100 50 25 6.25 1.25	571	1920	1.000	0.1	46.6
3	Ethacrynic acid	2000 1000 500 200 100 50 12.5	3.12	897	0.997	3.1	12.0
4	Hydroflumethiazide	2000 1000 500 200 100 50 12.5	8.37	−55.9	0.991	1.8	20.8
5	Amiloride	10000	32.1	0	1.000	2800	10.7
6	Chlorthiazide	4000 2000 1000 400 200 100	5.56	1960	0.994	28.9	10.4
7	Clopamide	2000 1000 500 200 100 50 12.5	20.7	1310	0.992	2.3	16.1
8	Furosemide	2000 1000 500 200 100 50 12.5	59.1	2010	0.999	1.1	33.0
9	Chlorthalidone	2000 1000 500 200 100 50	6.53	614	1.000	6.4	23.3
10	Hydrochlorthiazide	2000 1000 500 200 100	6.97	178	0.999	12.2	24.5
11	Bumetanide	1000 500 200 100 50 12.5 2.5	201	529	1.000	1.3	12.5
12	Indapamide	1000 500 200 100 50 12.5 2.5	278	2450	1.000	0.7	25.5
13	Triamterene	2000 1000 500 200 100 50 12.5	11.2	1300	0.996	1.2	30.7
14	Trichlormethiazide	10000 5000 2500 1000 500 250 62.5	1.27	165	1.000	15.5	12.1
15	Canrenoic acid	4000 2000 1000 400 200 100 25	15.8	−348	0.997	6.2	12.1
16	Bendroflumethiazide	1000 500 200 100 50 12.5 2.5	191	10600	0.994	0.6	29.9
17	Althiazide	2000 1000 500 200 100 50 12.5	9.38	55.9	0.999	1.1	33.7

LCL values are assumed as the lowest calibration level concentration used.

Table 2 reports gradient, intercept and R^2 values for each analyte regression line, together with calculated LOD and S/N values at the lowest calibration level. R^2 values ranged from 0.991 to 1.000 indicating good fit and linearity of the calibration curves. For most diuretics, LOD values proved significantly lower than the MRPL value. In particular, for most of the analytes (**2**, **3**, **4**, **7**, **8**, **11**, **12**, **13**, **16** and **17**) LOD values were lower than 5 ng/ml, while for all the other analytes the limits lie in the 5–30 ng/ml interval, with the exception of **5**, for which a LOD of about 2800 ng/ml was calculated. The S/N values reported in Table 2 represent another estimation of the method sensitivity toward the different analytes. These values inversely correlate with LODs, as expected. The individual sensitivity for diuretics is likely to depend on a combination of mass spectral features and reactivity toward the derivatizing agent. For example, the unsatisfactory sensitivity of the procedure toward **5** is possibly due to the presence of a large set of polar hydrogens in its structure, which on one side limits the yield of the complete derivatization product and, on the other, prevents the GC elution of a partially underivatized molecule. A different example is provided by the comparison of **14** and **16**, which have similar structures and polar hydrogens, but exhibit considerably different response factor (LODs are 15.5 and 0.6 ng/ml, respectively). In this case, the discrepancy is likely to depend on their different spectrometric properties.

3.3. Validation

The protocol developed in the present work involves three analytical steps, including (1) liquid/liquid extraction of the analytes from the matrix, referred as Method 1 in Section 2, (2) derivatization with methyl iodide at 70 °C for 2 h, (3) analysis of the resulting mixture by fast GC/EI-MS. Both steps (1) and (2) present only slight modifications of traditional and well-assessed methods, while in step (3) a fully innovative approach is proposed. Therefore, in the validation procedure particular attention has been paid to the instrumental determination step.

3.3.1. LOD, S/N at LCL, linearity range

The experimental results relative to limits of detection, S/N at lowest calibration level, as well as linearity ranges, has been presented and discussed in the preceding sections of this paper (in particular, Table 2).

