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Short communication

LC–high-resolution multiple stage spectrometric analysis of diuretic compounds Unusual mass fragmentation pathways

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ABSTRACT

The analysis of diuretic compounds has become of great concern because of their extensive use both in therapy and in illicit treatments (such as masking agents in sport doping and drug abuse). The variety of chemical structures of this class of drugs encouraged the development of new methods and techniques of analysis, especially as regards to acidic compounds. LC/MS has so grown to be the reference technique for this kind of analysis in forensic and anti-doping confirmation purposes.

Multiple stage MS permits identification of single drugs with high selectivity, but some unexpected pathways could weaken the entire process. In this work we aim to explain some unusual fragmentation steps using high-resolution MS^n.

For example, in the case of amiloride an intense product ion in MS^3 analysis generates an apparent loss of 10 Da. Water adduct formation and successive carbon monoxide elimination can explain this uncommon behavior, which was studied using different ion traps.

Bendroflumethiazide MS^n spectra show instead three successive HF losses, in spite of the presence of a radical site in the parent structure.

Homolytic cleavages with radical ion production occur also in the case of protonated positive ion of ethacrynic acid (loss of chlorine radical) showing that such fragmentation behavior is not so rare as generally reported.

Different ionization modes were studied and a tentative correlation with acidic-base properties was done. Multiple stage high-resolution mass spectra of positive and negative ions were discussed.

1. Introduction

Diuretics are a class of drugs which are banned in sport by the Medical Commission of the International Olympic Committee (IOC), since they may be abused by competitors to reduce weight quickly, to dilute urine or change pH in order to prevent detection of other drugs and to control the retention of water produced by anabolic steroids.

The analysis of diuretic compounds has become of great concern because of their extensive use both in therapy and illicit treatments such as masking agents in sport doping and drug abuse. They are usually analysed by gas chromatography/mass spectrometry (GC/MS) but due to their high polarity a derivatisation step is needed. Several years ago some papers were published comparing different derivatisation procedures before GC/MS analysis [1,2] even if extraction methylation is usually preferred [3]. More recently an improved derivatisation method of diuretics was reported [4] along with an application in fast gas chromatographic analysis [5]. However, the variety of chemical structure and polarity of this class of drugs encouraged the development of new methods and techniques of analysis, especially as regards to acidic compounds. In recent years LC/MS has assumed a role of great importance as a possible reference technique for this kind of analysis in forensic and anti-doping confirmation purposes [6–14]. The necessity to identify diuretics and their metabolites makes the interpretation of mass spectra obtained by collisionally activated dissociation particularly important. In fact to be able to know the origin and the assumed structure of fragment ions allow to obtain information about the administered drug and its related compounds. The possibility for LC/MS of operating in multiple stage MS mode should permit the identification of single drugs with high selectivity.

However, the interpretation and combination of the information coming from CAD experiments in order to obtain a complete or at least partial structure elucidation is not always straightforward. As a matter of fact negative ions formed by deprotonation of molecules structurally rigid show peculiar fragmentation pathways often involving homolytic cleavage and radical product ions

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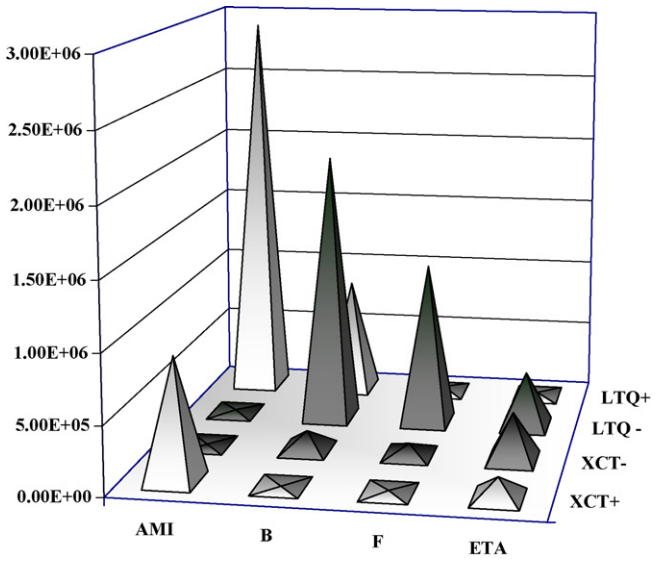


Fig. 1. Signal intensity of each compound obtained with LTQ and XCT in positive and negative ionization mode.

formation. Such behavior is not so common in a soft ionization source like ESI and requires complicate interpretation scheme. A study concerning the effect of the presence of functional groups of different acidity on the fragmentation of diuretics in negative electrospray ionization mass spectrometry has been reported [15]. In such a study a complex procedure based on selective alkylation and deuterium labeling was used to better elucidate the fragmentation pathways. Alternatively the use of a high-resolution mass spectrometer can simplify the approach to the experimental support of the supposable fragment losses.

