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Comparison of a preliminary procedure for the general unknown screening of drugs and toxic compounds using a quadrupole-linear ion-trap mass spectrometer with a liquid chromatography–mass spectrometry reference technique

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Abstract

Liquid chromatography–tandem mass spectrometry (LC–MS–MS) might be a complement to GC–MS and HPLC–diode array detection for the general unknown screening (GUS) of drugs and toxic compounds, particularly when using information- or data-dependent acquisition (IDA or DDA), an auto-adaptive MS–MS product-ion scan mode where, at each unit time, the m/z ratios above a given intensity threshold are selected for fragmentation. A new quadrupole-linear ion-trap mass spectrometer (LC-QqQlinear ion-trap) was evaluated for GUS using IDA. For the first detection step (so-called ''survey scan'') the single quadrupole ''enhanced'' MS mode (EMS), where ions are accumulated then filtered in the Q3-linear ion-trap, was used. The so-called ''enhanced'' parent ion scan mode (EPI) used at two alternated fragmentation energies gave the best signal intensity and the best mass spectral information when adding mass spectra obtained in low and high fragmentation conditions, respectively, both in the positive (+20 and +50 eV) and negative (−15 and −40 eV) modes. Solid-phase extracts of serum spiked with eight test compounds (chosen for their retention times distributed along the 30-min long chromatogram and for ionising in both the positive and negative modes) were analysed in parallel with this LC–MS–MS technique and with a reference LC–MS method run on a single-quadrupole instrument where low and high in-source fragmentation conditions in the positive and the negative ion modes are alternated. A C_{18}, 5 μm (150×1 mm I.D.) column and a gradient elution of acetonitrile in pH 3, 2 m*M* ammonium formate, were used for both. Higher signal-to-noise ratios were obtained with the LC-QqQlinear ion-trap instrument than with the reference technique, resulting in mass spectra devoid of contaminant ions and at least as informative as the reconstructed single-MS spectra. After optimisation of the IDA intensity threshold for the detection of tiny chromatographic peaks in noise, five out of the eight compounds (milrinone, lorazepam, fluometuron, piretanide and warfarin) could be unambiguously identified at the concentration of 0.1 mg/l in serum, in the positive or negative modes, or in both, versus only two by LC–MS. All of them could be identified at 1 mg/l by both techniques. These preliminary results show that the sensitivity and mass structural information brought by this new LC-QqQlinear ion-trap instrument may help design an efficient toxicological GUS procedure.
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Keywords: General unknown screening; Linear trap

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1. Introduction

The identification of xenobiotics in biological fluids generally involves a panel of automated immunological screenings (for the most common drugs) and chromatographic techniques, ideally coupled to specific detectors (mass spectrometers or UV-diode array detectors). Nevertheless, failures are not uncommon, particularly when polar compounds, with no or little UV absorbency are involved. However, mass spectrometry (MS) is more specific and reliable than diode array detection (DAD) and should always be preferred when possible. As gas chromatography (GC) is limited to volatile and thermally stable compounds, the coupling of MS with high-performance liquid chromatography (HPLC) has long been regarded as a possible means to increase the range of compounds amenable to MS.

Single mass spectrometry with in-source collision-induced dissociation (CID), tandem mass spectrometry (MS–MS) and MS–MS with information-dependent acquisition (IDA) were previously investigated as possible techniques to develop such a general unknown screening (GUS) procedure for drugs and toxic compounds [1]. However, single-MS techniques are repeatable and reproducible on a same but not on different types of instruments [2], while a simple MS–MS strategy is not really compatible with GUS, as a limited number of pre-defined ions must be selected before fragmentation [1]. Preliminary studies [3,4] showed the potential of IDA, an auto-adaptive MS–MS product-ion scan mode, for GUS. The principle of this technique is as follows: in a first step (so-called ‘‘survey scan’’), the MS–MS instrument is operated in the full-scan single-mass mode using the second mass filter (that may be a quadrupole, a time-of-flight or an ion-trap filter), in order to select all the ions above a predefined intensity threshold; instantly, the first mass filter is set to selectively transmit these high-intensity ions to the collision cell where fragmentation energy is switched on and the resulting fragments are analysed by the second mass filter in the scan mode; finally, after a short time the instrument switches back to the initial conditions, with the possibility to set a refractory period for the last ions selected. The major advantage of this approach is its high specificity and selectivity, as the spectra recorded come from a single parent ion. However, its main drawback is the difficult detection of signals of toxicological interest among background noise and the difficult setting of a given intensity threshold due to the intense and, above all, highly variable background noise produced by extracts of real samples (in particular forensic samples), or by gradient chromatographic elution. If this threshold is given a high value it may result in very poor sensitivity, and if it is given a very low value it may result in too much, ‘‘noisy’’ information.