3.3.2. Selectivity

The selected ion chromatogram profiles obtained from nine blank urine samples provided by different donors did not show the presence of any significant signal (S/N < 3) at the typical retention times of the studied compounds, indicating that the method is selective for all 17 diuretics and no interfering substances are present in the biological matrix.

3.3.3. Precision and accuracy

The International Standard for Laboratories [19] promulgated by WADA does not report any protocol for determining the precision and accuracy of test methods validated for non-threshold substances. For this reason, standard criteria for measuring intra- and inter-assay bias % and precision % were adopted and acceptance limits were set at ±30% and <30%, respectively. A large set of experiments was conducted by three operators, in order to evaluate accuracy and repeatability. The corresponding results are reported inTable 3.

Table 3. Accuracy (bias %), intra-assay precision (%), percentage recovery (ng/ml), recovery (%) for each analyte from three different operators

	Analytes	Real conc. (ng/ml)	Operator 1				Operator 2				Operator 3				Inter-assay precision (%)	Total recovery (%)
			Recovery (ng/ml)	Bias (%)	Recovery (%)	Intra-assay precision (%)	Recovery (ng/ml)	Bias (%)	Recovery (%)	Intra-assay precision (%)	Recovery (ng/ml)	Bias (%)	Recovery (%)	Intra-assay precision (%)		
1	Acetazolamide	250	290 ± 57	16.1	116.1	13.9	242 ± 69	−3.2	96.8	28.5	269 ± 6	7.6	107.6	2.1	28.9	106.8
2	Probenecid	250	225 ± 25	−10.1	89.9	8.8	226 ± 79	−9.6	90.4	34.9	221 ± 159	−11.7	88.3	72.1	13.1	89.5
3	Ethacrynic acid	250	229 ± 66	−8.5	91.5	18.1	292 ± 12	16.8	116.8	4.2	247 ± 62	−1.2	98.8	25.2	19.3	102.4
4	Hydroflumethiazide	250	269 ± 16	7.7	107.7	4.4	202 ± 19	19.1	80.9	9.5	278 ± 64	11.0	111.0	23.2	29.6	99.9
5	Amiloride	250	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
6	Chlorthiazide	250	251 ± 16	0.6	100.6	6.5	258 ± 64	3.3	103.3	24.7	220 ± 94	−12.1	87.9	42.6	8.3	97.2
7	Clopamide	250	275 ± 125	10.0	110.0	19.2	284 ± 55	13.6	113.6	19.5	317 ± 88	26.6	126.6	27.9	48.6	116.7
8	Furosemide	250	203 ± 20	−18.8	81.2	7.1	239 ± 82	−4.4	95.6	34.1	232 ± 185	−7.3	92.7	79.8	9.6	89.8
9	Chlorthalidone	250	290 ± 25	15.9	115.9	6.5	94 ± 18	−62.4	37.6	19.4	126 ± 26	−49.8	50.2	20.9	79.5	67.9
10	Hydrochlorthiazide	250	232 ± 13	−7.3	92.7	5.1	175 ± 4	−30.0	70.0	2.3	282 ± 44	12.8	112.8	15.6	23.6	91.8