A further unexpected complication comes from the possible presence of water or other solvent vapor inside ion trap which can interfere, by formation of adducts, with the fragmentation processes making their interpretation more difficult.

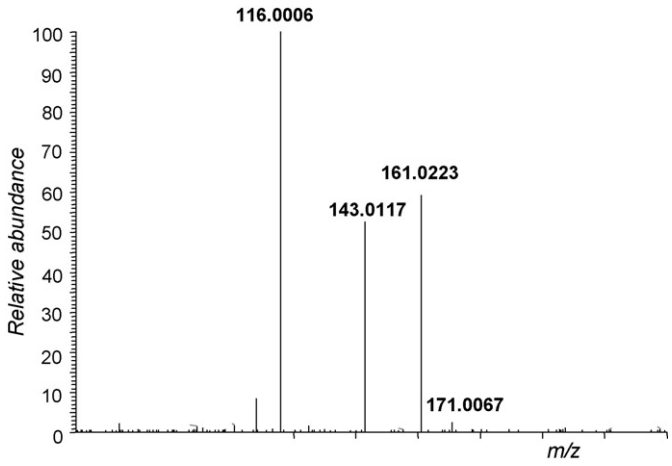


Fig. 2. MS^3 amiloride spectrum.

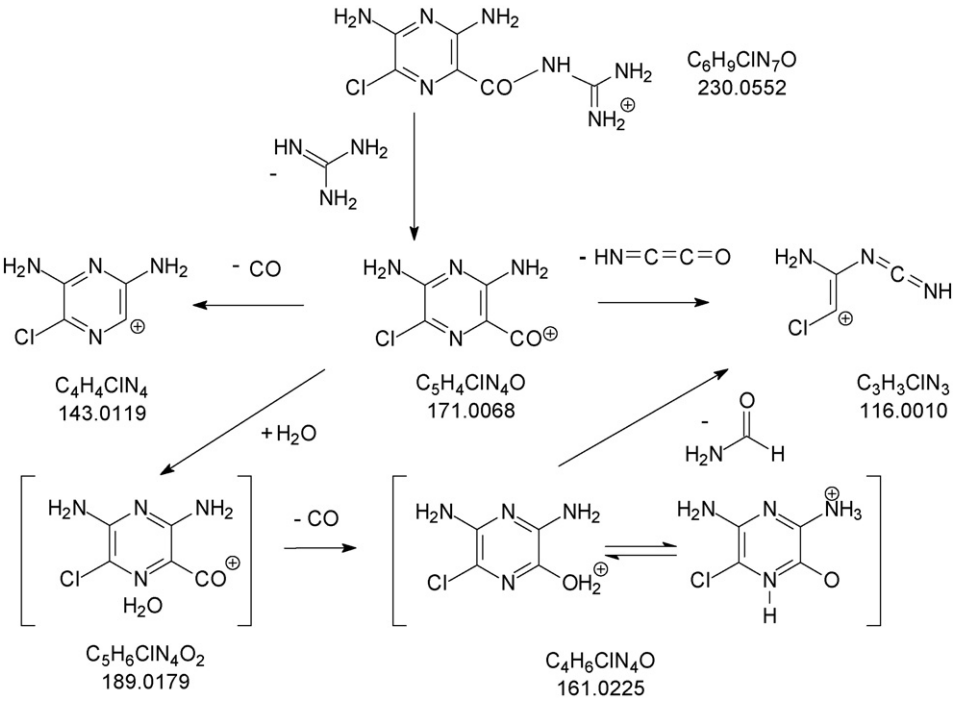
The group of diuretics studied in this work include compounds with different acid–base behavior: weakly acidic such as bendroflumethiazide (B) (pK_a 8.4 ± 0.5) [16] and acidic compounds such as furosemide (F) (pK_a 3.8 ± 0.5) [16] and ethacrynic acid (ETA) (pK_a 3.1 ± 0.5) [16] and basic compounds such as amiloride (AMI) (pK_a 9.0 ± 0.5) [16].

