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RECENT ADVANCES IN DOPING ANALYSIS (8)

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Suitability of HPLC/MS-MS for the detection of diuretics

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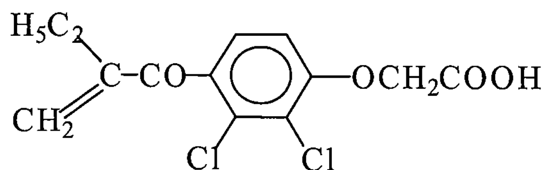
1. Introduction

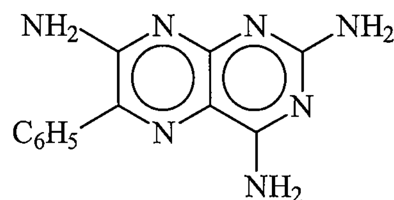
The common screening procedures for diuretics in doping analysis are GC/MS after methylation and HPLC/DAD. The methylation technique usually applied in GC/MS screenings is a time consuming procedure, requires purified extracts and produces non-uniform reaction products and/or artefacts (1). In some cases (eg benzothiadiazines) a differentiation between individual parent substances can be impossible.

The advantages of liquid chromatography of diuretics compared to GC/MS coupling consist in an adequate separation of the unchanged polar substances, but in spite of specific and intense UV spectra the required specificity and identification criteria are not fulfilled in combination with diode array detection. LC-API MS coupling appears to be a useful alternative (2) according to the polarity of diuretics. In case of API (thermospray) technique, ionisation mainly depends on the formation of ions in solutions and depends therefore on the chemical proporties of the substance to be analysed. Diuretics are typically characterised by the presence of polar (amino-, carboxy-, sulfonamide-) groups, which can undergo

protonation (positive ionisation) $-NH_2$ → $-NH_3^+$,
deprotonation (negative ionisation) $-COOH$ → $-COO^-$
or both $>SO_2(NH_2)_2$.

The favourite ionisation mode is consequently related to chemical properties like acidity of the substance or to extraction behaviour, and often predictable:





Ethacrynic acid: negative ionisation

triamterene: positive ionisation

If ions of both polarities can be formed from an analyte, negative mode is generally preferred due to its lower background.

2 Experimental

2.1 Chemicals and Reagents

The solvents of the mobile phase (water and acetonitrile, Baker) have the specification ‚gradient grade', all other chemicals are analytical grade:
ammonium acetate (Merck), ethylacetate (KMF), potassium carbonate (Baker), potassium dihydrogenphosphate and disodium hydrogenphosphate (LC Apolda).
Diuretics included in the investigations are chlorothiazide (Sigma), torasemide (Boehringer Mannheim), bemethizide (Syntheolabo), etozoline (Warner Lambert) and chloroamidophenamide (Merck).

2.2 Sample Preparation

2.5 ml urine, spiked with mefruside as internal standard (400 ng/ml urine), were adjusted to pH 6, using about 1 ml of phosphate buffer (0.5 m). After two extraction steps with 2.5 ml ethylacetate the organic layers were separated. The urine was than adjusted to pH 9-10 by addition of about 500 mg K_2CO_3 and extracted with 3 ml ethylacetate. The combined organic layers were evaporated to dryness under nitrogen stream and reconstituted in 50 µl of mobile phase A.

2.2 Instrumentation

An HP 1100 (Agilent technologies) equipped with a binary pump and a variable wavelength UV detector was applied for LC separation. The mass spectrometer API 2000 (PE biosystems) served as mass specific detector, coupled by an athmospheric pressure ionisation interface (TurboIon Spray). Quad-1-scan, selected ion recording (SIR) and multiple reaction monitoring (MRM) experiments were carried out at both polarities.

LC Parameters and interface parameters

column	XDB C8 (Zorbax) 4.6mm*150mm*5µm	
mobile phase	solvent A	0.2mmol/l NH_4Ac in water acetonitrile (95+5)
	solvent B	0.2mmol/l NH_4Ac in water acetonitrile (5+95)
flow	0.6 ml/min flow (split 1:1)	
gradient	0 - 1 min	45% B
	1 - 9 min	45→75% B
	9 - 12 min	75% B.
injection volume	15µl	
oven temperature	30°C	

interface temperature 450°C, gas 1 = 65, gas 2 = 80

MS parameters

The majority of the MS parameters is less sensitive and default settings of the instrumentation are appropriate, whereas some LC related parameters depend on gradient type and flow rates (s. above). The most important and substance specific voltages are declustering potential and collision energy, listed in table 1 for the diuretics examined.