	Analytes	Real conc. (ng/ml)	Operator 1				Operator 2				Operator 3				Inter-assay precision (%)	Total recovery (%)
			Recovery (ng/ml)	Bias (%)	Recovery (%)	Intra-assay precision (%)	Recovery (ng/ml)	Bias (%)	Recovery (%)	Intra-assay precision (%)	Recovery (ng/ml)	Bias (%)	Recovery (%)	Intra-assay precision (%)		
11	Bumetanide	250	280 ± 62	12.0	112.0	20.0	235 ± 93	−5.9	94.1	39.7	218 ± 175	−12.7	87.3	80.1	19.2	97.8
12	Indapamide	250	245 ± 32	−2.0	98.0	12.2	232 ± 86	−7.3	92.7	37.1	207 ± 162	−17.2	82.8	78.4	12.4	91.2
13	Triamterene	250	274 ± 23	9.7	109.7	6.8	66 ± 33	−73.5	26.5	50.2	229 ± 4	−8.4	91.6	1.9	64.3	76.0
14	Trichlormethiazide	250	215 ± 40	−14.1	85.9	18.5	259 ± 93	3.6	103.6	35.8	235 ± 98	6.2	93.8	41.8	9.3	94.5
15	Canrenoic acid	250	204 ± 34	−18.4	81.6	10.6	293 ± 17	17.3	117.3	5.8	187 ± 30	−25.4	74.6	16.3	27.1	91.2
16	Bendroflumethiazide	250	274 ± 41	9.7	109.7	13.2	200 ± 93	−19.9	80.1	46.5	203 ± 141	−18.7	81.3	69.5	27.2	90.4
17	Althiazide	250	270 ± 36	8.0	108.0	9.9	207 ± 9	−17.2	82.8	4.4	260 ± 33	4.0	104.0	12.5	29.5	98.3

Inter-assay precision (%) and total recovery (%) for each analyte.

Typically, the experimental bias (%) values were found in the range between −30% and +30%, fulfilling the accuracy criteria adopted, with the exception of three anomalous data (Table 3). In particular, operator 1 obtained bias (%) values lower than ±20% for all the analytes, while operator 2 obtained two outliers (namely, −62.4% for **9** and −73.5% for **13**), one result at the lower acceptance limit (−30.0% for **10**) and all the other data in the −20% to +20% interval. Also in the case of operator 3, bias (%) data were generally lower than ±20%, with one anomalous result (−49.8%, again for **9**). Also in the evaluation of intra assay precision (%), operator 1 obtained the closest results, with all precision values below 20%. Operators 2 and 3 obtained more disperse results, specifically for **2**, **8**, **11**, **12**, **14** and **16** (plus **13** only from operator 2 and **6** for operator 3). For the remaining compounds, both operators achieved precisions below 30%. In a general perspective, the repeatability of the analytical method proved satisfying, as the inter assay precision (%) was below 30%, for 13 out of 16 compounds detected at the MRPL level. The remaining diuretics (**7**, **9** and **13**) were still detectable in all the experiments performed, i.e. all samples would turn out positive in the standard screening procedure.

3.3.4. Extraction efficiency

From averaged quantitative results, the mean extraction recovery (%) was calculated for each operator (Table 3) and compared with the others. The total recovery (%) was obtained as the mean value from the nine determinations made for each analyte.

Mean extraction recoveries (%) ranged between 81.2% and 116.1% in the results from operator 1. The results from operators 2 and 3 were slightly worse, ranging in the interval 70.0–117.3% for operator 2 and 74.6–126.6% for operator 3. Two outliers were found by operator 2 (37.6% for **9** and 26.5% for **13**) and one by operator 3 (50.2% for **9**). These results confirm once more the difficulties already encountered in determining accuracy and precision for these two compounds (see above).

3.4. Derivatization

The experimental conditions traditionally used in the derivatization with methyl iodide [17] were investigated, in the effort to shorten the time requested for sample preparation. Along with fast GC, these experiments strive for the reduction of the total analysis time. Table 4 reports the results obtained using the derivatization temperature indicated in the literature (70 °C) [17] at 1 and 2 h incubation time, respectively, in comparison with those obtained at 100 °C and a drastically decreased incubation time (10 min). The concentrations were calculated from peak area responses using a calibration curve build on the traditional procedure (70 °C for 3 h); the results were then compared with the expected concentrations (250 ng/ml for each analyte). The data reported in Table 4 are mean values, calculated from derivatization of three different aliquots of the 17 analytes mixture, with the corresponding standard deviations.