The aim of this work was essentially to elucidate the fragmentation patterns obtained by CAD experiments on the diuretics considered, trying to explain some unusual fragmentation steps with the aid of high-resolution MS.

2. Experimental

2.1. Sample preparation

Drug standards were purchased from Sigma (Milan, Italy). They were dissolved at a concentration of 1.0 g/L in methanol and then



Scheme 1. Amiloride hypothetical fragmentations.

diluted with methanol to 100.0 mg/L and with methanol–water 50:50 (v/v) to 1.0 mg/L. HPLC grade water was obtained from MilliQ System Academic (Millipore, Vimodrone, Italy). Methanol HPLC grade was filtered through a 0.45 μm filter before use. All solvents and reagents were from Aldrich (Milan, Italy).

2.2. HPLC–MS conditions

Analyses were performed on two LC–MS systems: Agilent LC/MSD Trap XCT Plus with ESI interface and Dionex Ultimate 3000, coupled with ESI interface to a Thermo LTQ Orbitrap FTMS spectrometer.

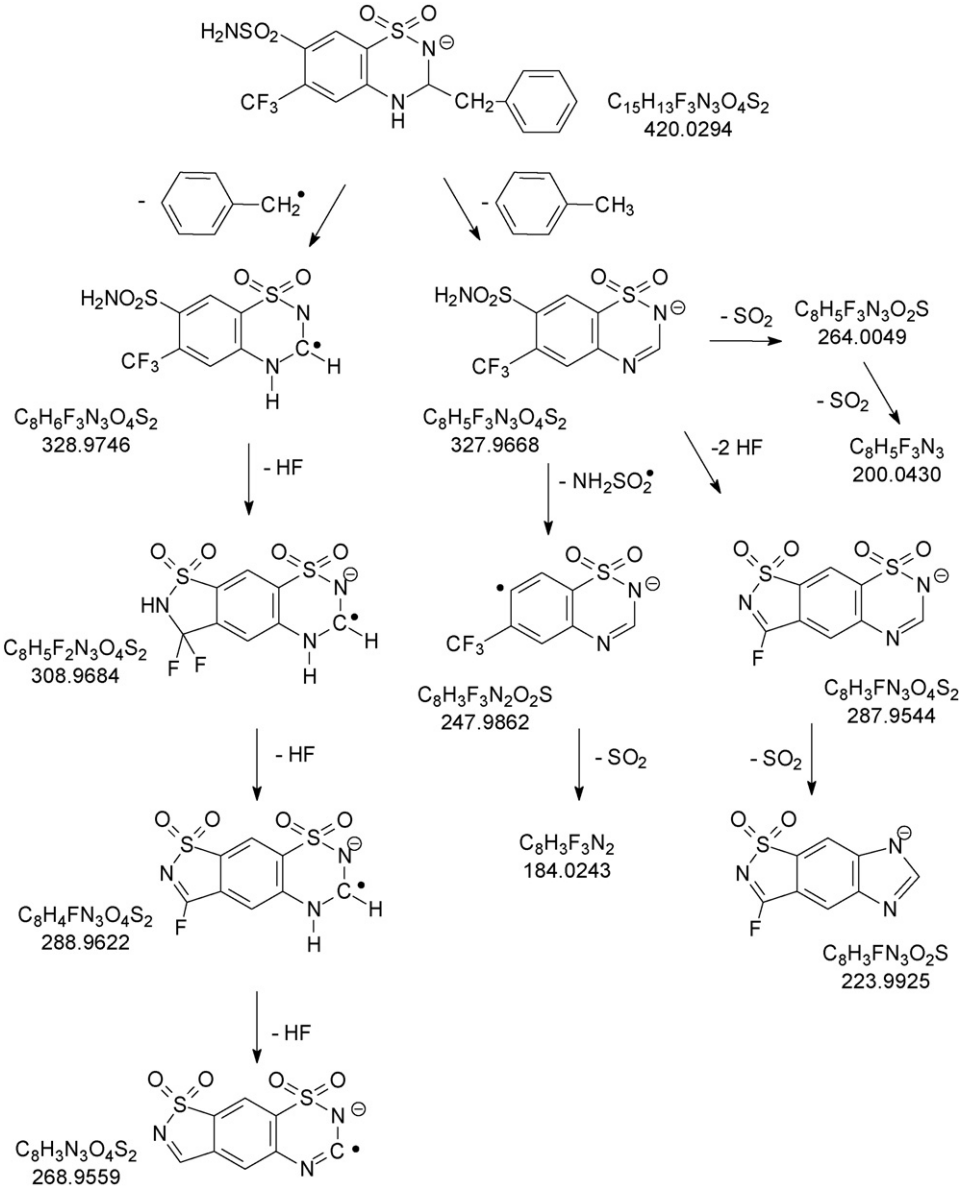
The chromatographic separations were run on a Phenomenex Luna C18 column (150 mm × 2 mm, 3 μm particle size). Injection volume was 20 μL and flow rate 200 μL/min. Gradient elution conditions were as follows: formic acid 0.05% in water/acetonitrile, from 90:10 to 10:100 in 22 min.

