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New approach for the detection of BSH and its metabolites using capillary electrophoresis and electrospray ionization mass spectrometry

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Abstract

Boron neutron capture therapy is a promising binary treatment for cancer. It is based on the nuclear fission that occurs when non-radioactive ^{10}B absorbs thermal neutrons. One of the two boron compounds currently used in clinical trials for this therapy is BSH. To ensure differentiated retention in the tumour versus normal tissue prior to treatment, routine analytical methods to determine pharmacokinetics must be available. For this purpose we have developed a new, easy and time saving approach, in which the separation of boron derivatives is performed by means of capillary electrophoresis (CE). The CE method allows analyses to be performed in short times (less than 18 min), sensitively (LOD 8 pg loaded on the capillary) quantitatively (LOQ 5 μg/ml) and with a high efficiency of separation. Moreover it is simpler than HPLC and more reproducible (intra- and inter-day values were ±1% and ±3%, respectively), and does not require a specific column of derivatization. Mass spectrometry analysis of boron derivatives in different samples was also performed to ensure correct attribution of the CE peaks.

Keywords: BSH; Boron

1. Introduction

Boron neutron capture therapy (BNCT) is a promising binary treatment for cancer. It is based on the nuclear fission [$^{10}B(^{1}n,\alpha)^{7}Li$] that occurs when non-radioactive ^{10}B (in nature 20% of elemental boron) absorbs thermal neutrons (0.025 eV) [1]. Provided that the amount of ^{10}B is in the range of 15–30 μg per gram of tumour, which seems to be the minimum amount able to sustain lethal tumour cell damage [2,3], its selective uptake would allow highly localized damage, as the range of high-LET (linear energy transfer) particles is limited to ~1 cell diameter [1]. Therefore, it is of primary importance that timing is correct for achieving the maximal cytotoxic effect upon malignant cells, together with the maximal tolerated dose to contiguous cells [4]. On the other hand correct timing can only be ensured

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if pharmacokinetics can be readily measured and if administered drugs can be measured in biological samples.

Although several ^{10}B containing compounds belonging to different families have been synthesised, there are currently just two products currently used in BNCT trials for the treatment of glioblastoma multiforme and melanoma: BSH (*closo*-undecahydro-1-mercaptododecaborate) disodium salt and BPA (boro-phenylalanine).

When compared to BPA, the sulfydryl containing polyhedral borane (cage) anion BSH shows several favourable characteristics: high boron content, low toxicity, a spherical geometry and previous use for BNCT clinical trials on patients at the high flux reactor of the European Commission in Petten (the Netherlands) [5,6].

Different analytical methods have been proposed, which are mainly used to assess the purity of synthetic BSH, its amount and the quantities of its oxidation impurities, namely BS-SB and BS-O-SB. Among these methods, thin layer chromatography [7] and capillary isotachophoretic methods [8,9] have been developed, but their resolution is insufficient for the determination of all contaminants. Thereafter, a short description of an HPLC method based on ion pair chromatography on octadecyl silica gel was published [10]. More recently, a new HPLC method based on the use of hydroxyethylmethacrylate sorbent Separon HEMA-BIO 300 and 100 m*M* sodium perchlorate in 0.01 *M* phosphate buffer as mobile phase has been described [11,12]. According to this procedure, common HPLC equipment with direct spectrophotometric detection is used; however a major limitation is the need for a special column, which is not generally commercially available [12]. Moreover, nuclear magnetic resonance (NMR) has been used to detect boron derivatives [13,14], but this approach is not quantitative and its sensitivity is very low.

To study in depth the biological behaviour of BSH, which has not yet been completely elucidated, we developed a new, easy and time saving method, which allows the quantitative determination of BSH and its impurities.

We propose a new analytical approach in which the separation of boron derivatives is performed by means of capillary electrophoresis (CE). The CE method allows analyses in short times, quantitatively and with high efficiency of separation. Moreover it is more simple and reproducible than HPLC, and does not require a specific column of derivatization.

