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Screening, confirmation and quantitation of diuretics in urine for doping control analysis by high-performance liquid chromatography–atmospheric pressure ionisation tandem mass spectrometry

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Abstract

A sensitive, selective, robust and fast method to identify 32 diuretics and masking agents in urine is described. The analytical procedure is reduced to a single XAD extraction step for sample preparation, followed by reversed-phase liquid chromatography in combination with atmospheric pressure ionisation/tandem mass spectrometry. This technique is, after minor modifications, suitable for screening analyses and confirmation of identity as well as quantitation of diuretics. Considerations relating to the stability and metabolism of the compounds are given if relevant for routine screening analyses.

Keywords: Doping control; Diuretics; Benzothiadiazine

1. Introduction

The identification of diuretics in doping control is routinely performed by GC–MS after methylation or by LC–DAD screening analyses [1]. The advantage of the former approach consists in the versatility of MS detection, combined with high sensitivity and selectivity. However, the derivatisation step is time consuming and the resulting methyl-derivatives may be unstable under the conditions of GC separations. Some of them (e.g. amiloride, benzthiazide, torasemide, etc.) decompose to artefacts during derivatisation, injection and/or on column. Optional derivatives may be identified [2,3], but can hardly be combined into a simple and robust screening procedure.

The disadvantage of LC–DAD screening is probably its limited sensitivity if potent diuretics (cyclothiazide, polythiazide, bumetanide) administered in low dosages, requiring detection limits below 1 μg/ml.

Many technical modifications for the detection of selected diuretics have been described recently, such as capillary electrophoresis [4,5], supercritical fluid chromatography [6], MALDI-TOF [7], fluorescence detection [5,8] and LC column switching [9], but these are mainly focused on specific and sensitive

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identification of selected substances rather than on screening purposes.

LC–MS has been applied successfully for the screening of pharmaceutical compounds in forensic toxicology [10–13] and can be considered appropriate also for the detection of polar and hydrophilic substances such as diuretics. An early approach to the analysis of diuretics by LC–MS using thermospray and particle beam interfaces [14] showed promising results. However, an application to routine analysis was impeded by technical limitations of these interfaces. The identification of selected benzothiadiazines [15] and of eight regulated sulphonamides [16] by LC-API–MS was previously described.

The goal was to establish a robust screening procedure for all relevant diuretic agents (according to the list of prohibited substances [17]) to overcome the limitations of GC–MS.

2. Experimental

2.1. Chemicals and reagents

The solvents of the mobile phase (water and acetonitrile; Baker) were specified as gradient grade, while all other chemicals were analytical grade: ammonium acetate (Merck), ethylacetate (KMF), potassium carbonate (Baker), potassium dihydrogenphosphate and disodium hydrogenphosphate (LC Apolda).

Diuretics included in the investigations were acetazolamide (SPOFA), altizide (IMIM, Barcelona, Spain), amiloride (Hennig Arzneimittel), bemetizide (Synthelabo), bendroflumethiazide (ICI Pharma), benzbromarone (KRKA, Yugoslavia), benzthiazide (IMIM), bumethanide (Thomae), butizide (IMIM), canrenone (Theraplix), chlorothiazide (Sigma), chlorthalidone (Spofa), chloramidophenamide (Merck), clopamide (Sandoz), cyclothiazide (Eli Lilly), diclofenamide (Dr. Mann Pharma), ethacrynic acid (Merck Sharp & Dohme), etozoline (Warner Lambert), furosemide (Hoechst), hydrochlorothiazide (Berlin-Chemie), hydroflumethiazide (Sigma), indapamide (Servier Itheria), mefruside (Bayer Leverkusen), metolazone (Heumann Pharma), piretanide (Cassella-Riedel Pharma), polythiazide (Pfizer-Roerig), probenecide (Merck Sharp & Dohme), quinetazone (Aquamor), torasemide (Boehringer Mannheim), triamterene (Schryver, Hamburg), trichlormethiazide (Merck) and xipamide (Beiersdorf).