Table 4. Comparison of three different derivatization conditions (incubation time and temperature) on the reaction yields

	Analytes	Real concentration (ng/ml)	10 min at 100 °C (ng/ml)	1 h at 70 °C (ng/ml)	2 h at 70 °C (ng/ml)
1	Acetazolamide	250	282 ± 38	241 ± 22	244 ± 18
2	Probenecid	250	135 ± 38	257 ± 72	259 ± 18
3	Ethacrynic acid	250	103 ± 60	261 ± 10	282 ± 65
4	Hydroflumethiazide	250	104 ± 26	247 ± 19	258 ± 31
5	Amiloride	250	N.D.	N.D.	N.D.
6	Chlorthiazide	250	N.D.	N.D.	191 ± 69
7	Clopamide	250	N.D.	221 ± 54	245 ± 52
8	Furosemide	250	73 ± 10	136 ± 59	174 ± 38
9	Chlorthalidone	250	51 ± 13	227 ± 60	186 ± 19
10	Hydrochlorthiazide	250	53 ± 14	146 ± 32	168 ± 9
11	Bumetanide	250	77 ± 25	151 ± 61	189 ± 26
12	Indapamide	250	111 ± 12	207 ± 46	243 ± 30
13	Triamterene	250	46 ± 10	252 ± 43	258 ± 54
14	Trichlormethiazide	250	N.D.	N.D.	311 ± 96
15	Canrenoic acid	250	No active hydrogens		
16	Bendroflumethiazide	250	54 ± 12	141 ± 43	178 ± 73
17	Althiazide	250	223 ± 7	215 ± 80	234 ± 87

The results are expressed as mean concentration values of three replicates with the corresponding standard deviation.

The quantitative results obtained in the experiments carried out with an incubation time of 10 min and temperature of 100 °C were generally lower than the corresponding ones obtained under the other derivatization conditions tested, even if most of the analytes were still detectable. On the other hand, the apparently higher yields recorded for **1** and **17** fall within the experimental error. Lastly, four of the analytes (**5**, **6**, **7** and **14**) were not detected using these drastic derivatization conditions. In general, it is concluded that 10 min at 100 °C represent an insufficient incubation time for the derivatization to reach completion, at least without further enhancement from microwave assistance [3].

Derivatizations occurring after 2 h incubation produce systematically higher results than those obtained from 1 h incubation (with the exception of chlortalidone), even if most differences fall within the experimental error. Moreover, **6** and **14** were not derivatized after 1 h incubation, while no major difference was detected between 2 and 3 h incubation. In fact, the results reported in the last column of Table 4 match the theoretical concentrations, within the experimental error. Amiloride (**5**) could not be detected in any conditions since its LOD is more than ten times higher than the MRPL concentration considered. In conclusion, the results obtained by reducing the derivatization time from 3 to 2 h confirm that all diuretics could be easily detected under the new conditions and also the quantitative determinations turned out reasonably close to the real values. This offers the chance to slightly reduce the duration of the sample pretreatment step.

3.5. Comparison among five different combinations of extraction procedures and derivatization conditions

In order to optimize and possibly speed up the whole sample preparation step, five different combinations of extraction procedures and derivatization conditions (tests A–E) were compared under severe conditions, i.e. performing the analyses on an urine sample spiked with a mixture of the 17 analytes each at the lowest calibration level (as defined in Table 2). In test A, the general experimental conditions described in the present work were adopted, in which liquid/liquid extraction of the analytes from the matrix (Method 1, Section 2) was followed by derivatization with methyl iodide for 2 h at 70 °C. In test B, liquid/liquid extraction was followed by derivatization with methyl iodide for 20 min at 100 °C. In tests C and D the extraction step was executed respectively by means of SPE cartridges (Method 2, Section 2) and ultrasonic assistance (Method 3, Section 2), while the derivatization was performed as in test A. In test E, a regular liquid/liquid extraction was followed by derivatization under microwave-assisted incubation (10 min at 900 W). Table 5 reports the results obtained from tests A–E and expressed as (i) S/N values at the LCL and (ii) calculated LOD values. The results show that the total series of 17 analytes was detected only in test A, since amiloride (**5**) was not detected with any of the other methods (B–E) at the LCL. Analyte **14** was not detected in the case of test B, while neither **6** nor **14** were detected in test E. Moreover, as many as seven analytes (**1**, **3**, **7**, **8**, **13** and **15**) could not be detected in test D. The lowest LOD values obtained for the various analytes were spread among different tests, including test A (**2**, **4**, **5**, **13**, **14** and **15**), test B (**3** and**7**), test C (**6** and **10**) and test E (**1**, **2**, **8**, **9**, **11**, **12** and **16**).

