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Short communication

Application of high-performance liquid chromatography–mass spectrometry to detection of diuretics in human urine

Y. Qin[a,b], X.B. Wang[b], C. Wang[a], M. Zhao[a], M.T. Wu[b], Y.X. Xu[b], S.Q. Peng[a,*]

[a]*College of Pharmaceutical Sciences, Peking University, Beijing 100083, China*
[b]*China Doping Control Center, National Research Institute of Sports Medicine, Beijing 100029, China*

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Abstract

A rapid, sensitive and reliable high-performance liquid chromatographic–mass spectrometric method for the detection of 25 diuretics in human urine has been developed. Atmosphere pressure chemical ionization (APCI) and electrospray ionization (ESI) modes were evaluated. A 2-ml volume of urine was extracted under basic conditions and separated on an Agilent Zorbax SB-C_{18} column (150×2.1 mm, 5 μm). The mobile phase consisted of formic ammonium–formic acid buffer (pH 3.5) and acetonitrile. The effects of capillary temperature, sheath gas pressure and compositions of mobile phase on the sensitivity were studied. The recoveries of most of the diuretics were 75–95%. In the full scan mode, the limits of detection of the 25 diuretics were 0.25–25 ng/ml for APCI and 0.6–250 ng/ml for ESI. Under the optimal conditions, 14 diuretics from authentic urine samples were detected successfully by LC–APCI-MS. To obtain more fragmentation information on the chemical structure for positive confirmation, tandem mass analysis was also investigated.

Keywords: Diuretics

1. Introduction

Diuretics have been banned in sport since the 1988 Olympic Games. As therapeutic agents, however, diuretics are used to eliminate tissue liquids and enhance renal excretion of salt and water [1]. In sport, diuretics are misused to reduce the body mass of athletes and to lower the urine concentration of other doping agents [2,3]. In addition, the use of carbonic anhydrase inhibitor diuretics results in an alkaline urine and consequently the reduction of the excretion of basic drug metabolites [4]. As one of the first class substances diuretics have been banned in sport and have to be monitored both in and out of competition.

With a wide variety of structures and individual physicochemical properties [5], e.g. the pK_a value covers a range of 3.9–9.5, the screening procedure for diuretics in human urine is mainly based on HPLC with UV detection [6–9] or gas chromatography–mass spectrometry (GC–MS) [10,11]. The screening of diuretics in urine using HPLC with spectral detection suffers from the disadvantage that it is not sufficiently specific and hence is limited in the qualitative information, which is required for a positive confirmation. Chromatography with mass

*Corresponding author. Tel.: +86-10-6209-2482; fax: +86-10-6209-2311.
E-mail address: sqpeng@mail.bjmu.edu.cn (S.Q. Peng).

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selection detection provides a specific means of screening for diuretics, in which the mass spectral fragmentation pattern provides the essential qualitative information for a positive confirmation. However, the polar nature of most diuretics makes it impossible to determine these drugs by direct GC without time-consuming chemical derivatization [10,11].

One successful alternative to GC–MS is LC–MS. LC–MS allows direct separation of parent compounds with concurrent structural information without chemical derivatization. In the literature a few diuretics in biological fluids have been analyzed by LC–MS but the reported sensitivity is relatively low [12–16]. In the present paper we describe a rapid, sensitive and reliable high-performance liquid chromatography–mass spectrometry method for the detection of 25 diuretics in human urine, of which the structures are listed in Fig. 1.

2. Experimental

2.1. Chemicals

The diuretics **2**, **7**, **8**, **10**,**12**, **16**, **19** and **22** were purchased from Sigma; **1**, **3**, **4**, **5, 6** and **14** were provided by Canada INRS-Santè Laboratory; **9**, **11**, **13**, **15**, **17**, **20**, **21** and **23** were provided by Korea Anti-doping Laboratory and **18**, **24** and **25** were provided by Köln Anti-doping Laboratory. HPLC Grade acetonitrile was purchased from Fisher. Deionized water was prepared with Milli-Q water purification system. Methanol, ammonium formate and formic acid were purchased from Beijing Chemical (China).

2.2. Equipment

Finnigan TSQ7000 series system mass spectrometer was used with Finnigan LC–MS interfaces (APCI and ESI). A Hewlett-Packard Model 1090 system was used for HPLC separation of the diuretics and delivery of the eluent to the mass spectrometer. The HPLC column used was Agilent Zorbax SB-C_{18} reversed-phase column (150×2.1 mm, 5 μm). An LC flow-rate of 0.25 ml/min was used in both the APCI and ESI modes. The mobile phase consisted of the buffer of 0.01 *M* of ammonium formate–formic acid (A, pH 3.5) and acetonitrile (B). The gradient program was that after 2 min 10% B was increased to 40% over 15 min, increased to 70% over 3 min and held for another 5 min. The MS data were collected and processed using a Finnigan ICIS data system. Full scan mass spectra from m/z 100–550 amu were obtained in 1 s with the electron multiplier voltage (EMV) set at 1000 V. The typical interface parameters for the best sensitivity were: for APCI, the vaporizer temperature 450 °C, the heated capillary temperature 250 °C and the sheath gas pressure 40 p.s.i. (1 p.s.i. = 6894.76 Pa); for ESI, the heated capillary temperature 300 °C, the sheath gas pressure 60 p.s.i. and the auxiliary gas flow 20 l/min. The spray voltage was kept at 4500 V in the ESI mode. The CID offset for tandem mass analysis (MS–MS) was set at 30 eV. The screen procedure was 0–5 min for the positive ion mode, 5–7 min for the negative ion mode, 7–8 min for the positive ion mode, 8–20.5 min for the negative ion mode and 20.5–25 min for the positive ion mode.