The aim of this preliminary study was to evaluate the feasibility of a GUS procedure with IDA, using a prototype of a new type of MS–MS instrument,

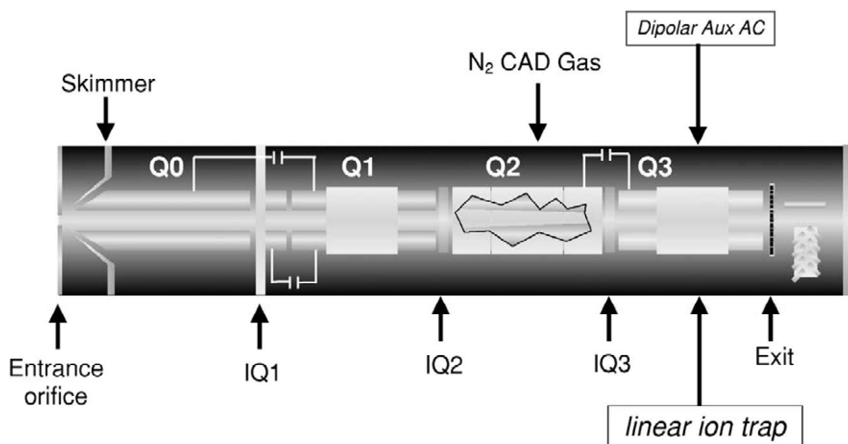

Fig. 1. Scheme of the QqQlinear-ion-trap instrument.