The effluent was delivered to the ion sources using nitrogen as sheath and auxiliary gas. For ESI LTQ mass analyzer in the negative ion mode, needle potential was 3.5 kV, tube lens offset 80 V, in the positive ion mode, needle potential was 4.5 kV, tube lens offset −80 V; heated capillary temperature was 270 °C. For ESI XCT in the negative ion mode needle potential was 4.5 kV, capillary exit 116 V, in the positive ion mode, needle potential was 4.5 kV, capillary exit −99 V; drying gas temperature 325 °C.

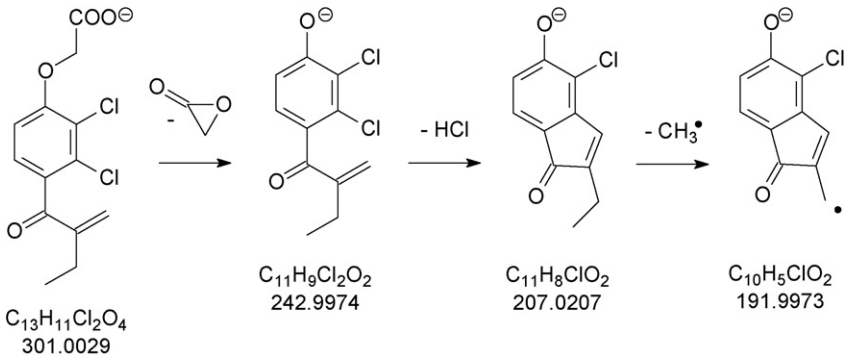
The acquisition method used was first optimized in the tuning sections for each compound de-protonated ion (capillary, magnetic lenses and collimating multipole voltages) to maximize sensitivity. Collision energy (CE) was generally chosen to maintain about 10% of the precursor ion. IT mass spectra were collected in full scan mode in the range 50–500 *m*/*z* and in the tandem MS mode in various ranges depending on precursor ion *m*/*z* value. Mass width accuracy in high-resolution mode was ±5 ppm.

3. Results

For optimum detection of the four diuretics by mass spectrometry, it was necessary to adjust the mass spectrometric conditions



Scheme 2. Bendroflumethiazide hypothetical fragmentations.



Scheme 3. Ethacrynic acid hypothetical fragmentations.

after LC separation. The chromatographic method was a conventional gradient separation on a RP C18 column with an acidic eluent able to increase the retention of the acidic compounds. A good separation of the studied substances was obtained and the order of elution was amiloride (t_R 2.0 min), furosemide (t_R 13.1 min), bendroflumethiazide (t_R 14.3 min) and ethacrynic acid (t_R 17.9 min).