In order to ensure the correct attribution of peaks obtained through CE, mass spectrometric analysis of the boron derivatives in different samples was also performed. The favourable characteristics of the analytical procedure described in this paper will allow the establishment of protocols to routinely determine blood and urine pharmacokinetics in treated patients, the knowledge of which is very important for further development of BNCT.

2. Experimental

2.1. Chemicals

^{10}B enriched (99%) BSH and BSSB standards were obtained from Katchem (Prague, Czech Republic).

Sodium phosphate was supplied from J.T. Baker (Deventer, Holland). Methanol, acetonitrile and water were HPLC grade.

2.2. Apparatus

Capillary electrophoresis separations were carried out using a Model 270A apparatus from Applied Biosystem (San Jose, CA, USA) equipped with a 46 cm (to the detector)×50 μm I.D. fused-silica capillary. The running buffer was 100 m*M* phosphate (pH 7.0). The voltage was −300 V/cm; injection was by aspiration for Model 270A (1–2 s corresponding to ~4–8 nl). The temperature was 30 °C and detection was performed at 204 nm. The capillary was rinsed with fresh buffer by 2-min aspiration after each run.

Mass spectrometry analyses were performed by direct infusion (flow rates 15 μl/min) into an LCQ_{DECA} ion trap mass spectrometer (Thermo Finnigan, Milan Italy) equipped with an electrospray interface. The following instrumental parameters were used for ESI-MS detection of boron derivatives: capillary voltage −23 V, capillary temperature 300 °C, sheath gas flow 70 (in arbitrary units), ion spray voltage 4.20 kV.

2.3. Sample preparation

2.3.1. Drug samples

BSH solutions in 0.9% sodium chloride were prepared as follows: (a) pharmaceutical form A (final concentration: 26.6 mg BSH/ml): 771.4 mg of BSH were dissolved under stirring in 29.0 ml of 0.9% NaCl previously degassed by flushing argon for 10 min. The pH of the resulting solution was 7.3. After filtration through a 0.2-μm filter, argon was bubbled again into the preparation, and 1-ml fractions of the solution obtained were stored protected from light at −20 °C; (b) pharmaceutical form B (final concentration: 6.65 mg BSH/ml; high BSSB content): 212.8 mg BSH were dissolved in 32.0 ml 0.9% NaCl solution. The measured pH value was 7.4. For the rest, samples were prepared as reported for pharmaceutical form A.

Before the analyses, both pharmaceutical preparations were diluted 100–500-fold with water.

2.3.2. Urine samples

Urine samples were obtained from patients participating in the EORTC phase I trial 11961 ''Postoperative treatment of glioblastoma with BNCT at the Petten irradiation facility'' [15,16]. BSH was dissolved in a 0.9% NaCl solution to obtain a total volume of 500 ml for 100 mg BSH per kg body weight (proportionally less if less BSH is to be infused). The infusion rate was defined to be 1 mg BSH per kg body weight per minute. The patients received 100 mg BSH per kg body weight prior to the first BNCT treatment. Then the amount of BSH was adjusted to guarantee an average ^{10}B-concentration of 30 ppm over the entire irradiation treatment.

Urine samples were collected before BSH administration. After each BSH infusion, the 24-h urine were collected and a sample was taken from this volume. Urine samples were injected on CE capillary after centrifugation at 4000 *g* for 5 min.

2.4. Standard solutions

BSH and BSSB were dissolved in water ([c]=1 mg/ml). These solutions were diluted with water to obtain different concentration (5–300 μg/ml) and 2-s injections were performed.

3. Results and discussion

The 95% ^{10}B enriched form of BSH is used as sodium salt ($Na_2B_{12}H_{11}SH$) in cancer radiation treatment (BNCT). In Fig. 1 the molecular structure of the sodium borocaptate monomer (BSH) is shown. Usually, the determination of BSH and its dimer (BSSB) has been carried out by ion pair HPLC [12].