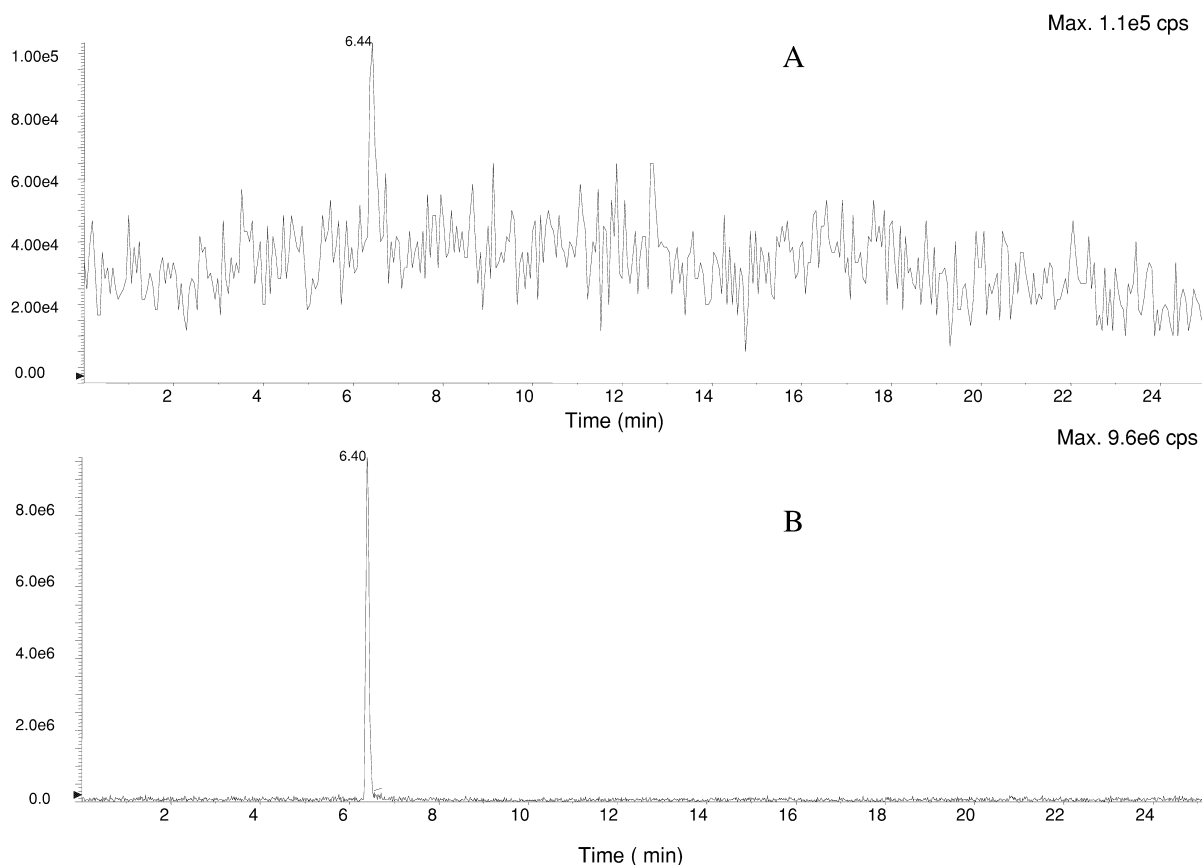

Fig. 2. Comparison of product ion scan (A) and enhanced product ion scan (B) modes for the detection of 1 ng milrinone on-column using the QqQ-linear-ion-trap instrument.

so-called quadrupole-linear ion-trap mass spectrometer (QqQlinear ion trap or QTRAP), and to compare the results with those of a previously reported LC–MS GUS procedure [5,6] taken as reference.

2. Materials and methods

The mass spectrometer used was a prototype Applied-Biosystem/Sciex (Concord, Canada) LC-QqQlinear ion-trap instrument (now commercially available under the trade name QTRAP), based on a triple quadrupole ion path in which the last quadrupole is equipped with entrance and exit electronic lenses giving it the properties of an ion-trap. When operated in the quadrupole/ion-trap configuration, this systems offers MS^3 possibilities and several high sensitivity modes (owing to ion accumulation in the trap) (Fig. 1).

Ionization and mass spectral conditions were optimized using standard solutions of eight test compounds (milrinone, clindamycine-phosphate, lorazepam, fluometuron, piretanide, warfarin, simvastatin, phenobarbitone), chosen for belonging to eight different classes of compounds (cardiotonics, antibiotics, benzodiazepines, pesticides, diuretics, anticoagulants, antihypertensive and antiepileptic drugs, respectively), for their retention times distributed along the 30-min long chromatogram (hence with various lipid solubility and polarity) and for being detected both in the positive and negative modes (except for simvastatin which was only detected in the positive mode); however, these compounds were not necessarily of major toxicologi-