2.2. Sample preparation

A total of 2.5 ml urine, adjusted to pH 6–7 and spiked with mefruside as internal standard (400 ng/ml urine), was cleaned by solid-phase extraction using XAD columns (type 2, 0.1–0.2 mm; Serva), washed with 5 ml water and eluted with 2 ml methanol. The methanolic extract was evaporated to dryness under nitrogen stream and reconstituted in 50 μl of mobile phase (45% solvent A+55% solvent B).

2.3. Instrumentation

An HP 1100 (Agilent Technologies) liquid chromatograph equipped with a binary pump and a variable wavelength UV detector was applied for LC separation. The mass spectrometer API 2000 (PE Sciex) was supplied with an atmospheric pressure ionisation interface (TurboIon Spray Ion Source). Full scan, selected ion recording (SIR) and multiple reaction monitoring (MRM) experiments were carried out using positive and negative modes.

2.3.1. LC parameters

An XDB C8 (Zorbax, 4.6 mm×75 mm×3.5 μm) column, protected by an XDB C18 (Zorbax, 4 mm×4 mm×5 μm) guard column, was applied for chromatographic separation. The following binary mobile phase gradient was formed by solvent A (0.2 m*M* NH_4ac in water+acetonitrile (95+5)) and solvent B (0.2 m*M* NH_4ac in water+acetonitrile (5+95)) at a constant flow-rate of 0.4 ml/min: 45% B (0–1 min), 45→75% B (1–9 min) and 75% B (9–12 min). The injection volume was 10 μl.

2.3.2. MS parameters

The spray conditions of the API interface were turbo gas temperature 400°C, nebuliser gas pressure 55 p.s.i., and heater gas pressure 80 p.s.i.

Default settings of the API 2000 were appropriate to the majority of MS parameters. The substance specific voltages declustering potential (DP) and

Table 1
Summary of analytical parameters of the diuretics screening[a]

	Concentration in control urine, μg/ml (Fig. 2)	RT (min)	RRT	Precursor ion, *m/z* (amu)	Product ion, *m/z* (amu)	DP (eV)	CE (eV)
Chlorothiazide	1	1.92	0.42	294	214	−61	−38
Furosemide	1	2.01	0.35	329	285	−41	−14
Acetazolamid	1	2.29	0.40	221	83	−31	−26
Ethacrynic acid	1	2.33	0.41	301	243	−26	−10
Quinethazone	1	2.38	0.42	288	245	−51	−20
Chloroaminophenamide	+[b]	2.47	0.43	284	205	−66	−28
Piretanide	1	2.47	0.43	361	205	−51	−30
Hydrochlorothiazide	1	2.52	0.44	296	269	−51	−20
Bumetanide	0.1	2.60	0.46	363	319	−71	−12
Amiloride	1	2.63	0.49	230	171	+31	+25
Probenecide	1	2.65	0.46	284	240	−46	−14
Torasemide	0.5	2.65	0.46	347	262	−41	−14
Chlorthalidone	1	2.70	0.47	337	190	−61	−18
Triamterene	1	2.73	0.49	254	104	+71	+51
Xipamide	1	2.97	0.52	353	274	−86	−30
Hydroflumethiazide	1	3.02	0.53	330	239	−56	−30
Diclofenamide	1	3.20	0.56	303	239	−56	−20
Clopamide	1	3.33	0.58	344	189	−91	−34
Trichlomethiazide	1	3.88	0.68	380	306	−21	−12
Mefruside (OXO_Mb)	+[b]	4.02	0.71	395	189	−71	−38
Etozoline (OXO_Mb)	+[b]	4.09	0.74	299	253	+21	+15
Metolazone	1	4.20	0.74	364	257	−76	−24
Benzbromarone	+[b]	4.47	0.97	423	251	−66	−38
Altizide	−[b]	4.79	0.84	382	341	−16	−16
Benzthiazide	1	4.97	0.87	430	308	−81	−36
Butizide	−[b]	5.13	0.90	352	205	−61	−30
Indapamide	1	5.16	0.91	364	189	−66	−34
Cyclothiazide1	−[b]	5.58	0.98	388	322	−21	−40
Mefruside (parent), internal standard	400 ng/ml	5.70	1.00	381	189	−71	−38
Cyclothiazide2	−[b]	5.70	1.00	388	322	−21	−40
Polythiazide	1	6.34	1.11	438	324	−16	−34
Bendroflumethiazide	1	6.39	1.12	420	289	−86	−24
Canrenone	1	7.85	1.42	341	107	+81	+41

[a] The sign of collision energy (CE) and declustering potential (DP) indicates the polarity of ionisation. Product ions are $[M+H]^+$ and $[M-H]^-$, respectively in all cases.