Table 1: substance specific MS parameters

substance	precursor ion	product ion	declustering potential	collision energy
etozoline (oxo-metabolite)	298 + 1	253	21	15
chlorothiazide	295 – 1	214	-61	-38
bemethizide	401 – 1	294	-66	-26
chloroamidophenamide	285– 1	205	-51	-32

3. Results

3.1 Screening approach

3.3.1 Identification of parent compounds

The majority of diuretics is –at least partially– excreted in urine as parent compound. The most abundant ion of all substances is in API-MS the $[M+1]^+$ or $[M-1]^-$, as the typical ionisation consists in protonation or deprotonation. We did not observe the formation of dominant fragment ions or adducts of the diuretics observed (except etozoline, fig. 1). The design of a screening method is

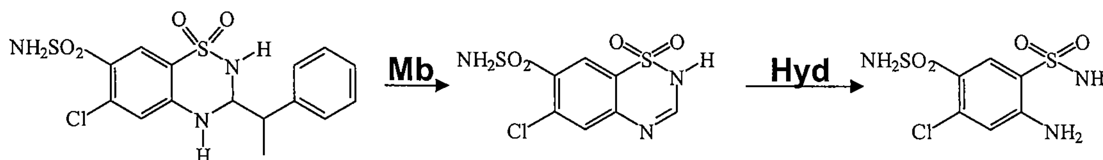
therefore straight forward: Based on the molecular ions, an automatic procedure (substance specific autotune) takes control about optimisation of all parameters of fragmentation reactions.

3.3.2 Metabolites, adducts, hydrolysis product and artifacts

Some diuretics are completely converted into metabolites, which are easily identified, (etozoline, mefruside → oxo-metabolite) but substance specificity is not always given. In a few cases it is not possible to conclude from the metabolite to the diuretics applied (bemethizide →chlorothiazide, spironolactone → canrenone).

In contrast to the typical behaviour, oxo-etozoline (fig. 1) forms a variety of abundant fragments (mainly cleavage of ethyl ester group), sodium adducts, di- and trimers in combination with Na-addition.

The parent compount of bemethizide was undetectable, not even after direct injection of urine. The presence of chlorothiazide and its hydrolysis product (Hyd) chloroamidophenamide indicate a rapid and complete metabolism (Mb) of the compound. (fig. 2)



3.2 Confirmation of results

The most intense fragmentation reaction is applied for screening purposes, whereas the optimisation procedure routinely includes more than one reaction. Therefore, the technical parameters necessary to trace several fragmentation reactions for confirmatory analyses do not require extra effort. The reproducibility and robustness of the technique are sufficient to provide the required accuracy to comply with conventional identification criteria (3 fragments with $<20\%$ abundance deviation).

3.3 Comparison of methods

The preference for a screening experiment is certainly MRM, due to its high specificity. Typical target concentrations in the low ppm range do not require sensitivity optimisation and a reduction of sample preparation effort (limitation to XAD extraction) is possible. A comparison of LC detection by UV detector, single MS and tandem MS is shown in figure 3. Although UV spectra of most of the diuretics are intense and specific, the identification of some of them may cause problems. Especially some potent thiazide diuretics, applied at dosages of few mg only (polythiazide, cyclothiacide) require the specificity of MS detection.

3.4. Quantification

The LC/API-MS screening methods described above can be applied to quantify diuretics. The calibration curve for two diuretics prooved to be linear over 3 decades (fig.4). An external calibration of spiked urine extracts was applied to eliminate recovery fluctuations and to check the influence of technical parameters (injection volume, MS stability etc.).

4. Conclusions

Technical

1. LC/API-MS-MS is a routine technique with excellent reproducibility, specificity and sensitivity
2. By injecting real target substances, the tuning procedure provides substance specific parameters
3. Ionisation is characterised by regular occurance of abundant molecular ions $[M+1]^+$ or $[M-1]^-$ and poor fragmentation
4. The solvent influence (eg formation of adducts) is less important. The main chromatographic limitation consists in the balance between spray optimisation (optimum flow <0.3ml/min) and LC prerequisites (flow>0.5ml/min for typical LC applications).
5. A good linearity of MRM calibration permits quantification over several orders of magnitude.