2.3. Preparation of standard solutions

A stock standard solution of an individual diuretic with a concentration of 1–5 mg/ml was prepared by dissolving the corresponding diuretic in methanol and storing at 4 °C. The stock standard solution of the mixture of 25 diuretics including **13** (internal standard) was prepared by mixing each stock standard solution of the individual diuretic, of which the final concentrations for **1**–**25** were 64, 58, 46, 114, 357, 224, 19, 128, 96, 129, 114, 71, 89, 108, 143, 90, 428, 50, 193, 143, 150, 108, 129, 99.3 and 64 ng/μl, respectively.

2.4. Urine sample collection

The studied substances were chosen according to the list of banned substances in the Olympic Movement Anti-doping Code, such as **1–3**, **7**–**10**, **12**, **16**, **19**, **21**–**23** and **25**, which need to be detected emphatically in routine analysis. In accordance with the principle of Public Health Bureau of Beijing the healthy volunteers were administered orally a single dose of 5, 25, 50, 10, 40, 2.5, 40, 5, 50, 5, 20, 50, 50 and 50 mg of diuretics **1–3**, **7**–**10**, **12**, **16**, **19**, **21**–**23** and **25**, respectively and urine samples were collected at regular intervals.

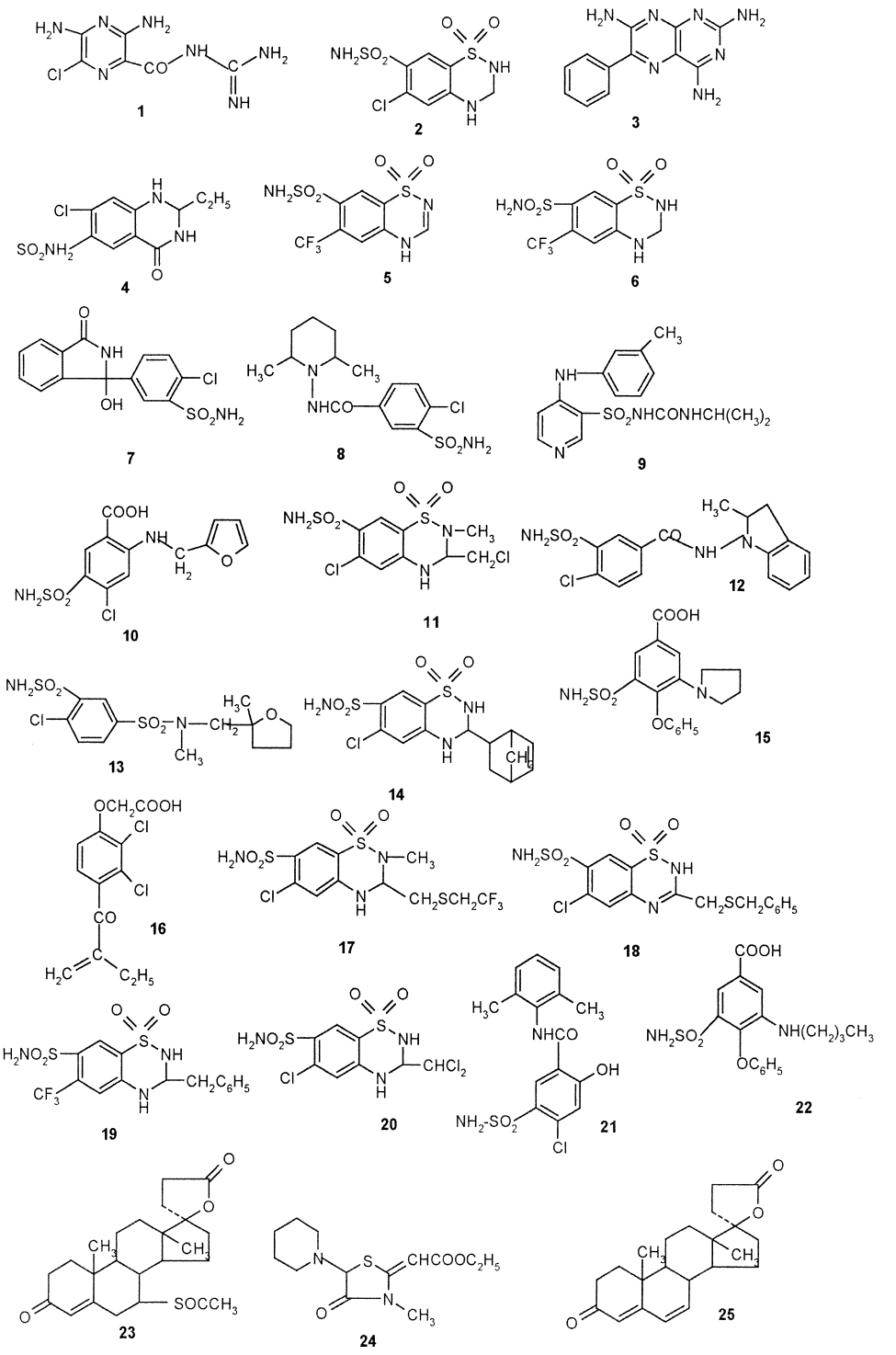

Fig. 1. Chemical structures of the diuretics. **1** = Amiloride; **2** = Hydrochlorothiazide; **3** = Triamterene; **4** = Quinethazone; **5** = Flumethiazide; **6** = Hydroflumethiazide; **7** = Chlortalidone; **8** = Clopamide; **9** = Torasemide; **10** = Furosemide; **11** = Methyclothiazide; **12** = Indapamide; **13** = Mefruside; **14** = Cyclothiazide; **15** = Piretanide; **16** = Etacrynic acid; **17** = Polythiazide; **18** = Benzthiazide; **19** = Bendroflumethiazide; **20** = Trichlormethiazide; **21** = Xipamide; **22** = Bumetanide; **23** = Spironolactone; **24** = Etozolin; **25** = Canrenone.