[b] + or – instead of concentration signifies the presence of unknown metabolite concentration or the absence of the respective compound in the control sample.

collision energy (CE) were optimised for each compound and are listed in Table 1.

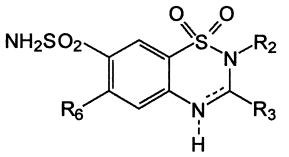
3. Results

3.1. Identification of diuretics by GC–MS

GC separation of diuretics requires derivatisation of the polar compounds. The most frequent technique is methylation, carried out as bulk, flash or extractive reaction. Other derivatives are available, but proved to be less homogeneous and therefore limited to special problems (e.g. silylation of amiloride [3]).

Methylation of amino and carboxy groups is not always complete and reproducible; problems occur especially in the group of benzothiadiazines (Table 2), e.g. unpredictable products (hydrochlorothiazide), irreproducible hydrolysis products (chlorothiazide),

Table 2
Benzothiadiazines available as diuretics may be characterised depending on their substituents in 2, 3 and 6 position (R_2, R_3, R_6) and the saturation of the 3–4 bond



Name	R_3	R_2	R_6	3–4 Bond
Chlorothiazide	H	H	Cl	Double
Hydrochlorothiazide	H	H	Cl	Single
Cyclothiazide	Norborn-5-en-2-yl	H	Cl	Single
Polythiazide	CH_2–S–CH_2–CF_3	CH_3	Cl	Single
Butizide	CH_2–$CH(CH_3)_2$	H	Cl	Single
Altizide	CH_2–S–CH_2–CH=CH_2	H	Cl	Single
Bemetizide	1-Phenylethyl	H	Cl	Single
Benzthiazide	CH_2–S–CH_2–C_6H_5	H	Cl	Double
Trichlomethiazide	$CHCl_2$	H	Cl	Single
Hydroflumethiazide	H	H	CF_3	Single
Bendroflumethiazide	CH_2–C_6H_5	H	CF_3	Single

methylation products and/or artefacts depending on GC and derivatisation conditions (cyclothiazide, polythiazide etc.), or no signal at all (benzthiazide).

These limitations of GC–MS identification of benzothiadiazines are influenced by chromatographic conditions. Depending on the topical status, either substance-specific fragments with identical mass spectra (Fig. 1) and/or identical artifacts are detected for the compounds altizide, bemetizide, butizide and polythiazide. Therefore, an unequivocal differentiation of incorporated substances may be impossible, although they are excreted unchanged.

3.2. Suitability of LC–MS technique

3.2.1. Liquid chromatography

The choice of adequate reversed-phase LC conditions was determined by a compromise between optimum flow-rate for electrospray, gradient reproducibility, robustness against relatively ''dirty'' extracts, selectivity of separation and chromatographic capacity, and economic considerations (turnaround time, solvent consumption). With a RP-C8 column a reproducible water/acetonitrile gradient was necessary to cover the polarity range from lower molecular polar compounds (acetazolamide) to lipophilic substances, e.g. the steroid canrenone. A 4.6-mm I.D. column was chosen for robustness when dealing with the dirty extracts of urinary matrix, with an optimum flow-rate for turbo ion spray of 0.4 ml/min and a moderate gradient (45→75% acetonitrile). Both solvents contained 2 m*M* ammonium acetate to establish constant ionisation conditions.

In addition to the main purpose of LC — to separate the target compounds from an excess of polar ionisation-inhibiting compounds which are eluted in the front peak — a suitable chromatographic separation of all diuretics could be achieved (Fig. 2).