Detection of diuretics

1. The majority of diuretics (26 species) is detectable in negative mode, only 4 diuretics require positive ionisation. The polarity may be changed in routine experiments.
2. Unchanged parents of most diuretics (except bemethizide, mefruside, etozoline, spironolactone) were detected.
3. A limited stability (vs. hydrolysis and oxidation) of benzothiadiazines was observed.

Acknowledgement

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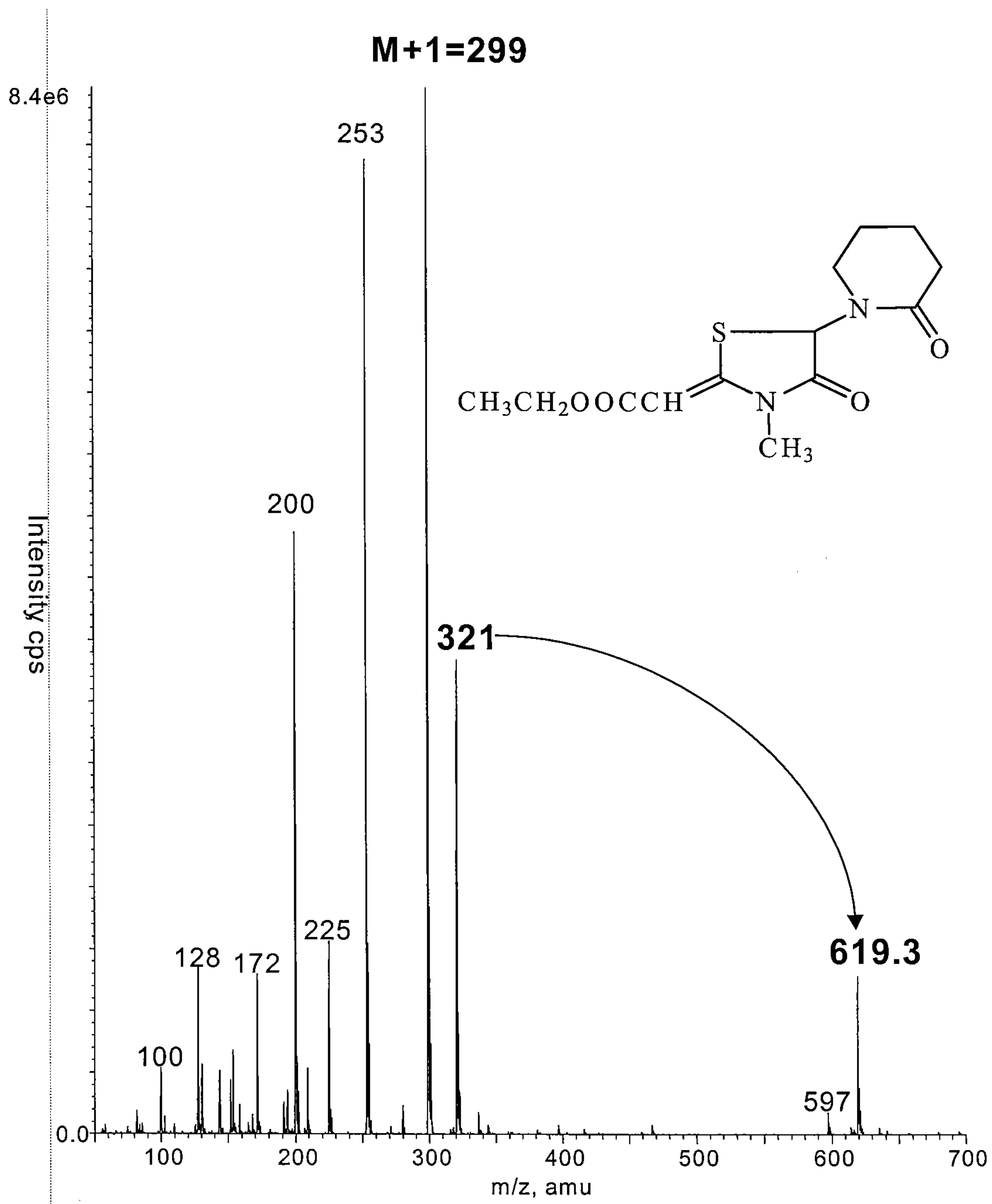

figure 1: API mass spectrum of oxo-etozoline. Extra to the molecular ion (M+1) there is a formation of sodium adducts, dimers and fragments, which is not the typical behaviour of diuretics in API/MS.