2.5. Sample extraction

Based on the literature [15,18], a simple and rapid alkaline extraction was used. After addition of 0.5 g of solid buffer ($NaHCO_3$–K_2CO_3, 3:2, pH 9.0) to 2 ml of urine, the mixture was shaken with 4 ml of ethyl acetate containing 1 μg of **13** (I.S.) for 15 min and centrifuged at 3000 *g* for 5 min. The organic layer was separated and evaporated to dryness under a gentle stream of nitrogen at 50 °C. The residue was dissolved in 300 μl of initial mobile phase (A–B, 90:10) and 10 μl of this solution was injected into the HPLC system.

2.6. Extraction recovery

The recoveries of 25 diuretics (including **13**) were evaluated by comparing the peak heights found in urine samples spiked with known amounts of the diuretics, which had been processed through the entire extraction procedure, to the peak heights obtained from the same concentration in the reference solution (adjusted for reconstitution). The recoveries of spiked human urine were evaluated at all three QC concentrations (spiked with 2, 10 and 40 μl of stock standard mixture in 2 ml of urine) in replicates of six. These samples were extracted as described previously except without the internal standard.

2.7. Assay validation

By adding different volumes (final concentration: 1 ng/ml−1 μg/ml) of the stock standard solution of 25 diuretics to the urine of a healthy volunteer who had never consumed diuretics, the linearity and limits of detection were established. Calibration curves of the peak heights versus the sample concentration ratios, and fitted to the linear regression $y = ax + b$, were constructed. The limit of detection was defined as the concentration that gave a basepeak signal-to-noise ratio of 2:1 in the full scan mode.

The intra- and inter-day variabilities were determined by replicate analysis of urine samples spiked with 25 diuretics, of which the concentration was the same as that for the recovery test, on the preparation day and different days, respectively. The precision was obtained from the calculation of the relative standard deviations (RSDs).

3. Results and discussion

3.1. HPLC separation of the mixture of 25 diuretics

In the routine HPLC method for screening diuretics, separation was carried out with gradient elution on a reversed-phase column and the mobile phase consisted of phosphate buffer (pH 3.5) and acetonitrile [7]. However, the high ionic strength and the nonvolatile mobile phase in the HPLC method is not suitable for the electrospray interface. A mobile phase consisted of 0.01 *M* ammonium formate–formic acid buffer (pH 3.5) and acetonitrile was particularly suitable to LC–ES-MS. A typical separation of a 10-μl sample (50 μl of the stock standard solution of 25 diuretics was diluted with initial mobile phase to 1 ml) is shown in Fig. 2. The retention times, relative retention times and characteristic ions of 25 diuretics are presented in Table 1. The data from the separation indicated that most of the diuretics can be isolated within 25 min. Even if some retention times were very close (e.g. **3** and **4**, **8**

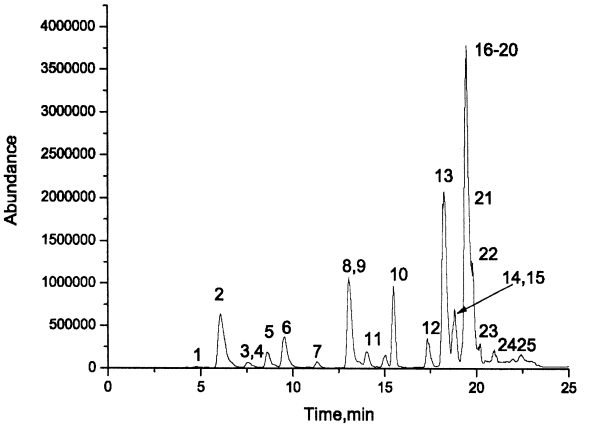

Fig. 2. Total ion current (TIC) of LC–APCI-MS analysis for the standard solution of the mixture of 25 diuretics. Vaporizer temperature 450 °C; heated capillary temperature 250 °C; sheath gas pressure 40 p.s.i.; the procedure was 0–5 min for the positive ion mode, 5–7 min for the negative ion mode, 7–8 min for the positive ion mode, 8–20.5 min for the negative ion mode, and 20.5–25 min for the positive ion mode.

Table 1
Retention time (t_R), relative retention time (t_{RR}) and characteristic ions in APCI and ESI for 25 diuretics[a]