3.2.2. Ionisation, polarity and fragmentation

LC–MS detection was carried out using electrospray ionisation technique (TurboIonspray interface of the API 2000), which is compliant with typical LC flow-rates (0.2–1 ml/min) and predominantly forms the protonated molecules $[M+1]^+$ (positive mode) or the deprotonated molecules $[M-1]^-$ (negative ionisation). Positive and negative ions formed in solutions may be detected at either polarity, and both options can be combined within one experiment. The necessity to cover both ionisation modes is obvious because the class of diuretics consists of strong acids (ethacrynic acid) as well as some basic compounds

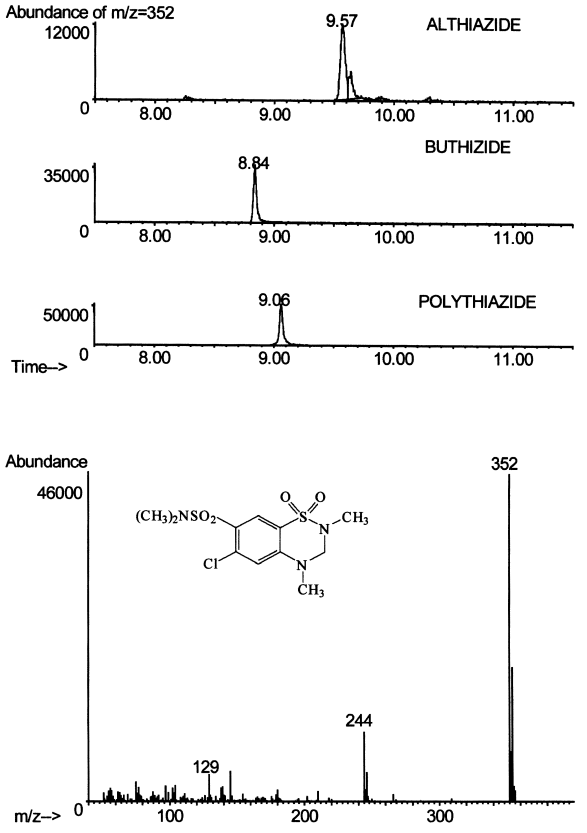
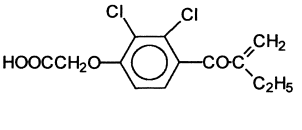
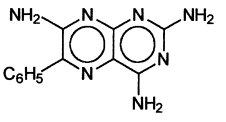


Fig. 1. Identification of benzothiadiazines by GC–MS causes difficulties, mainly related to derivatisation and chromatography. The occurrence of non-uniform reaction products and artefacts reduces sensitivity and reproducibility of the screening. The detection of altizide, butizide, cyclothiazide, polythiazide, etc. is based on the identification of substance-unspecific core fragments ($m/z = 352$, shown above).

(triamterene). The former will logically form $[M-H]^-$ acid-anions rather than $[M+H]^+$ and vice versa.



Ethacrynic Acid



Triamterene

The quasi-molecular ions proved to be the base peak of most of the observed mass spectra, moreover the relevant voltage (declustering potential) is optimised to a value high enough for sufficient declustering while fragmentation of the molecule is not yet excited.

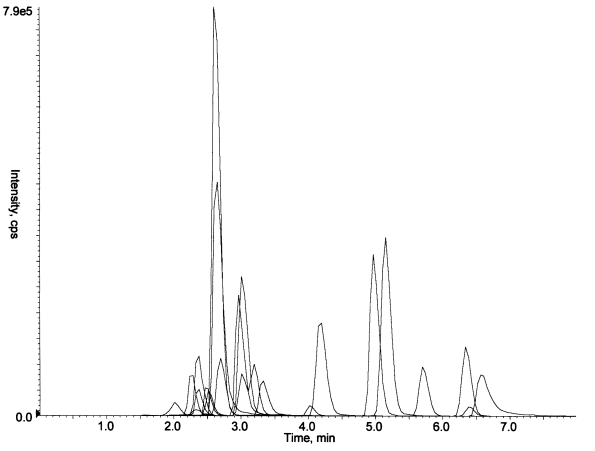


Fig. 2. LC–MRM chromatogram of the positive diuretics' control urine. All substances of interest are detectable at relevant urinary concentrations, and peak shapes and peak-to-noise ratios are very satisfactory. Retention times, MS parameters and composition of the control urine are listed in Table 1.