By using CE, it has been possible for the first time to separate and quantify BSH and BSSB in pharmaceutical preparations. Different CE conditions have been tested and the best separation was obtained using 100 m*M* phosphate buffer, pH 7.0. A typical electropherogram of a standard mixture of BSH and BSSB is shown in Fig. 2. These compounds are well resolved within 18 min with a standard deviation of the migration times of 0.5% (n=10) and 0.8% (n=7) for intra- and inter-day determinations, respectively. The limit of detection (LOD) at 204 nm was ~1 μg/ml (S/N=3), while limit of quantitation (LOQ) was 5 μg/ml (S/N=10). The detector response was linear in the range 5–300 μg/ml (n=5), and the regression coefficient was higher than 0.998. Repeatability of the quantitative data, in the linear range, was around ±1% and ±3% for intra- (n=5) and inter-day (n=6) analyses, respectively.

Stability of BSH and BSSB has been also evaluated. Different standard solutions of BSH and BSSB ([c]=0.05 mg/ml) have been stored at environmental and 4 °C temperature in presence or absence of light, respectively. Under these conditions, it has been possible to observe that BSSB is stable at 4 °C and in the dark, while at 25 °C and in the presence of

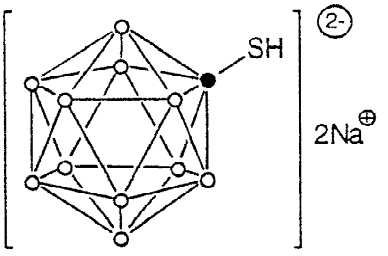

Fig. 1. Structure of BSH (*closo*-undecahydro-1-mercapto-dodecaborate) disodium salt. The black point in the structure evidences the boron atom bound to the –SH.

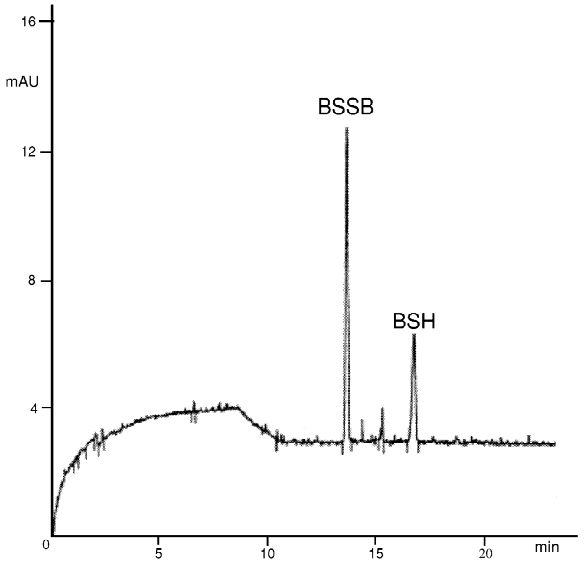

Fig. 2. Typical electropherogram at 204 nm of BSH and BSSB standards.

light the BSSB peak decreases ~50% after 48 h. In contrast, the peak of BSH shows no difference.

The developed method was applied for determining the concentration of BSH and BSSB in different pharmaceutical preparations. In this way it has been possible to detect the eventual presence of BSSB impurity. As an example, Fig. 3 shows the electropherogram of a BSH drug (pharmaceutical preparation A), which is normally used for intravenous infusion. In this case, it is possible to observe the

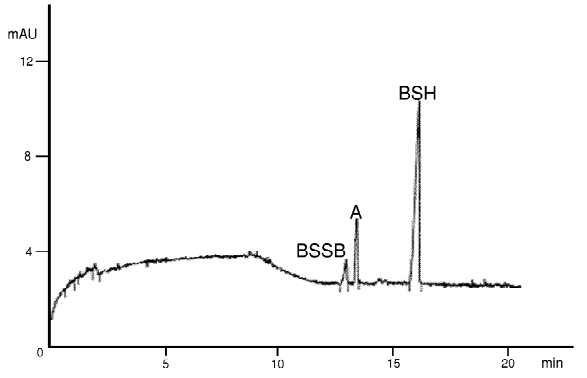

Fig. 3. Electropherogram at 204 nm of BSH pharmaceutical preparation A.