Diuretics	t_R	t_{RR}	Characteristic ions	
	(min)		ESI	APCI
1	4.82	0.262	230 (100) $[M+H]^+$, 195(10)	230(100) $[M+H]^+$, 232(35), 195 (25)
2	6.34	0.344	296 (100) $[M-H]^-$, 342 (5)	296(100) $[M-H]^-$, 298(36), 260(30), 284(20), 250(18)
3	7.61	0.413	254 (100) $[M+H]^+$, 295(6)	254(100) $[M+H]^+$, 295 (8)
4	7.59	0.412	290 (100) $[M+H]^+$, 292(40), 307(30), 273(10), 348(30)	290(100) $[M+H]^+$, 292(35),307(10),273(20), 348(20)
5	8.67	0.471	328 (100) $[M-H]^-$, 288 (10)	328(100) $[M-H]^-$, 288 (30)
6	9.58	0.520	330 (100) $[M-H]^-$, 393 (30), 376 (70)	330(100) $[M-H]^-$, 310 (60), 290 (30)
7	11.51	0.625	337 (100) $[M-H]^-$, 383 (60), 400 (20), 451 (10)	337(100) $[M-H]^-$, 383(10), 320(80), 339 (39), 303 (40)
8	13.34	0.725	344 (100) $[M-H]^-$, 390 (30), 407 (30), 458 (40)	344(100) $[M-H]^-$, 346 (38), 233 (10), 310 (20)
9	13.54	0.735	347 (100) $[M-H]^-$, 393(5),	347(100) $[M-H]^-$, 262(10)
10	15.72	0.854	329 (100) $[M-H]^-$, 375 (5), 285 (8)	329(95) $[M-H]^-$, 294 (100), 285 (26), 234 (10), 249 (7)
11	15.09	0.820	359 (100) $[M-H]^-$, 421 (20), 472 (40), 322 (25)	359(50) $[M-H]^-$, 322 (100)
12	17.62	0.957	364 (100) $[M-H]^-$, 410 (20), 478 (20)	364(100) $[M-H]^-$, 366 (40), 330 (60), 233 (10)
13 (I.S.)	18.41	1	381 (100) $[M-H]^-$, 444 (30), 495 (10)	381(100) $[M-H]^-$, 347 (20)
14	18.85	1.024	388 (100) $[M-H]^-$, 451 (30), 434 (15)	388(100) $[M-H]^-$, 353 (40), 434 (50)
15	18.90	1.027	361 (100) $[M-H]^-$, 407 (20),	361(100) $[M-H]^-$, 317 (35),407(30)
16	19.53	1.061	301 (100) $[M-H]^-$, 347 (1), 243 (40), 266 (10)	301(55) $[M-H]^-$, 243(100), 245(70), 209(15), 266(13)
17	19.38	1.053	438 (100) $[M-H]^-$, 440 (35), 484 (5)	438(100) $[M-H]^-$, 440(36), 398 (50), 418 (30)
18	19.45	1.056	430 (100) $[M-H]^-$, 432 (35), 307 (5)	430(100) $[M-H]^-$, 432(35), 307 (60)
19	19.54	1.061	420 (100) $[M-H]^-$, 483 (10), 466 (5)	420(100) $[M-H]^-$, 310 (30), 380 (20)
20	19.61	1.065	379 (100) $[M-H]^-$, 424(20)	379(100) $[M-H]^-$, 343 (20), 307 (15)
21	19.83	1.077	353 (100) $[M-H]^-$, 355(34)	353(100) $[M-H]^-$, 355(35), 319(40)
22	20.13	1.093	363 (100) $[M-H]^-$, 409(20)	363(100) $[M-H]^-$, 307(10)
23	21.08	1.145	434 (20) $[M+H+H_2O]^+$, 341 (100)	434(30) $[M+H+H_2O]^+$, 341 (100)
24	21.41	1.163	285 (100) $[M+H]^+$	285(100) $[M+H]^+$, 127(10)
25	22.38	1.216	341 (100) $[M+H]^+$, 382 (20)	341(100) $[M+H]^+$, 323 (30), 382 (5)

[a] **1**, **3**, **4** and **23**–**25** in the positive ion mode; the others in the negative ion mode; ESI parameters: heated capillary temperature 300 °C, sheath gas pressure 60 p.s.i., auxiliary gas flow 20 l/min; APCI parameters: vaporizer temperature 450 °C, heated capillary temperature 250 °C, sheath gas pressure 40 p.s.i., auxiliary gas flow = 20 l/min.

and **9**) or overlapped (**16**–**20**), the special characteristic ions made them still distinguishable. As no athlete takes so many diuretics simultaneously, these peaks should normally not occur together. The retention times and the characteristic ions allowed individual compounds to be identified from the complex components contained in human urine.

3.2. ESI-MS analysis of diuretics

As the mobile phase contained ammonium formate–formic acid buffer and acetonitrile, a characteristic mass spectrum showed a protonated molecular ion $[M+H]^+$ and solvent adduct ions such as $[M+NH_4]^+$, $[M+H+ACN]^+$ and $[M+NH_4+ACN]^+$ in the positive ion mode, or a deprotonated molecular ion $[M-H]^-$ and solvent adduct ions such as $[M-H+HCOOH]^-$, $[M-H+NH_4COOH]^-$ in the negative ion mode, with few fragmentation.

Compared with GC–MS, the LC–MS mass spectra, especially for the solvent adducts ions, are more dependent on the experimental conditions. The sensitivity of the technique based on TIC generally increases with an increase of the capillary temperature. The capillary temperature may affect the abundance of $[M-H]^-$ and $[M-H+HCOOH]^-$ ions significantly. Increasing the capillary temperature the intensity of $[M-H]^-$ ion was increased. In addition, as the sheath gas cannot be heated in this system, the dry nitrogen gas was used as the auxiliary gas to sweep the chamber of solvent vapors and help the aerosol to entrance the heated capillary. With the increase of the auxiliary gas flow and pressure of the sheath gas the intensity of $[M-H]^-$ ions was enhanced. This was not the case for the HCOOH adducts.

As pointed out in Table 1, under the optimal ESI conditions the spectra for most diuretics in the mixture of 25 diuretics were characterized by a dominant $[M+H]^+$ (positive mode) or $[M-H]^-$ (negative mode) with a few fragments. The thiazide diuretics (**2**, **5**, **6**, **11**, **13**, **14**, **17**–**20)** and loop diuretics (**9**, **10**, **15**, **16**, **22**) called forth a good response in the negative ion mode. On the other hand, potassium-sparing diuretics including **1**, **3** and **23**–**25** exhibited good sensitivity in the positive ion mode only. The sensitivities of thiazide type diuretics (**4**, **7, 8**, **12, 21**) were excellent in both the negative and positive ion modes. According to the TIC in Fig. 2 the positive ion mode in the screening procedure could be chosen for **1** in 0–5 min, for **3** and **4** in 7–8 min and for **23**–**25** in 20.5–25 min. The negative ion mode could be selected for the detection of the others in 5–7 min and 8–20.5 min, respectively. However, the absolute retention times drift with the change of HPLC conditions, the time of switching polarity had often to be calibrated with a standard mixture. Alternatively, simultaneous recording of signals of the positive and negative ion could be used in the screening procedure. In this case, 20–40% sensitivity will be lost, and the detection limit will increase about 2 times.