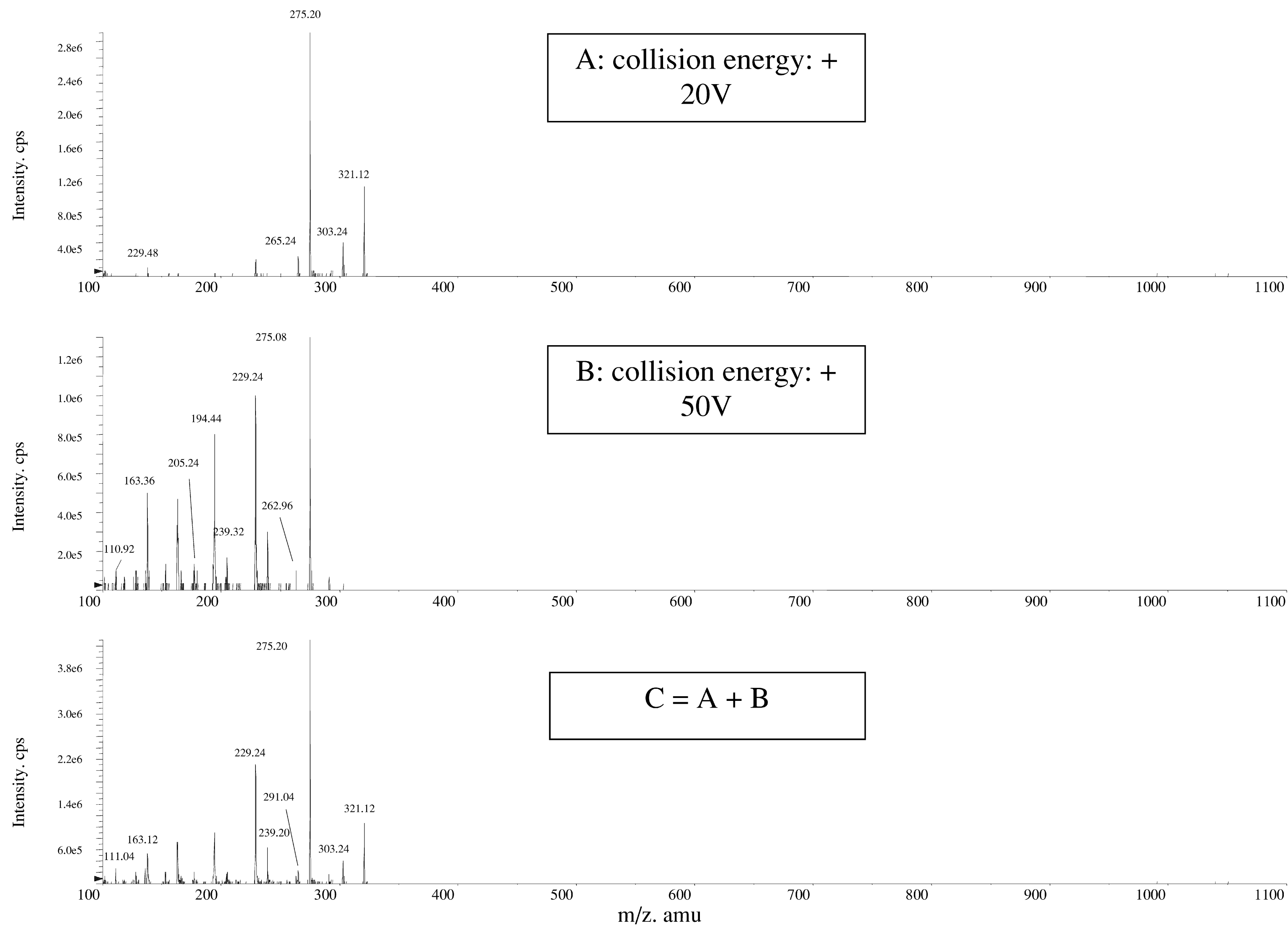

Fig. 3. Reconstructed MS–MS spectrum of lorazepam (C), obtained by adding product ion mass spectra obtained at fragmentation energy of +20 (A) and +50 (B) eV, respectively.