Most of the diuretics observed did not undergo intense fragmentation under default API conditions and adducts were not formed in considerable amounts. One exception for both processes was the metabolite of etozoline (Fig. 3). In addition to several fragmentation reactions the formation of oligomers and their corresponding sodium adducts of

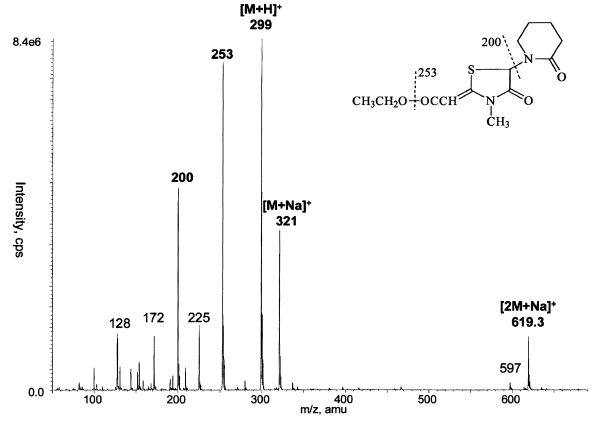


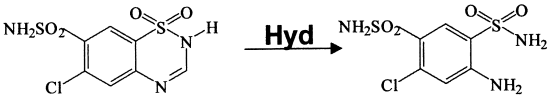
Fig. 3. API mass spectrum of oxo-etozoline in 2 mmol ammonium acetate buffer. In contrast to the typical behaviour of other diuretics, the formation of intense fragments, dimers and sodium adducts was observed in chromatograms of real samples.

the parent compounds are observed, e.g. $[M+H-99]^+$ (loss of 2-oxo-piperidine), $[M+H-46]^+$ (loss of ethanol), $[M+Na]^+$, $[2M+H]^+$, and $[2M+Na]^+$.

However, if required for confirmation analysis using single stage MS, fragmentation could be enforced by variation of the declustering potential.

3.2.3. Stability (benzothiadiazines)

There were two major processes observed, which diminish the stability of benzothiadiazines in the analytical process. The most important reaction is hydrolysis, which is relevant for all benzothiadiazines and produces chloroamidophenamide (or the respective methyl derivative for polythiazide and other 3-methylated compounds) [18].



This reaction takes place in any aqueous solution of diuretics and is not supposed to be a metabolic reaction. The ratio of parent-compound to hydrolysis product is mainly governed by storage conditions of the urine sample.

Secondly, oxidation of sulfide side-chain substituents (altizide, benzthiazide, polythiazide) to sulfoxides and sulfones was observed (Fig. 4). This reaction was caused by impurities in the organic extractand (ethylacetate) and can be widely suppressed e.g. by distillation of the organic solvent. However, both reactions may prevent the formation of adequate methyl derivatives in GC–MS.

All benzothiadiazines excreted in urine can be positively identified by LC–MS as intact compounds, in addition to the hydrolysis product. Two isomers of cyclothiazide are detectable showing identical MS but distinct retention times (Table 1).

3.2.4. Metabolism (mefruside, etozoline, spironolactone, bemetizide)

The consideration of metabolism was focused on those diuretics which are not excreted in urine as parent compounds. Without detailed investigations, the most dominant metabolite served as the key substance for identification. Corresponding compounds are:

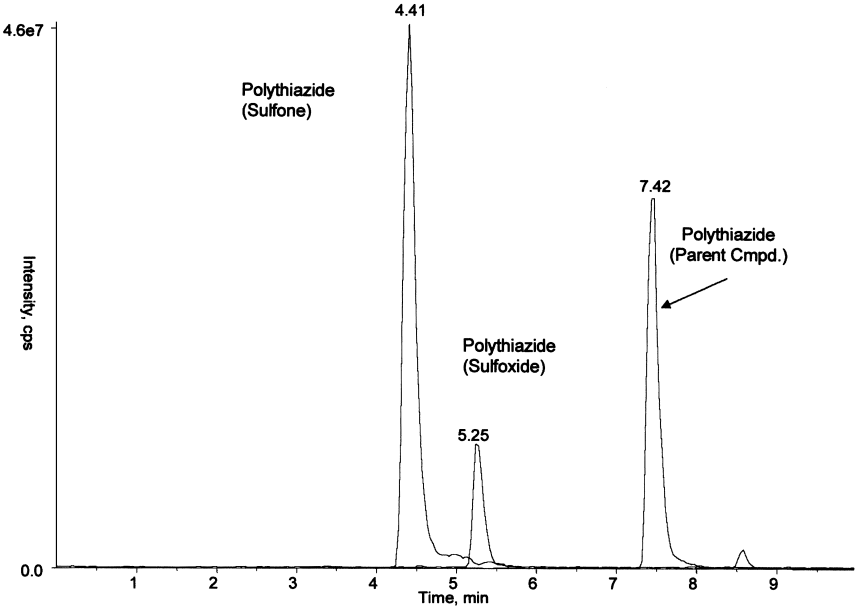


Fig. 4. The presence of traces of oxidant impurities in organic solvents caused the sulfoxidation of diuretics with sulfide substituents in 3 position (benzthiazide, altizide, polythiazide).

- mefruside and etozoline, which form oxo-metabolites
- spironolactone which is mainly converted to canrenone
- bemetizide which is mainly excreted as chlorothiazide.

Additional metabolites are detectable (hydroxy metabolites of canrenone), but the identification of the substance administered is not possible for the latter two cases.

3.2.5. Screening procedure

Typical therapeutic urinary concentrations of diuretics are well above 100 ng/ml, therefore robustness, reliability, effectivity and costs are more important criteria than ultimate detection limits. Due to the high specificity of MS detection, sample preparation could be reduced to a minimum. A simple solid-phase extraction using XAD columns proved to be sufficient to increase concentration and reduce the amount of ionic matrix compounds. The methanolic extract of the XAD columns is redissolved with solvent A and injected into LC. Analytical MS parameters were optimised using a substance specific auto tune procedure (''quantitative optimization''), which is done by injecting standard solutions of the target substances by a syringe pump. All relevant voltages are automatically optimised: the most important of them (declustering potential and collision energy) are listed in Table 1, together with the respective ions and LC retention times. Fig. 1 shows the LC–MS–MS chromatogram (MRM mode) of a control urine containing 25 compounds, which gives a good impression about the excellent specificity of the assay.

3.2.6. Confirmation

Several fragment ions are included in the process of substance related optimisation. Therefore, a number of suitable fragmentation reactions are routinely available for MRM confirmation experiments, which should be the best option for verification of screening results. According to the considerably high concentrations of diuretics, any other technique (selected ion recording, full scan (quad 1), product or precursor ion scan) should be applicable as well.

The example of polythiazide at a concentration of 1 ng/ml (Fig. 5) demonstrates the possibility of confirming the identity of the substance by monitoring three fragmentation reactions (loss of HF, 2HF and $S–CH_2–CF_3$, respectively). The ratio of the