Table 5. Comparison among S/N at LCL and calculated LOD values for the methyl derivatives of the 17 analytes, obtained from five different combinations of extraction procedures and derivatization conditions

	Analyte	Test A		Test B		Test C		Test D		Test E	
		S/N at LCL	LOD	S/N at LCL	LOD	S/N at LCL	LOD	S/N at LCL	LOD	S/N at LCL	LOD
1	Acetazolamide	27.3	11.0	61.2	4.9	83.3	3.6	N.D.	N.D.	100.0	3.0
2	Probenecid	46.6	0.1	3.4	1.1	18.8	0.2	3.5	1.1	37.5	0.1
3	Ethacrynic acid	12.0	3.1	18.8	2.0	8.2	4.6	N.D.	N.D.	8.3	4.5
4	Hydroflumethiazide	20.8	1.8	13.9	2.7	8.7	4.3	2.5	15.2	19.7	1.9
5	Amiloride	10.7	2800	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
6	Chlortiazide	10.4	28.9	2.7	109.1	30.3	9.9	9.8	30.6	N.D.	N.D.
7	Clopamide	16.1	2.3	17.0	2.2	8.2	4.6	N.D.	N.D.	8.3	4.5
8	Furosemide	33.0	1.1	22.1	1.7	18.8	2.0	N.D.	N.D.	93.8	0.4
9	Chlortalidone	23.3	6.4	60.0	2.5	18.8	8.0	2.5	60.0	166.7	0.9
10	Hydrochlorthiazide	24.5	12.2	46.2	6.5	125.0	2.4	5.5	54.1	9.3	32.1
11	Bumetanide	12.5	1.3	0.8	9.9	12.5	0.6	2.6	2.9	18.8	0.4
12	Indapamide	25.5	0.7	2.2	3.4	3.8	2.0	1.8	4.2	37.5	0.2
13	Triamterene	30.7	1.2	19.7	1.9	7.8	4.8	N.D.	N.D.	22.1	1.7
14	Trichlormetiazide	12.1	15.5	N.D.	N.D.	4.1	45.2	3.8	50.0	N.D.	N.D.
15	Canrenoic acid	12.1	6.2	6.8	11.1	9.6	7.8	N.D.	N.D.	5.5	13.6
16	Bendroflumethiazide	29.9	0.6	2.3	3.3	4.2	1.8	1.9	4.0	15.0	0.5
17	Althiazide	33.7	1.1	18.8	2.0	15.0	2.5	1.9	20.1	10.7	3.5

Test A: liquid–liquid extraction, derivatization 70 °C 2 h; Test B: liquid–liquid extraction, derivatization 100 °C 20 min; Test C: SPE extraction, derivatization 70 °C 2 h; Test D: ultrasonic-assisted extraction, derivatization 70 °C 2 h; Test E: liquid–liquid extraction, microwave-assisted derivatization 900 W 10 min.