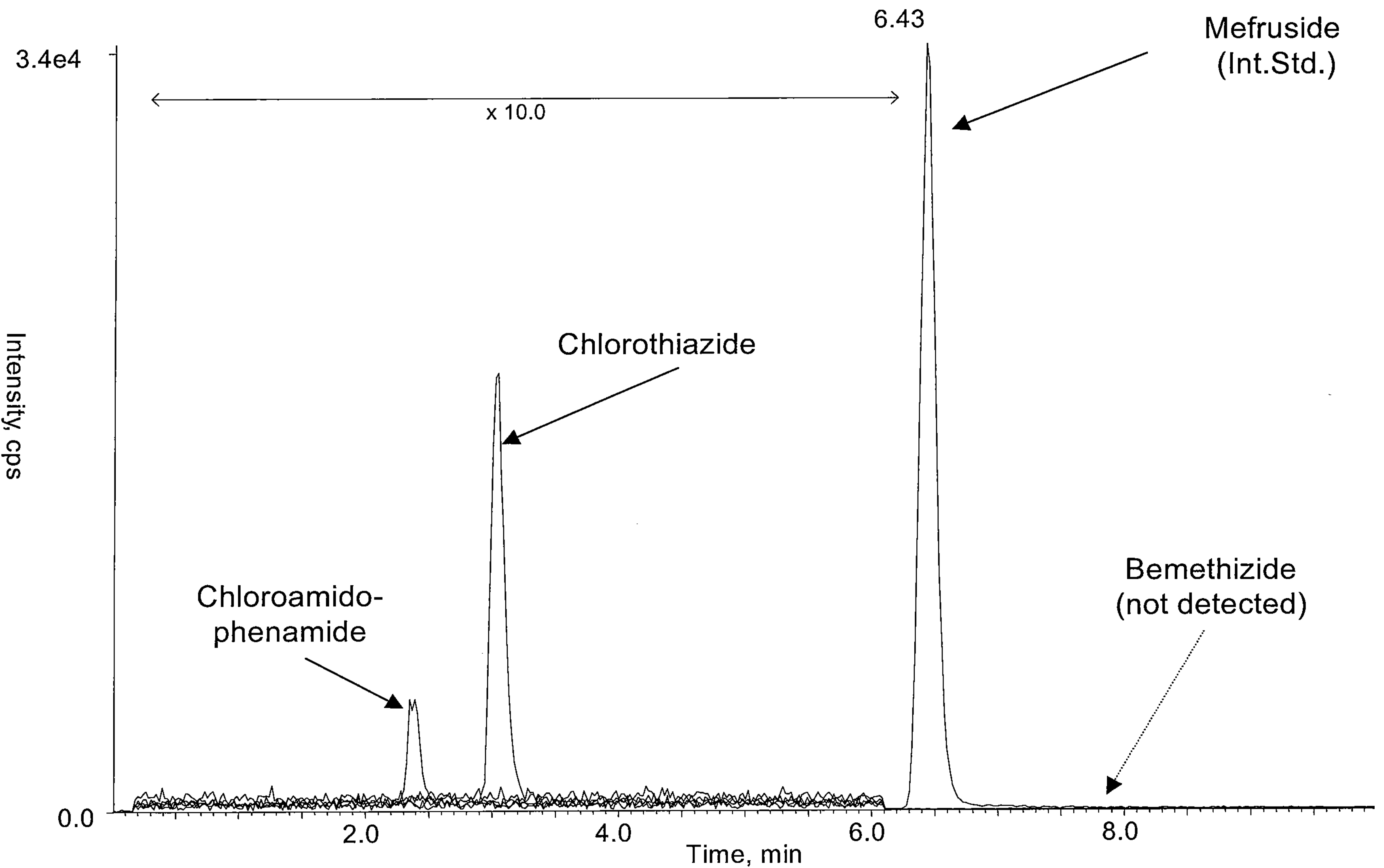

figure 2: LC/MS screening of a bemethizide excretion study. Contrary to the parent compound (not detected in any sample) there was chlorothiazide and its hydrolysis product detected.