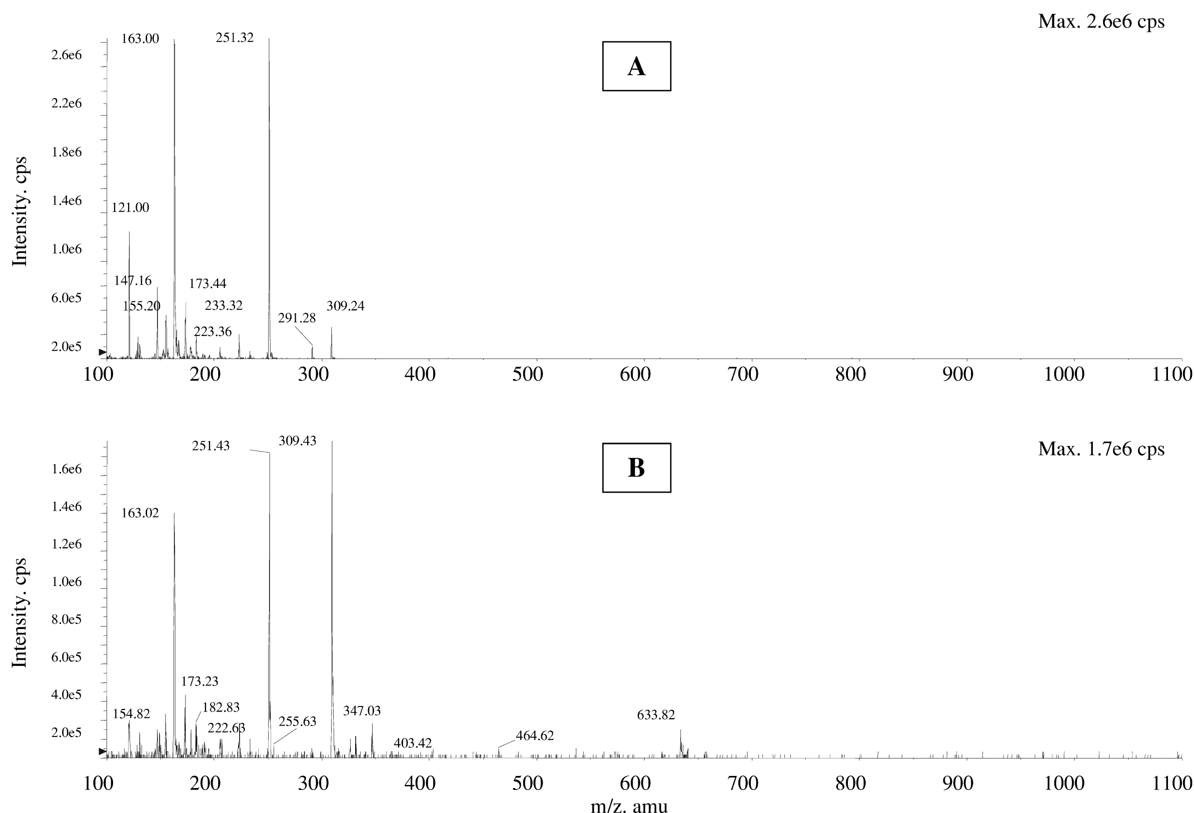

Fig. 4. Comparison of the positive reconstructed MS–MS spectrum obtained in the EPI mode using IDA (A) with the single MS reconstructed spectrum (B) of warfarin (M_r=308.1).

cal interest, as the choice was limited when taking in account the last criterion. Glafenin was used as internal standard (I.S.), as in our previously published LC–MS technique [5,6].

The detection of the compounds eluted from the chromatographic column was performed in the ''enhanced MS'' (EMS), single quadrupole mode (so-called ''survey scan'') where ions are accumulated then filtered in the Q3-linear ion-trap.

Two MS–MS modes for on-the-fly fragmentation of the ions above the user-defined IDA intensity threshold were compared: the usual product ion scan mode (PIS) and the ''enhanced'' product ion scan mode (EPI) where ions are trapped in the third quadrupole before filtration. The MS^3 mode was excluded as it requires to select fragments of interest in order to fragment them again, which is contrary to the concept of general unknown screening.

This technique was compared to a reference LC–ES-MS method [5,6], using a single-quadrupole API 100 LC–MS instrument (Applied-Biosystems/Sciex) equipped with a pneumatically assisted electrospray (Ionspray) ionisation source, where four in-source collision-induced fragmentation conditions are alternated: i.e., low and high fragmentation in the positive and the negative ion modes (+20, +80, −20 and −80 V, respectively). Masses were scanned from m/z 100 to 1100, with a 0.2 u step-size and a dwell-time of 0.06 ms. The main other parameter settings were as follows: in the positive mode, ionisation voltage was +5500 V, Q0 +10 V, ring +275 V; in the negative mode, ionisation voltage was −4500 V, Q0 −10 V, ring −250 V. Positive and negative reconstructed spectra were obtained by adding, in the centroid mode (merge distance 1 u, minimum width 1 u) spectra at +20 and +80 V on the one hand and spectra at −20 and −80 V on the other. Finally, the 30-min long chromatogram corres-