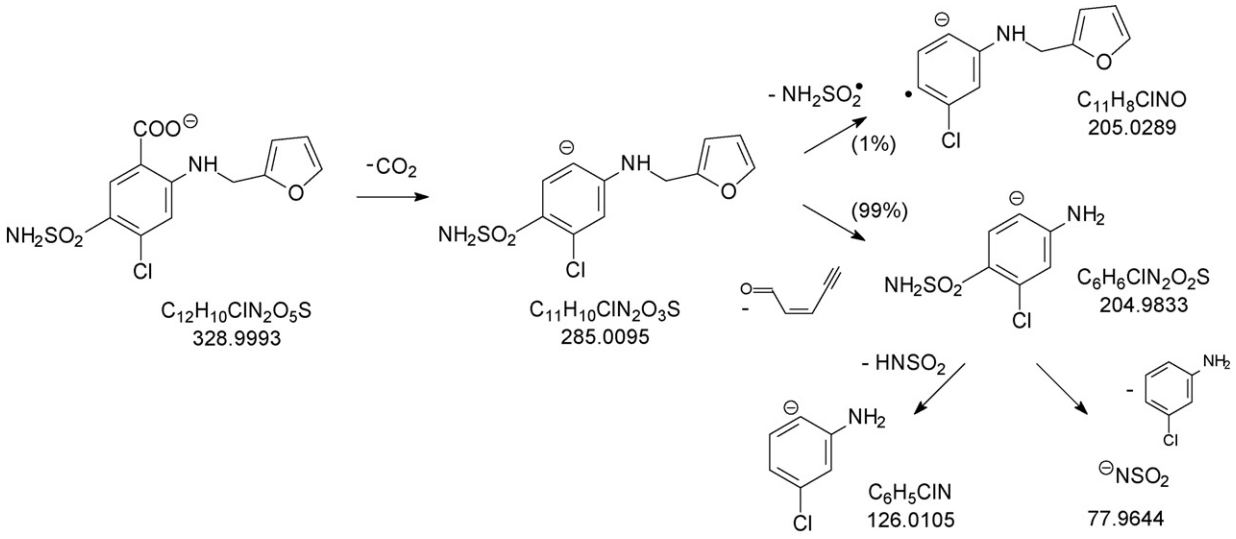
Different ionization modes were studied and a tentative correlation with acidic-base properties was done. Fig. 1 represents the signal intensity of each drug, using the two different instruments. Amiloride does not have acid functions and so positive ionization mode is preferable. On the contrary the ethacrynic acid does not have basic functionality and so the negative ionization mode gives the better sensitivity, although in the positive mode there is a low signal due to $[M+K]^+$ adduct.

Bendroflumethiazide and furosemide own in principle both functionalities. They own potentially basic sites whose conjugate acids have pK_a values below 0 (aryl-ethylamine in furosemide, p$K_a = -1.0 \pm 0.5$; nitrogen in 4 position in bendroflumethiazide, p$K_a = -1.0 \pm 0.5$ [16]) and the ionization in water should take place only in extremely acid environment. In effect their positive ions show slight signal intensity. Acquiring spectra in the negative ion mode gave good results for all of the acidic drugs. The relative intensity of these signals should however take into account the intrinsic solubility and ESI source engineering because they do not correlate exactly with the pK_a data.

In the case of amiloride an intense base peak at m/z 230.0552 $[M+H]^+$ was revealed. Such precursor ion was isolated and then subjected to collisional activation in two different ion traps. The product ion spectrum clearly shows the formation of the m/z 171.0068 acylium ion by the loss of guanidin. MS^3 spectrum evidenced the ion m/z 161.0225 due to an apparent elimination of 10 Da (Fig. 2). By the accurate mass data measurement [$\Delta m = -9.9844 = 18.0100$ (H_2O) -27.9944 (CO)] it was possible to assign this loss to a concerted water addiction/carbon monoxide elimination reaction inside the ion trap. This result was confirmed on XCT trap which differently from LTQ shows evidence of a peak at m/z 189 $[M+H_2O]^+$ even more intense than the m/z 171 ion. This can be explained by the formation of an ion–neutral molecule complex with the residual H_2O in the ion trap by the ion at m/z 171. The reverse process (189 → 171) also occurs. The equilibrium reached by these two opposite processes determines the relative abundance of the m/z 171 and 189 ions. Analogous cases were reported in the literature [17,18]. We evaluated also the effect on peaks abundance by varying ion trap parameters dedicated to avoid space charge effects (automatic gain control, AGC on LTQ and ion charge control, ICC on XCT) but no meaningful effect on spectra was observed. Moreover only peak abundance showed to change by collision energy modulation without significant spectrum modification in each one of the two instruments.

The successive fragmentation is related to this fragmentation behavior by successive loss of formamide. Different ion traps shows the same unusual loss pattern (Scheme 1).

Bendroflumethiazide was detected as negative deprotonated ion $[M-H]^-$ at m/z 420.0294. In the MS^2 spectrum it is noteworthy



Scheme 4. Furosemide hypothetical fragmentations.

the presence of two chemical species with one mass unit difference. m/z 327.9668 ion is due to the loss of toluene and m/z 328.9746 ion was attributed to the homolytic breakdown to form a benzyl radical (Scheme 2). MS^3, MS^4 and MS^5 spectra of radical ion m/z 328.9746 were not leading to even electron product ions but showed three consecutive HF losses. The stability of the radical species of high electron delocalization probably allows this type of elimination. In the parallel pathway involving the even electron ion at m/z 327.9668 a homolytic fragmentation is unexpectedly favored in MS^3 spectrum. An aminosulphonyl radical was lost to form a m/z 247.9862 radical ion which successively lost SO_2 confirming the high stability of the radical structure. Alternative competitive pathways involving only even electron ion species are present. The main one involves two successive losses of SO_2 groups, the minor one a double HF elimination which form an ion structurally analogous to m/z 288.9622 radical ion detected in MS^4 conditions from the parent radical ion at m/z 328.9746. The behavior of such even electron ion (m/z 287.9544) is however different because instead of eliminating the last HF molecule it loses the residual SO_2 group.