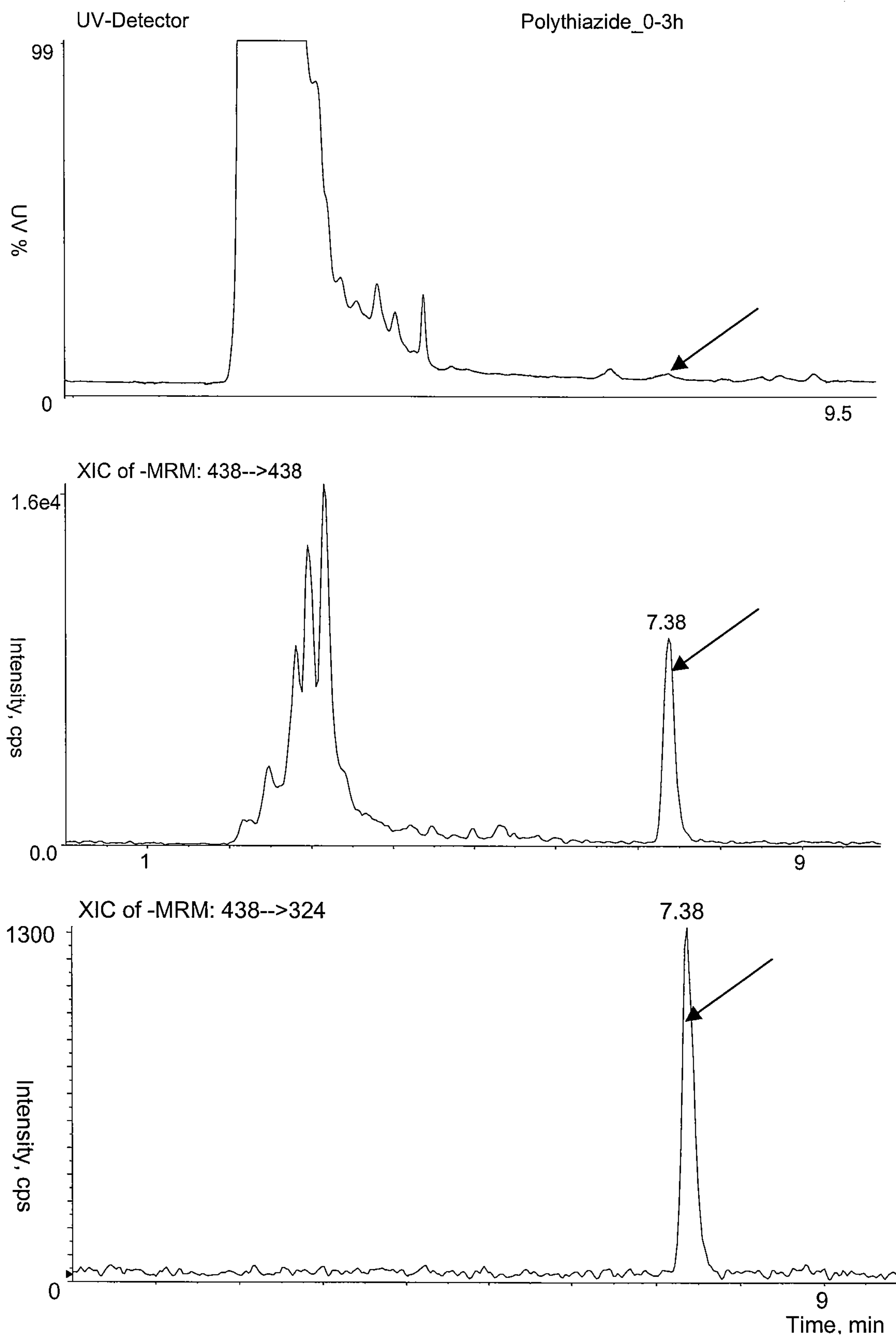

figure 3: Comparison of LC coupled to DAD (top) MS and MS/MS (bottom), applied to the identification of polythiazide in a control urine.