presence of BSSB impurity and an additional contaminant (peak A). In addition, the BSH and BSSB concentrations in the pharmaceutical preparations were determined. In particular we found levels of BSH and BSSB of 20.1 and 1.72 mg/ml, respectively, and the C.V. was in the range of 2–5%. The difference between the detected content of BSH and the theoretical value (26.6 mg/ml BSH) declared by the supplier is quite high. A possible explanation for this finding is the presence of the additional contaminant (peak A). Using the calibration curve of BSH we managed to estimate a concentration of 3.84 mg/ml for this impurity (peak A). In the pharmaceutical preparation B the measured content of BSH, BSSB and peak A were 6.47, 28.03 and 2.09 mg/ml, respectively.

MS measurements were performed to confirm the attribution of the peaks obtained in CE analysis. Specifically boron derivatives have been analysed by means of electrospray mass spectrometry. Fig. 4 shows typical mass spectra for BSH (Fig. 4a) and BSSB (Fig. 4b). For BSH the main ions m/z 187.4 and 397.6 correspond to $[M_1+Na]^-$ and $[(M_1Na)_2+Na]^-$, respectively. In addition, bicharged species $[M_1]^{2-}$ (m/z 82.3) was also detected. In the MS analysis of BSSB it has been possible to detect two different sodium adduct ions m/z 395.7 ($[M_2+3Na]^-$) and m/z 186.4 ($[M_2+2Na]^{2-}$).

To confirm the identification of these compounds, tandem mass spectrometry experiments (MS/MS) were performed. In particular, the ion m/z 187.4 produced the fragment m/z 131.6 (Table 1), due to elimination of the NaS residue. In the same way, the ion m/z 397.6 produced the fragments m/z 187.4 (MS/MS^2) and m/z 131.6 (MS/MS^3). In the contrast, the dimer (BSSB) is very stable and its fragmentation is more complex. In Table 1 the fragments obtained from MS/MS analysis of BSH and BSSB are shown.

The MS approach was applied to analyse the 1000-fold diluted pharmaceutical preparations. This allowed the identification of the monomer (BSH) and has given further prove of the presence of an unexpected impurity (Fig. 5; m/z 245.2 = BSH + 58), which could correspond to that observed in CE (peak A). In fact, MS/MS studies (Table 1) of ion m/z 245.2 $[M_3+Na]^-$ produced the fragments m/z 187.4 (MS/MS^2) and m/z 131.7 (MS/MS^3). A possible