The components of the mobile phase have a key influence upon the electrospray ionization. Using flow injecting without separation, the effect of the organic solvent was studied. Addition of acetonitrile to the mobile phase might enhance the efficiency of ion vapor and increase the relative abundance of a molecular analyte ion. The concentration of H^+ ion in the mobile phase is very important to the relative abundance of molecular analyte ion in the negative ion mode, as well as in the positive ion mode. Many diuretics in the negative ion mode gave an excellent response even though an acidic mobile phase (ammonium formate buffer) was used. In accordance with literature results [17], the addition of acetic acid or formic acid to the mobile phase may increase the TIC intensity of the analyte ion obviously. In the present study, formic acid was used to substitute acetic acid in the mobile phase to adjust the pH to satisfy the separation requirement, which could give lower background and higher sensitivity.

3.3. APCI-MS analysis of the diuretics

In APCI-MS analysis of 25 diuretics, similar results were observed as in the ESI spectra. The corresponding solvent adduct ions such as $[M+H+ACN]^+$, $[M+H+2ACN]^+$, $[M-H+HCOOH]^-$, $[M-H+2HCOOH]^-$ were also observed at a relatively low heated capillary temperature. The sensitivity of the diuretics in the APCI mode depended also on the same factors, such as capillary temperature, vaporizer temperature and mobile phase, etc. When the heated capillary temperature was increased the base peak of the diuretics was from $[M-H+$

$HCOOH]^-$ ions converted into $[M-H]^-$ ions. Via a series of chemical reactions, a high voltage created a corona discharge through which more characteristic ions were formed. When the vaporizer temperature was high enough, some fragments, such as m/z 243 of **16**, resulted from the elimination of CH_3COOH from $[M-H]^-$ could be observed.

Generally, more fragments could be found under the optimal conditions in the APCI mode (Table 1). All the compounds except **10**, **11, 16** and **23** showed $[M-H]^-$ ions or $[M+H]^+$ ions which are the base peak for some of them. For **23** the base peak at m/z 341 may be due to the loss of the thioacetyl group. The mass spectra of the diuretics containing the sulfanilamide group gave peaks related to the loss of SO_2NH_2, e.g. m/z 249 for **10**. The peak at m/z 323 of **25** could arise from the loss of water from the $[M+H]^+$. The fragments (m/z 322, m/z 418 and 398, m/z 343 and 307) generated by the loss of the halogen atom from the corresponding deprotonated molecular ion were also observed in the spectra of **11**, **17** and **20**, respectively. Thus, the abundant fragment ions in the mass spectra provided additional and useful structural information, useful for identification purposes.

Table 2
Linear range and limit of detection (LOD) of the diuretics in LC–APCI-MS and LC–ESI-MS[a]

Diuretics	APCI		ESI	
	Linear range (ng/ml)	LOD (ng/ml)	Linear range (ng/ml)	LOD (ng/ml)
1	14.0–1000	14.00	250.0–1000	250.0
2	4.0–1000	4.00	38.0–1000	38.0
3	6.0–1000	6.00	3.0–1000	3.0
4	2.8–1000	2.80	35.0–1000	35.0
5	2.0–1000	1.50	5.0–1000	5.0
6	0.7–1000	0.70	8.0–1000	8.0
7	1.0–1000	1.00	2.4–1000	2.4
8	1.0–1000	1.00	10.0–1000	10.0
9	2.0–1000	2.00	3.0–1000	3.0
10	4.0–1000	4.00	10.0–1000	10.0
11	4.0–1000	4.00	7.0–1000	7.0
12	3.5–1000	3.50	4.0–1000	4.0
13	0.5–1000	0.50	12.5–1000	12.5
14	4.0–1000	4.00	6.0–1000	6.0
15	1.5–1000	1.50	8.0–1000	8.0
16	25.0–1000	25.0	7.5–1000	7.5
17	1.0–1000	1.00	2.0–1000	2.0
18	1.0–1000	0.50	2.0–1000	2.0
19	1.0–1000	0.25	1.2–1000	1.2
20	2.5–1000	2.50	35.0–1000	35.0
21	1.5–1000	1.50	1.0–1000	1.0
22	2.5–1000	2.50	3.5–1000	3.5
23	3.2–1000	3.20	60.0–1000	60
24	2.0–1000	2.00	1.0–1000	0.6
25	4.0–1000	4.00	10.0–1000	10.0

[a] **1**, **3,4** and **23**–**25** in positive ion mode; the others in negative ion mode; APCI parameters: vaporizer temperature 450 °C, heated capillary temperature 250 °C, sheath gas pressure 40 p.s.i.; auxiliary gas flow 20 l/min.

3.4. Recovery and precision

Recoveries of urine samples for 25 diuretics were evaluated at three QC concentrations in replicates of six. By monitoring $[M+H]^+$ or $[M-H]^-$ ions except m/z 341 for **23**, the recoveries of 20 diuretics reached 75–95%, for **10** and **22,** recoveries were 60–70%, and recoveries of acidic diuretics **15** and **16** only reached 45–60%. Owing to the poor lipophilicity, the extraction recovery of **1** was only 23–33%, which was in accordance with that reported in the literature [18]. These data suggest that the extraction recoveries were structure independent.