By considering that most of the analytes could be detected at low concentration level with any of conditions adopted, with the exception of ultrasonic extraction (D), and further improvement of the experimental conditions is likely to be feasible for SPE extraction and microwave-assisted derivatization, it is confirmed that speeding up the sample preparation steps is an achievable task, as the studies of Goebel et al. [7], Amendola et al. [3] and Beyer et al. [20] already demonstrated.

On the other hand, the conservative sample preparation procedure adopted in the present study proved to provide the most effective and robust conditions to highlight the consistency of the novel instrumental approach.

3.6. Comparison between GC/EI-MS and GC/ECNI-MS data

The analytical procedures published in the scientific literature for the GC/MS screening of diuretics and masking agents report the use of EI as the MS ionization technique of choice. Most of the experiments described in the present work were also performed in EI, so that direct comparison with literature results and analytical performances is possible, taking into account that the main focus of the present study is addressed to the optimization of the chromatographic conditions.

The presence of electron withdrawing substituents and/or large systems of conjugated double bonds in most of the 17 diuretics considered, induced us to evaluate electron capture negative ionization (ECNI) as an alternative ionization technique. In particular, some samples were analyzed using EI and ECNI consecutively (i.e. within few hours), so that straightforward comparison of the sensitivity for the two ionization techniques toward the different analytes could be established. In one-half of the experiments, the order in which the two techniques were applied on the same set of samples was reversed, in order to exclude the occurrence of sample degradation phenomena. At a first glance, the profiles of the summed ion chromatograms obtained in the two ionization modes (Fig. 3) show that approximately the same peaks are present, even if the relative peak heights are considerably different. Table 6 reports the most abundant ions observed in ECNI mass spectra (used in SIM experiments), together with experimental LOD data values and the corresponding LODs obtained in EI from aqueous standard solution mixtures. Bendroflumethiazide (**16**) could not be detected in ECNI, at a concentration of 100 ng/ml. On the other hand, ECNI provided lower LOD values than EI, with the exception of **9** and **12**, while for **2** and **11** the same LODs were obtained for both ionization techniques.