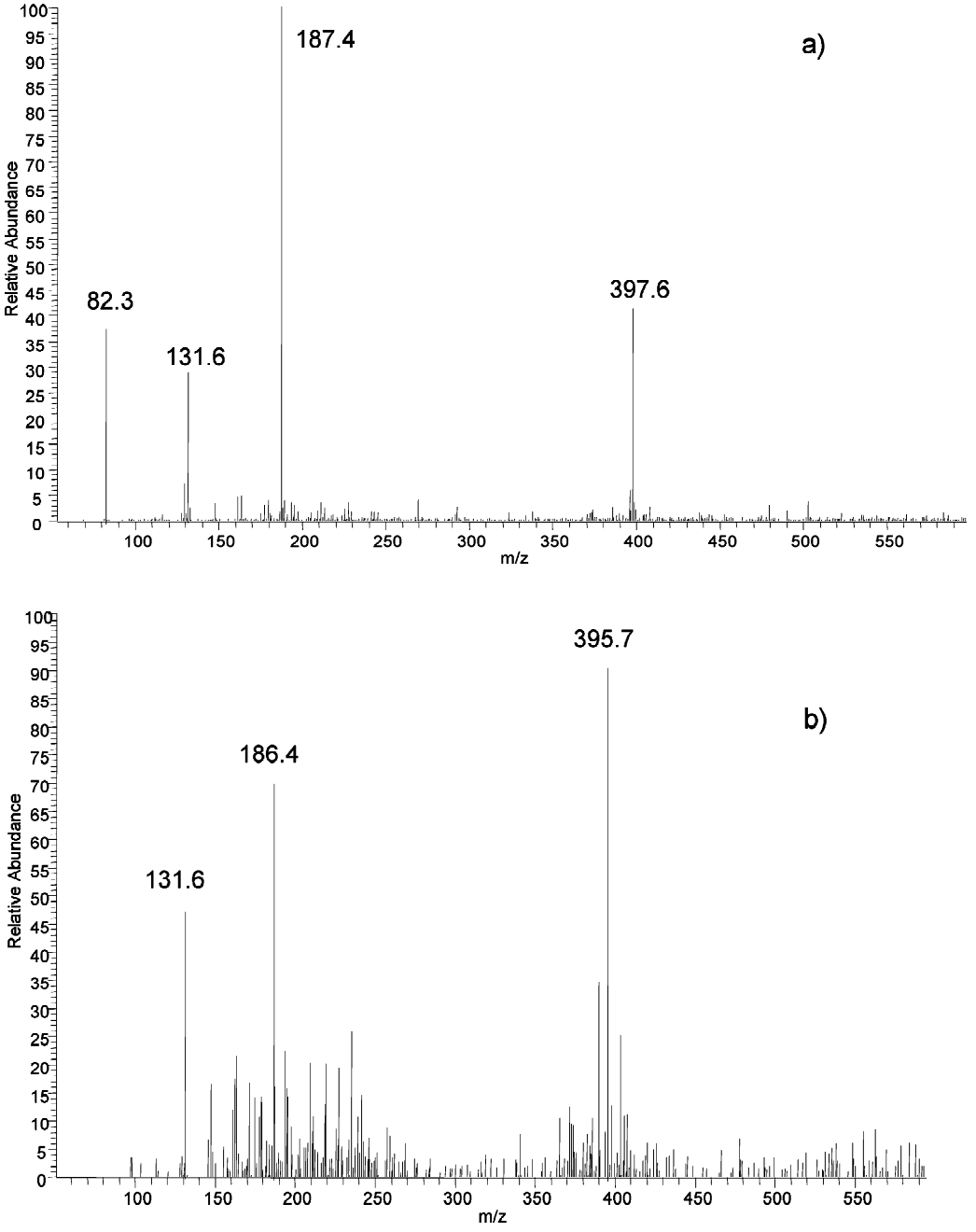

Fig. 4. Typical mass spectra of (a) BSH and (b) BSSB standards.

interpretation of this finding is the presence in the formulation of either the glycine or the acetate derivative of BSH. The ion m/z 395.7 corresponding to BSSB is present at a very low level and its identification is possible only by isolation and fragmentation.

Table 1
Fragments obtained from MS/MS analyses of boron derivatives

Parent	MS/MS^n	Product
397.6 $[(M_1Na)_2+Na]^-$	$n=2$	187.4
	$n=3$	131.6
187.4 $[M_1+Na]^-$	$n=2$	131.6
82.3 $[M_1]^{2-}$	$n=2$	131.6
395.7 $[M_2+3Na]^-$	$n=2$	391.9
186.4 $[M_2+2Na]^{2-}$	$n=3$	337.7
245.2 $[M_3+Na]^-$	$n=2$	187.4
	$n=3$	131.6

M_1 corresponds to $[^{10}B_{12}H_{11}SH]^{2-}$ (BSH). M_2 corresponds to $[^{10}B_{12}H_{11}SS^{10}B_{12}H_{11}]^{4-}$ (BSSB). M_3 corresponds to the glycin or acetate derivative of BSH.