The precision was determined by the evaluation of a typical production run. The human urine samples spiked with diuretics at a given concentration were evaluated in replicates of six. The RSD values for all inter- and intra-day samples peak height was $<17\%$. The intra-day data indicated that the RSD of the retention times and the relative retention times was within 0.69%. The inter-day precision of the retention times and the relative retention times did not exceed the 2% RSD.

Thirty urine samples from athletes were screened for interference at retention times of these diuretics by characteristic ions. No significant interferences were observed in diuretics-free urine samples.

3.5. Limit of detection

By adding different volumes of the stock solution of 25 diuretics to human urine of healthy volunteer who had never consumed the diuretics, the linearity and limit of the detection were examined in the full scan mode. The base peak ions were selected as the monitor ions in the detection of the diuretics. The linear responses of 25 diuretics were observed over the range 1.0–1000 ng/ml (Table 2). All of the

correlation coefficients were >0.99. By separation with the C_{18} column, the limits of the detection were 0.25–25 ng/ml for the APCI and 0.6–250 ng/ml for the ESI modes in human urine, which were obviously lower than that in the HPLC screening method with UV detection [6,10]. The minimum detectable quantities (MDQ) in the present work ranged from 0.017 to 1.7 ng for APCI, which are also lower than the MDQ of 50 ng in the reported LC–MS method [15]. Furthermore, if the selected ion monitoring (SIM) mode is chosen to screen the diuretics, the limit of detection would be decreased about 5–10 times. Comparing the ESI and APCI modes, in most cases the higher sensitivity, more fragments, and less consumption of nitrogen gas observed in the APCI mode means that APCI could be advantageously applied to the routine analysis of the diuretics in doping control.

3.6. Application to authentic urine samples

Since diuretics are excreted mainly in their unchanged form in urine, their screening and confirming procedures will focus on the direct detection of the unchanged diuretics from the human urine. Under the optimal conditions of LC–APCI-MS, **1**–**3**, **7**–**10**, **12**, **16**, **19**, **21**–**23** and **25** containing authentic urine samples were all detected based on the retention times and characteristic ions. The retention times, relative retention times and characteristic ions from Table 3 indicate that no significant differences were observed between the authentic urine samples and the standard solution of the mixture of diuretics except **23** (Table 1). The results are satisfactory for the common regulation of identity criteria for confirmation analysis in doping control [20]. After administration of **23,** the unchanged drug was hardly found in human urine but as the metabolite **25**. As a result, **23** could be detected by metabolite **25** in authentic urine samples. The APCI-MS spectra and chromatograms of **2**, **10** and **12** containing authentic urine samples are shown in Figs. 3–5, respectively. Some data suggested that a few metabolites could also be detected along with the unchanged diuretic, one of which was identified as the hydrogen elimination product of **12** (Fig. 5, A2) [19].

In general, the combination of a diode array detection system and mass spectrometry may be useful in LC–MS for screening the diuretics. After DAD eluting the peak width of the diuretic in MSD would be a little broader. The diode array detector of the LC instrument could be used to provide characteristic UV spectra to assist in screening large number of urine samples. If the suspicious UV

Table 3
APCI mass spectra data from authentic urine samples after administration of the diuretics[a]

Diuretics	Dose (mg)	Collected time (h)	t_R (min)	t_{RR}	Characteristic ions
1	5	3	4.87	0.263	230(100), 232(38), 195(25)
2	25	4	6.44	0.336	296(100), 298(40), 260(24), 284(20), 250(17)
3	50	4	7.63	0.415	254(100), 295(10)
7	10	6	11.34	0.617	337(100), 383(10), 339(36), 320(75), 303(45)
8	40	6.5	13.25	0.720	344(100), 346(36), 233(10), 310(15)
9	2.5	6	13.42	0.728	347(100), 262(10)
10	40	2	15.75	0.848	329(90), 294(100), 285(28), 234(9), 249(5)
12	5	20	17.71	0.952	364(100), 366(38), 330(55), 233(8)
16	50	1	19.44	1.056	301(50), 243(100), 245(68), 209(20), 266(15)
19	5	6.5	19.49	1.058	420(100), 310(30), 380(30)
21	20	4.5	19.84	1.074	353(100), 355(37), 319(35)
22	50	2.5	20.19	1.100	363(100), 307(12)
23	50	5.5	21.44	1.170	341(100), 323(35), 382(12)
25	50	6	22.42	1.223	341(100), 323(32), 382(10)

[a] **1**, **3, 23**and **25** in positive ion mode, the others in negative ion mode; APCI parameters: vaporizer temperature 450 °C, heated capillary temperature 250 °C, sheath gas pressure 40 p.s.i., auxiliary gas flow 20 l/min.