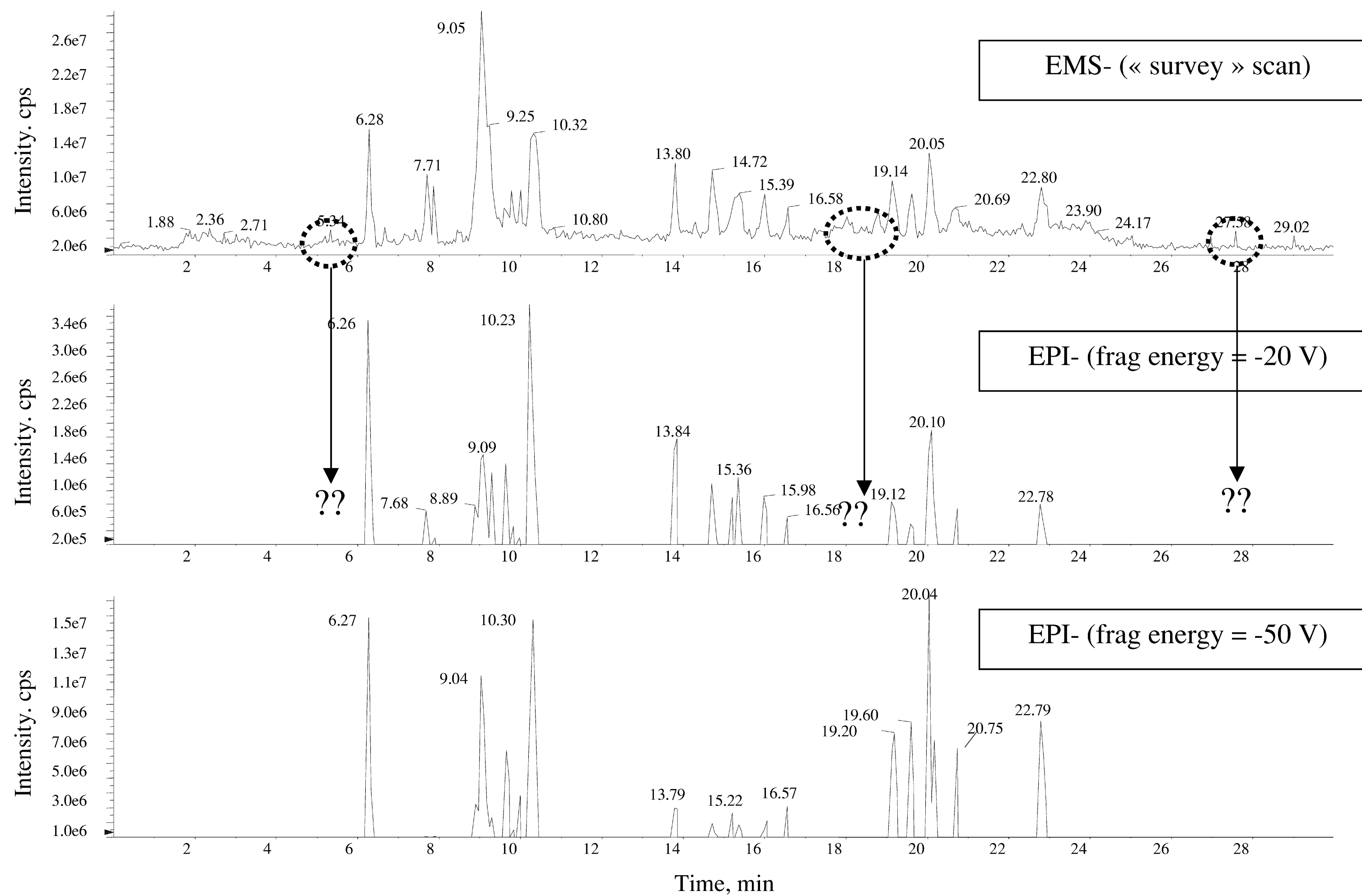

Fig. 5. Example of the influence of an IDA threshold of 200 000 counts/s on the detection of tiny chromatographic peaks.