Homolytic fragmentation with radical ion production is not so rare as generally reported, in fact also in the cases of negative ion fragmentation of ethacrynic acid a radical ion was obtained: m/z 191.9973 by loss of methyl radical (MS^4, Scheme 3). However, in all the cases examined one deals with charge-remote fragmentation, whose occurrence often involves homolytic cleavage [19]. In all cases the high resolving power was an important support to the correct identification of fragments. In particular it was useful to discriminate between approximate isobaric losses in fragmentation of similar compounds. In the fragmentation pathway of furosemide the main MS^2 product ion at m/z 285.0095, generated by a decarboxylation from the protonated precursor ion at m/z 328.9993, as already reported in literature [15], shows a loss of a neutral unsaturated molecule C_5H_4O (80.0257 u) and not of the aminosulphonyl radical (79.9801 u) on the basis of the successive MS^4 step (Scheme 4). Anyway furosemide $HRMS^3$ spectrum allows to verify the minority presence of the aminosulphonyl radical elimination (0.8%).

As one can see, a series of particular fragmentation behavior has been reported. The use of mass spectrometer with high resolving power permitted certain identification of product ions formed so allowing to improve the analytical characterization of a heterogeneous class of drugs.

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References

[1] R. Ventura, J. Segura, J. Chromatogr. B: Biomed. Appl. 687 (1996) 127–144.
[2] D. Carreras, C. Imaz, R. Navajas, M.A. Garcia, C. Rodriguez, A.F. Rodriguez, R. Cortes, J. Chromatogr. A 683 (1994) 195–202.
[3] A.M. Lisi, G.J. Trout, R. Kazlauskas, J. Chromatogr. B: Biomed. Appl. 563 (1991) 257–270.
[4] L. Amendola, C. Colamonici, M. Mazzarino, F. Botrè, Anal. Chim. Acta 475 (2003) 125–136.
[5] V. Morra, P. Davit, R. Capra, M. Vincenti, A. Distilo, F. Botrè, J. Chromatogr. A 1135 (2006) 219–229.
[6] C. Goebel, G.J. Trout, R. Kazlauskas, Anal. Chim. Acta 502 (2004) 65–74.
[7] A. Vonaparti, M. Kazanis, I. Panderi, J. Mass Spectrom. 41 (2006) 593–605.
[8] E.N.M. Ho, D.K.K. Leung, T.S.M. Wan, N.H. Yu, J. Chromatogr. A 1120 (2006) 38–53.
[9] F. Albu, C. Georgica, V. David, A. Medvedovici, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 816 (2005) 35–40.
[10] M. Thevis, W. Schanzer, J. Chromatogr. Sci. 43 (2005) 22–31.
[11] K. Deventer, P. Van Eenoo, F.T. Delbeke, Rapid Commun. Mass Spectrom. 19 (2005) 90–98.
[12] Y. Qin, X.B. Wang, C. Wang, M. Zhao, M.T. Wu, Y.X. Xu, S.Q. Peng, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 794 (2003) 1011–1017.
[13] K. Deventer, F.T. Delbeke, K. Roels, P. Van Eenoo, Biomed. Chromatogr. 16 (2002) 529–535.
[14] J. Caslavska, W. Thormann, J. Chromatogr. B 770 (2002) 207–216.
[15] M. Thevis, W. Schanzer, H. Smickler, J. Am. Soc. Mass Spectrom. 14 (2003) 658–670.
[16] ADME Boxes software version 2.0, http://www.ap-algorithms.com/.
[17] A.B. Attygalle, N. Kharbatia, J. Bialecki, J. Ruzicka, A. Svatoš, E.J. Stauber, Rapid Commun. Mass Spectrom. 20 (2006) 2265–2270.
[18] Z. Guan, J.M. Liesch, J. Mass Spectrom. 36 (2001) 264–276.
[19] J. Adams, Mass Spec. Rev. 9 (1990) 141–186.