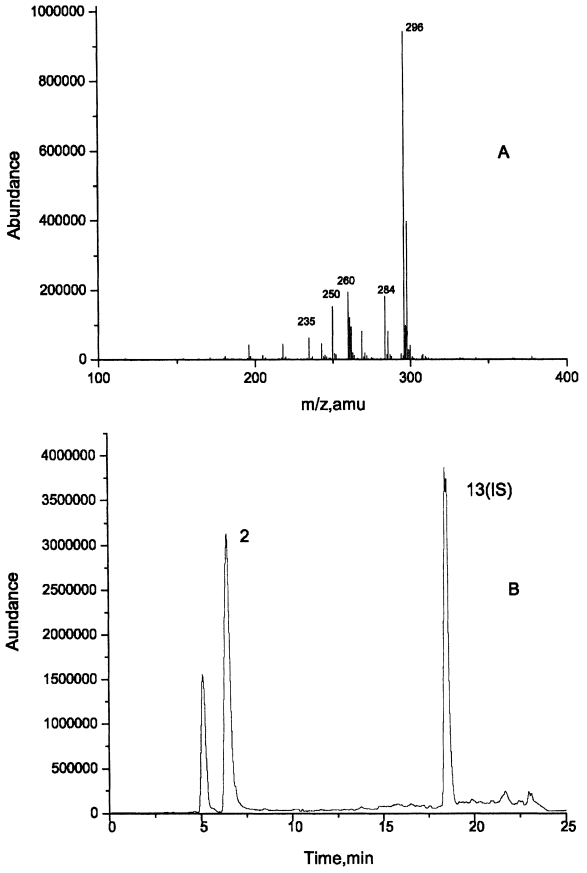

Fig. 3. Mass spectrum (A) and chromatogram (B, TIC) obtained from human urine 4 h after the oral administration of a 25-mg dose of compound **2.** Vaporizer temperature 450 °C, heated capillary temperature 250 °C, sheath gas pressure 40 p.s.i.; APCI negative mode.

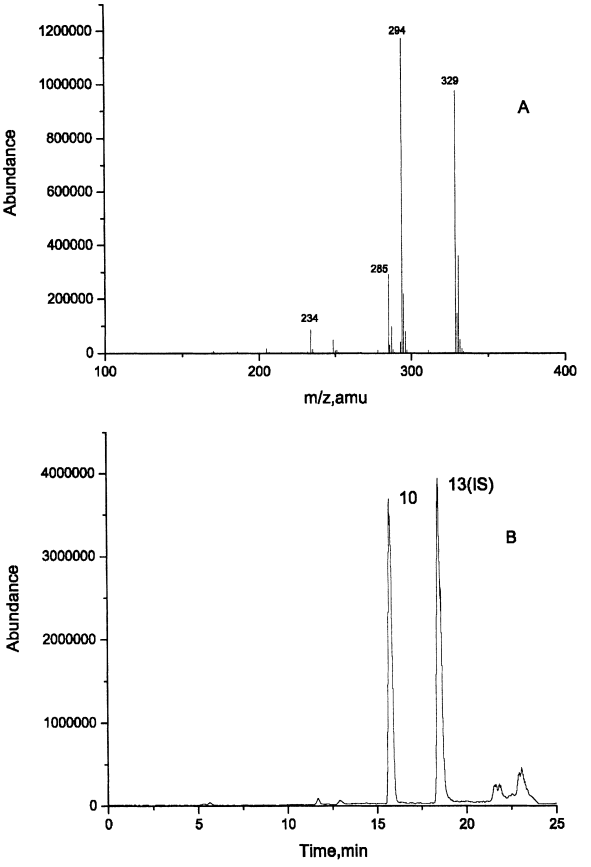

Fig. 4. Mass spectrum (A) and chromatogram (B, TIC) obtained from human urine 2 h after the oral administration of a 40-mg dose of compound **10**. Vaporizer temperature 450 °C, heated capillary temperature 250 °C, sheath gas pressure 40 p.s.i.; APCI negative mode.

spectral matches were observed at a certain retention time, the APCI-MS detection system can be used for confirmation purposes.

3.7. MS–MS spectra analysis

Compared with EI, ES mass spectra yield typically protonated or deprotonated molecules with little structural information. When more chemical information is needed the proper choice will be tandem mass spectrometry (MS–MS). The increase of CID offset may obtain more products ions from the parent ions. For instance, when the parent ion was chosen as m/z 301 $[M-H]^-$ for **16,** with CID offset altering from 10 to 50 eV the base peak m/z 301 $[M-H]^-$ were replaced by m/z 243 $[M-H-CH_2COO]^-$ or m/z 242 $[M-2H-CH_2COO]^-$; and more fragment ions, such as m/z 207 $[M-H-CH_2COO-HCl]^-$, m/z 192 $[M-H-OCH_2COO-Cl]^-$ and m/z 69 $[CH_3CH{=}CHCO]^-$ were observed. Table 4 presents the MS–MS spectra data of 25 diuretics at 30 eV, in which $[M+H]^+$ or $[M–H]^-$ ions were chosen as parent ions except m/z 341 for **23**. Besides some fragmentations that were mentioned previously in ES-MS, more specific fragmentations were also observed in tandem mass analysis. The parent ions of 15 compounds still could keep the base peak at 30