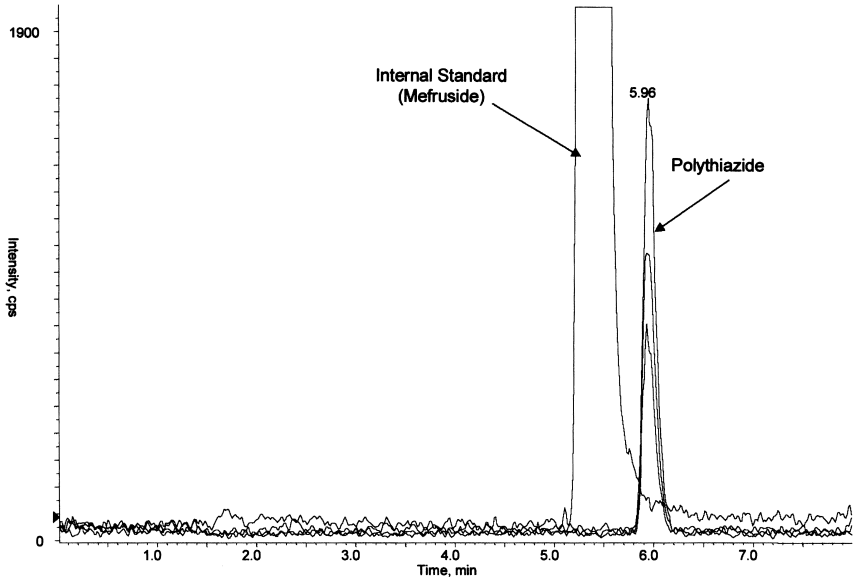


Fig. 5. Recording of three diagnostic fragmentation reactions (438→418, 438→398, 438→324) permits the confirmation of polythiazide identity. The chromatogram represents a concentration of polythiazide in urine of 1 ng/ml.

fragmentation intensities remains constant over four decimal powers of concentration (less than 5% standard deviation of relative peak heights between 1 ng/ml and 10 μg/ml).

Common regulation of identity criteria for confirmation analyses in doping control [19] requires the correspondence of three ion traces within ±20% of relative intensity, regardless of the mass spectrometric technique applied (low resolution, high resolution or tandem MS). However, the extra analytical dimension of MS–MS versus MS experiments reduces the background and identification requirements can be adopted more easily.

This comparison is shown in Fig. 6, were the detection limit of polythiazide, detected in urine by API-MS–MS was lower than 1 ng/ml, which is by far better than other LC detection techniques such as

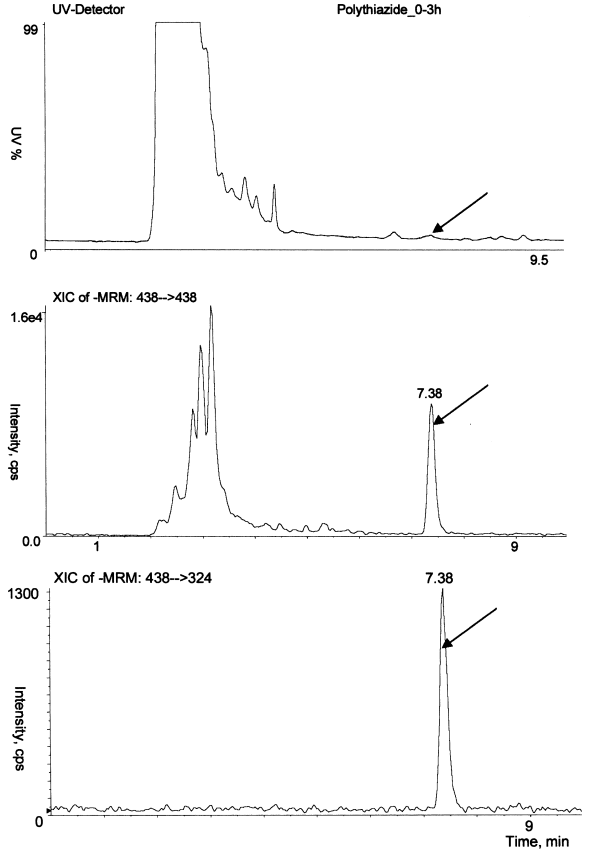


Fig. 6. Comparison of simultaneous detection of polythiazide by UV detector (220 nm), unfragmented precursor ion (438→438) and fragmentation reaction (438→324) recording.

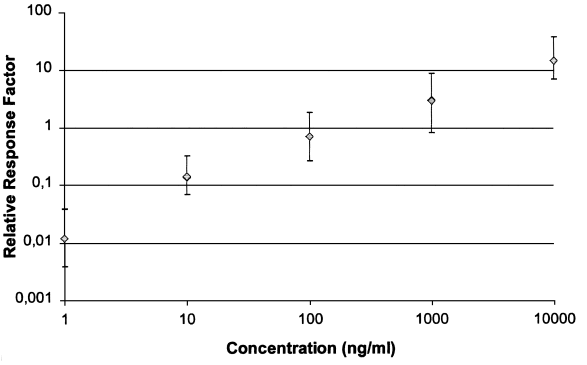


Fig. 7. A robust calibration curve of polythiazide may be established over a wide concentration range. The quantitation permits a detection limit better than 1 ng/ml urine which is more than adequate for diuretics.