3.1. Detection of ^{10}boron derivatives in human urine

The described analytical approaches used for studying standard and pharmaceutical preparations of boron derivatives were then applied to the analysis of biological sample. Urine samples of cancer patients treated with 1 mg BSH per kg body weight per minute, were analysed both by CE and ESI-MS. Fig. 6 shows a typical CE electropherogram of a 20-fold diluted urine sample collected after 24 h. The identification of BSH was performed by comparing the retention time of a spiked sample with that of the standard.

Further confirmation was achieved by analysing urine samples by direct infusion into electrospray mass spectrometry. Fig. 7a reports a typical mass spectrum of a 100-fold diluted urine sample. The main ion m/z 187.4 corresponds to $[M_1+Na]^-$, where the attribution was confirmed by its MS/MS fragmentation spectrum (Fig. 7b). In addition, in the urine mass spectrum the ion m/z 245.2 is present and corresponds to the glycine or acetate derivative (Table 1).

Probably, the ion m/z 245.2 is the same as that observed in the pharmaceutical formulation infused into the patients (Figs. 5 and 7a).

4. Conclusions

CE and ESI-MS methods allow the separation and characterization of ^{10}B-enriched compounds in different matrices, such as pharmaceutical preparations and urines. The capillary electrophoretic method is reproducible, rapid and allows quantitative analyses.

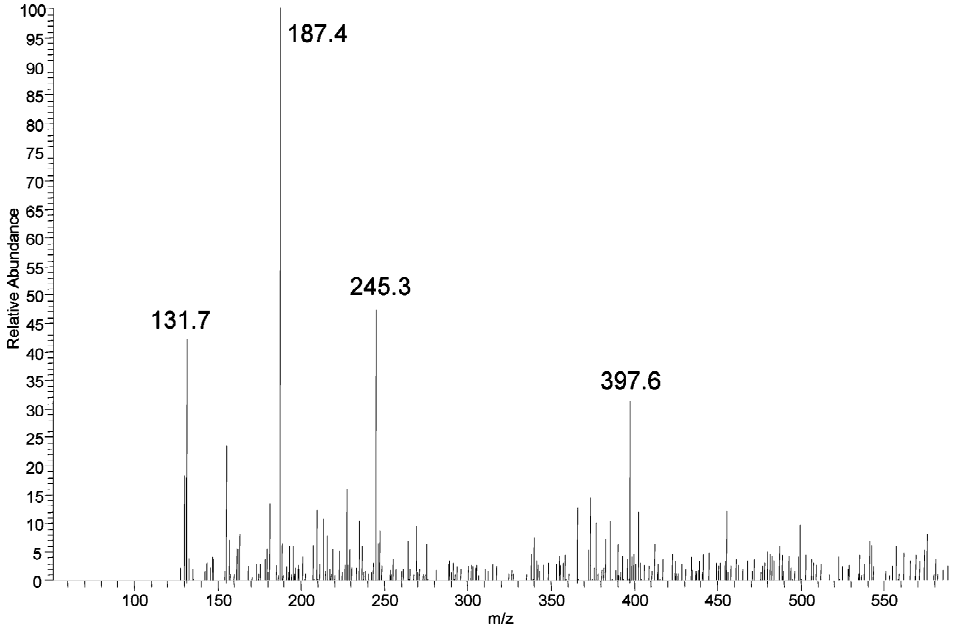

Fig. 5. Mass spectrum of the same sample analyzed by CE and reported in Fig. 3.