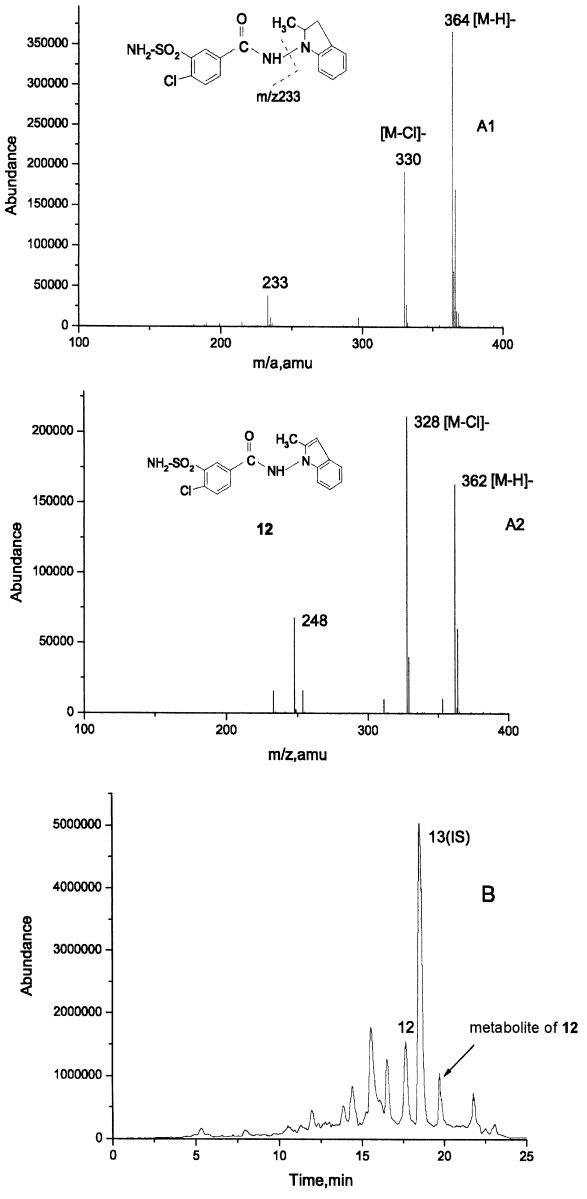

Fig. 5. Mass spectra (A1, A2) and chromatogram (B, TIC) obtained from human urine 20 h after the oral administration of a 5-mg dose of compound **12**. A1 = **12**; A2 = metabolite of **12**. Vaporizer temperature 450 °C; heated capillary temperature 250 °C; sheath gas pressure 40 p.s.i.; APCI negative mode.

eV of CID offset (Table 4). However, for some substances, such as **12,** the fragment ion m/z 132 (product ion from the methylindole group) was easy to produce as the base peak and the relative abundance of the deprotonted molecular ion was very low (1%).

The fragments generated by loss of the HCN from the deprotonted molecular ion appeared in some compounds: m/z 269 for **2**, and m/z 303 for **6**. For **10**, **15** and **22,** the ions corresponding to the $[M-H]^-$ ion minus 44 appeared with a relative abundance higher than 60%, which could be formed after the loss of CO_2 group. Similar to mass spectra, the tandems mass spectra of the diuretics with the sulfanilamide group usually gave the peaks related to the loss of SO_2NH_2, e.g. m/z 208 for **4,** m/z 248 for **5**, and m/z 273 for **21**, etc; for **2** and **6** the ions corresponding to the $[M-H]^-$ ion minus 64 could result from the loss of the SO_2 group. Sometimes collision made unstable side chains dissociate, e.g. m/z 308 for **18**, resulting from the elimination of SCH_2-Ph from $[M-H]^-$; m/z 207 for **22**, formed by the loss of phenyl and sulfanilamide groups; **7** could be divided into two fragment ions of m/z 190 and m/z 146; m/z 328 for **19**, formed by the loss of benzyl group; m/z 262 for **9**, resulted from the loss of $CONHCH(CH_3)_2$ group; and for **24**, m/z 200 could result from the loss of the piperidine group. Therefore the MS–MS spectra from collision-induced dissociation (CID) fragmentation do provide suitable structural information for a positive confirmation.

4. Conclusions

The present LC–MS technique offers an advantageous choice for fast analysis of diuretics without derivatization. The fragmentation characteristics of the APCI mode make it the better choice for diuretics identification in doping control. When a suspicious substance is observed, MS–MS can also be used to provide further information on the chemical structure to a positive confirmation. Compared with the routine HPLC and GC–MSD method, the described LC–MS method in the present work was more convenient and sensitive for the screening and confirming diuretics in human urine. Compared with the reported LC–MS method [15,16], this procedure provided a higher sensitivity and feasibility in routine analysis of diuretics. The present method was successfully applied to 2002 annual reaccredidation test of the IOC in our laboratory, and hydrochlo-

Table 4
MS–MS spectra data of the 25 diuretics[a]

Diuretics	Parent ion	Daughter ions
1	230	230(20), 171(100), 143(15), 60(68)
2	296	296(100), 269(80), 205(55), 232(2)
3	254	254(100), 237(20)
4	288	288(100), 245(40), 208(5)
5	328	328(100), 248(20), 288(2)
6	330	330(100), 303(50), 239(70), 266(10)
7	337	337(100), 190(75), 146(40), 256(20), 80(40)
8	344	344(100), 233(2)
9	347	347(5), 288(10), 262(100), 195(2)
10	329	329(25), 285(100), 205(90)
11	359	359(25), 323(100), 259(30), 195(5), 159(5)
12	364	364(1), 132(100)
13	381	381(100), 345(10), 317(8), 189(6), 153(4)
14	388	388(100), 322(15), 269(20), 205(15)
15	361	361(100), 317(60), 205(35), 80(40)
16	301	301(5), 243(100), 207(1), 192(1)
17	438	438(80), 324(100), 418(60), 398(40), 355(25)
18	430	430(50), 308(100), 339(2)
19	420	420(100), 289(60), 328(25), 239(10)
20	379	379(35), 307(60), 243(100), 343(10), 207(5)
21	353	353(100), 273(15), 170(5), 78(2)
22	363	363(100), 319(70), 207(60), 80(40)
23	341	341(100), 323(10), 283(120), 107(40)
24	285	285(10), 200(100), 84(25), 184(10), 130(5)
25	341	341(100), 323(10), 283(20), 107(35)

[a] **1**, **3** and **23**–**25** in positive ion mode, the others in negative ion mode; APCI parameters: CID offset 30 eV, vaporizer temperature 450 °C, heated capillary temperature 250 °C, sheath gas pressure 40 p.s.i., auxiliary gas flow 20 l/min.

rothiazide (**2**) was screened and confirmed in the test samples. In summary, the developed HPLC–MS may replace the routine method in the area of antidoping control as a useful analytical tool in the future.

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