UV or single stage MS. The latter was approximated by recording of the unfragmented $[M-H]^-$ ion after passing the collision cell. This signal is less sensitive than single stage selected ion recording (which cannot be recorded simultaneously) but usually represents a similar signal-to-noise ratio.

3.2.7. Quantitation

Quantitative analysis of diuretics is not part of the routine doping control tests. Even in the case of positive findings they are not mandatory, because any administration to athletes is prohibited. However, the possibility of estimating quantitative urinary concentrations of diuretics is demonstrated using polythiazide, which is a potent diuretic. Low therapeutic dosages require relative low cut-off values of the assay. A calibration curve was examined from 1 ng/ml to 10 μg/ml in a urinary matrix in parallel with the confirmation analyses described above (Section 3.2.6). Although the curve shape is not linear over the wide concentration range, reproducibility of individual injections is sufficient (Fig. 7).

4. Conclusions

The described LC-API–MS–MS technique permits the simultaneous screening of a large number of diuretics in urine matrices with a minimum of sample preparation. This technique can be easily extended to confirmation and/or quantitation analy-

ses. Negative ionisation is generally preferred, due to the acidic properties of most diuretics and the lower background, however positive mode cannot be avoided if basic diuretics are to be covered by the assay. The option of scan-by-scan polarity changes involves technical problems (sensitivity loss and limited stability). Therefore two subsequent LC runs per sample are recommended for routine purposes, which appears to be the only technical restriction of the method, which is sensitive, specific and robust enough to be applied under routine conditions.

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References

[1] R. Ventura, J. Segura, J. Chromatogr. B 687 (1996) 127.
[2] J. Segura, R. Ventura, C. Jurado, J. Chromatogr. B 713 (1998) 61.
[3] H. Bi, S.F. Cooper, M.G. Cote, J. Chromatogr. 582 (1992) 93.
[4] J. Jumppanen, H. Siren, M.L. Riekkola, J. Chromatogr. A 652 (1993) 441.
[5] E. Gonzalez, A. Becerra, J.J. Laserna, J. Chromatogr. B 687 (1996) 145.
[6] K. Dost, D.C. Jones, G. Davidson, Analyst 125 (2000) 1243.
[7] J.P. Huang, C.H. Yuan, J. Shiea, Y.C. Chen, J. Anal. Toxicol. 23 (1999) 337.
[8] C.Y. Gradeen, D.M. Billay, S.C. Chan, J. Anal. Toxicol. 14 (1990) 123.
[9] M.T. Saarinen, H. Siren, M.L. Riekkola, J. Liq. Chromatogr. 16 (18) (1993) 4063.
[10] H.H. Maurer, J. Chromatogr. B 713 (1998) 3.
[11] W. Weinmann, N. Lehmann, C. Muller, A. Wiedemann, M. Svoboda, Forensic Sci. Int. 113 (2000) 339.
[12] K. Heinig, J. Henion, J. Chromatogr. B 732 (1999) 445.
[13] M. Gergov, J.N. Robson, E. Duchoslav, I. Ojanpera, J. Mass Spectrom. 35 (2000) 912.
[14] R. Ventura, D. Fraisse, M. Becchi, O. Paisse, J. Segura, J. Chromatogr. B 562 (1991) 723.
[15] S.D. Garbis, L. Hanley, S. Kalita, JAOAC Int. 81 (1998) 948.
[16] M.T. Combs, M. Ashraf-Khorassani, L.T. Taylor, J. Pharm. Biomed. Anal. 19 (1999) 301.
[17] IOC, The Olympic Movement Anti-Doping Code, IOC, 2000, http://www.olympic.org/ioc/e/org/medcom/medcom_antidopage_e.html
[18] D. Thieme, J. Große, R. Lang, R.K. Mueller, A. Wahl, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, Vol. 8, Sport und Buch Strauß, Köln, 2000.
[19] IOC, Criteria For Reporting Low Concentrations of Anabolic