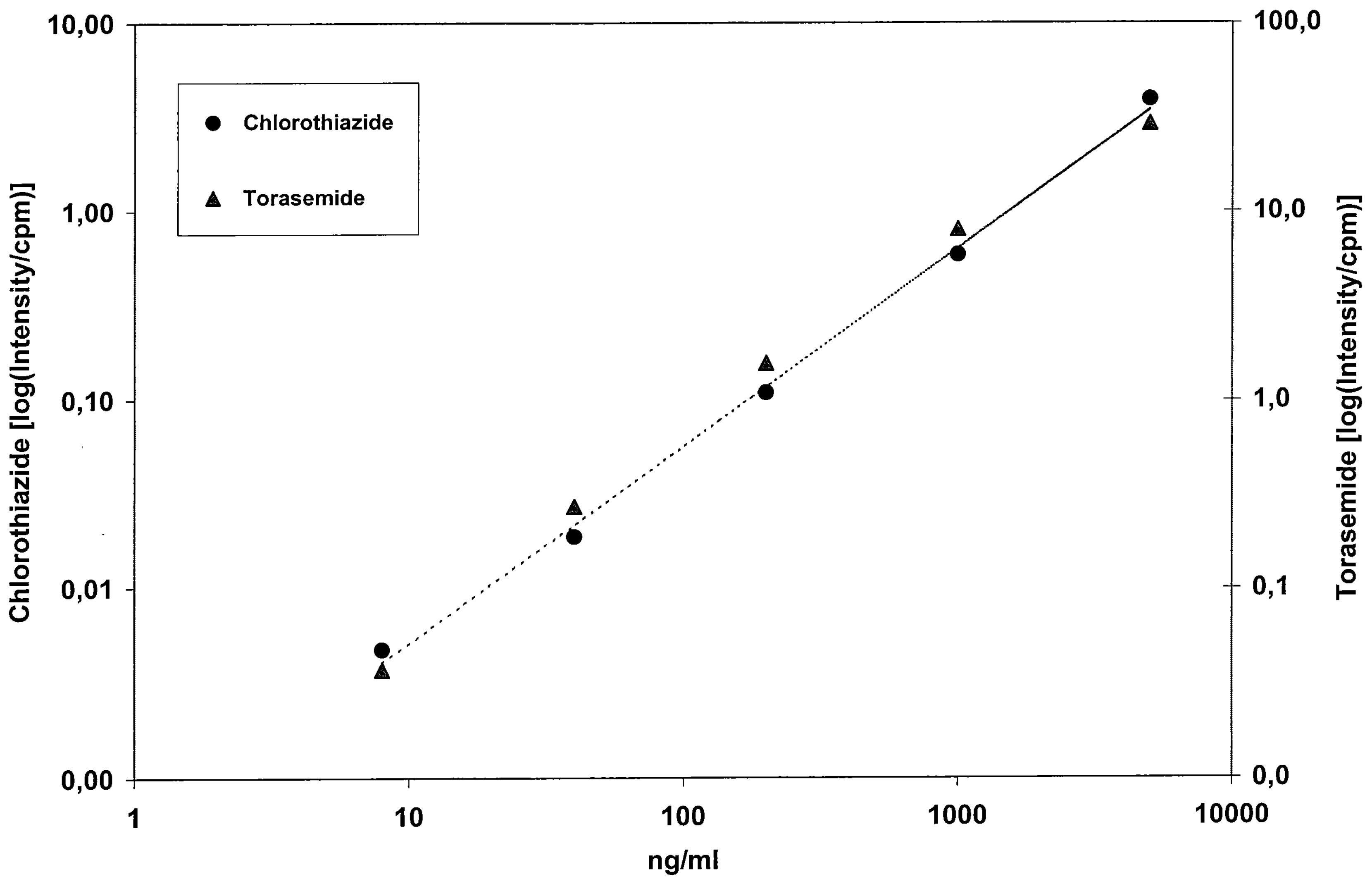

figure 4: External calibration of torasemide and chlorothiazide in a urine matrix