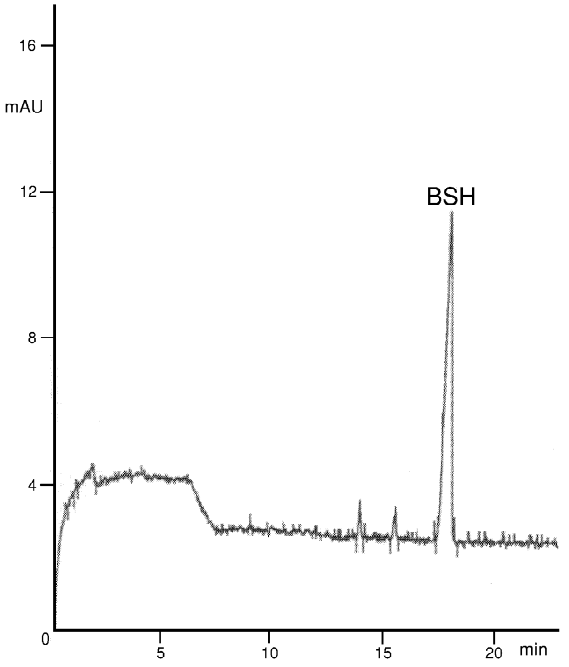

Fig. 6. Electropherogram of 20-fold diluted urine sample.

Using ion trap mass spectrometry (ITMS) it is possible to reach a limit of detection (LOD) of 20 ng/ml. In addition, ESI-MS/MS detection permits the complete characterization of analytes. These approaches may be very important for studies concerning the pharmacokinetics of ^{10}B-enriched compounds used in the treatment for cancer disease. The developed method and the preliminary results obtained for urine sample are very interesting, but more experiments are necessary. In particular, further well defined sampling in patients is required, and direct coupling of CE with ITMS is desirable to combine efficient separation (CE) and high sensitivity (ITMS).

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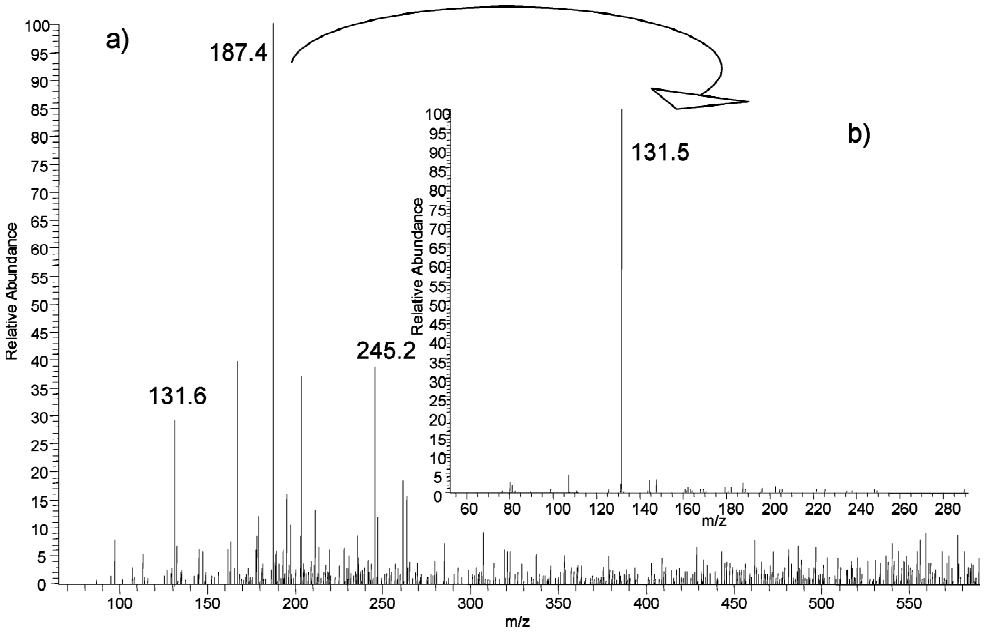

Fig. 7. (a) Mass spectrum of 100-fold diluted urine sample of a treated patient; (b) MS/MS^2 of ion m/z 187.4.

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