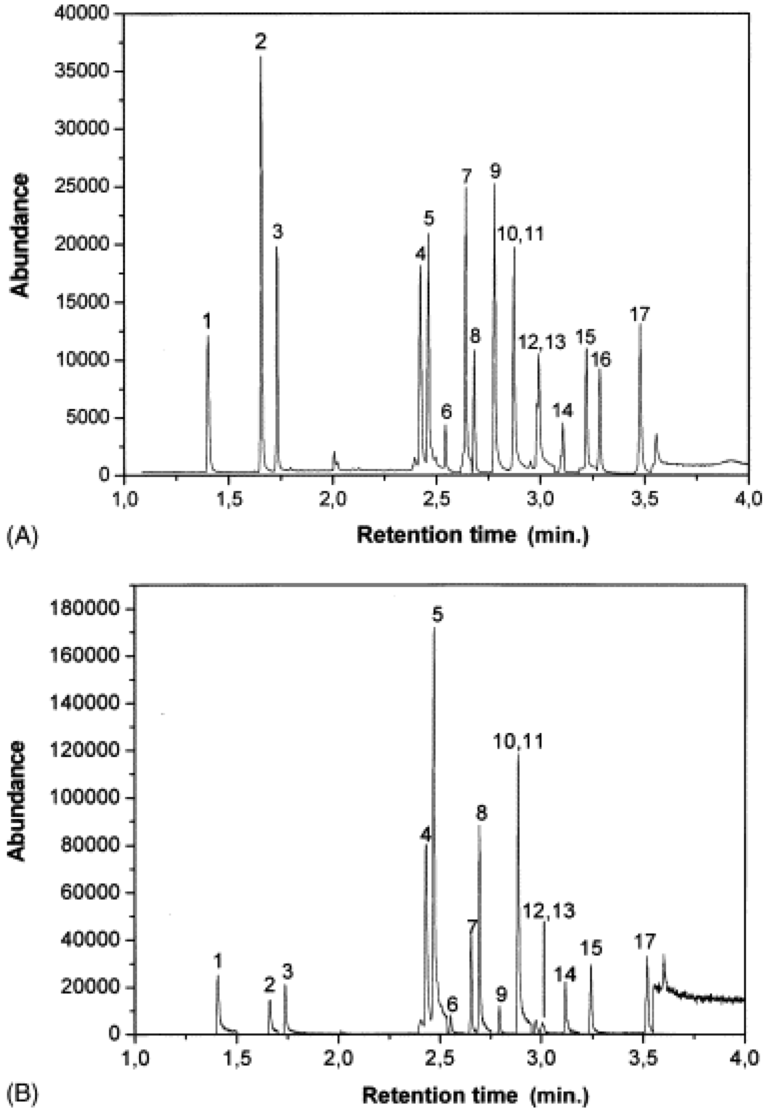

Fig. 3. Fast GC/MS SIM chromatograms of the methyl derivatives of the 17 analytes in (a) EI mode and (b) ECNI mode at the fourth calibration level concentration (i.e. 100–200 ng/ml for most analytes).

Table 6. ECNI characteristic ions and comparison between experimental LOD values in ECNI and EI for the analytes methyl derivatives

	Analytes	Ions (*m/z*) ECNI	LOD (ng/ml) ECNI	LOD (ng/ml) EI
1	Acetazolamide	220, 222	2	10
2	Probenecid	199, 299	0.2	0.2
3	Ethacrynic acid	207, 243	2	5
4	Hydroflumethiazide	343, 387	1	2
5	Amiloride	254, 268	100	2000
6	Chlorthiazide	312, 340	5	50
7	Clopamide	266, 330	2	5
8	Furosemide	329	1	2
9	Chlorthalidone	351	5	2
10	Hydrochlorthiazide	310	2	20
11	Bumetanide	269, 362, 406	2	2
12	Indapamide	275, 364	5	2
13	Triamterene	337	5	5
14	Trichlormethiazide	358	10	50
15	Canrenoic acid	340	2	20
16	Bendroflumethiazide	ND	ND	1
17	Althiazide	370, 396	1	2

These ECNI-MS results are very encouraging, especially for amiloride (**5**) and trichlormethiazide (**14**), for which LOD values were lowered below the MRPL required from WADA. Easy switching between the two ionization techniques, whenever possible, in combination with two consecutive fast GC/MS runs, may provide a rapid way to perform sensitive screening of all seventeen diuretics in human urine samples.

4. Conclusions

The introduction of fast GC in GC/MS antidoping screening procedures drastically reduces the time needed for the instrumental determination step of the analytical protocol, without sacrificing the chromatographic resolution nor the accuracy and precision of the analysis. This technique is made available to benchtop quadrupole mass spectrometers by the modern electronics controlling the quadrupole mass analyzer, which has considerably shortened dwell times and rest periods, enabling sufficient data-point sampling along the GC peaks. These principles have found clear demonstration in the present study, where fast GC/MS was applied in a protocol aimed to the determination of diuretics and masking agents in human urine. Optimal sensitivity, selectivity and range of linearity were observed for this class of analytes, together with good repeatability of quantitative determinations, taking into account that accurate concentration measurements are out of the scope of screening procedures applied to antidoping control.

A parallel reduction of the time requested for preliminary sample treatments appears to be possible, as we partially demonstrated, by reproducing the experimental conditions previously developed by other authors[3], [7] and [20].

It is predictable that the development of fast GC/MS procedures will be extended to the other screening methods used in anti-doping controls. In fact, the ability of fast instrumental processing of samples seems crucial in this analytical area, where high productivity is requested, both for method validations and high sample throughput.

We also made evident that ECNI may find increased application in the mass spectrometric determination of diuretics, both in combination with EI, during the screening step of anti-doping controls, as well as within confirmation protocols developed for specific diuretics.

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