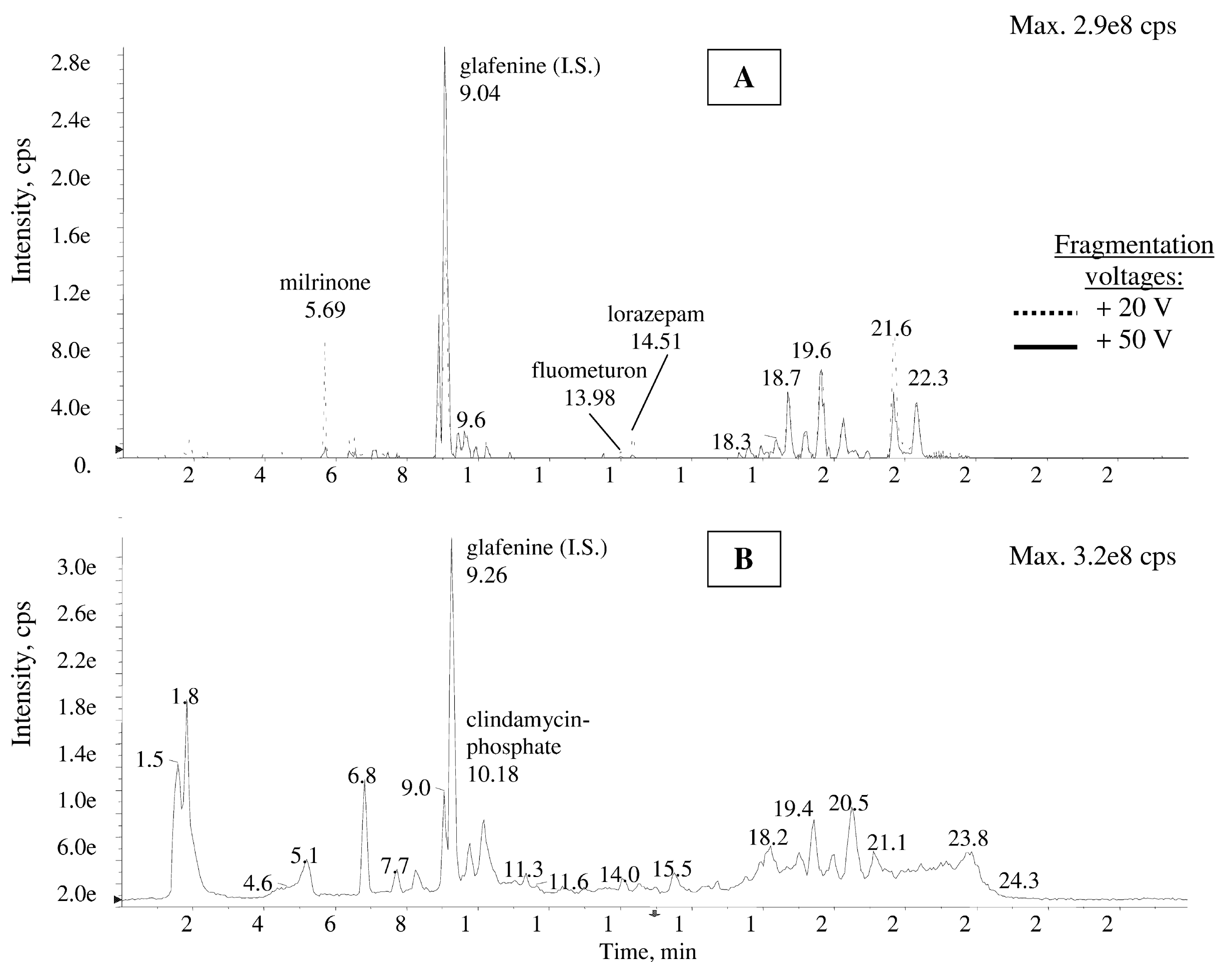

Fig. 6. Analysis of a serum sample spiked at 0.1 mg/l with eight test compounds by the LC–MS–MS technique developed (A) and the reference LC–MS technique (B) in the positive ionisation mode.

ponded to 589 iterations of a 3.055 s long loop alternating these four conditions, resulting in four chromatograms.

For this method comparison, extracts of a serum pool spiked with the eight test compounds at 0.1 and 1 mg/l, respectively, were analysed in parallel. Solid-phase extraction involved Oasis MCX, mixed-mode phase extraction cartridges containing both hydrophobic polymers and sulfonic acid (cation exchange) functional groups. A 1-ml volume of serum spiked with glafenin (I.S.) at 1 mg/l and the eight compounds at 0.1 or 1 mg/l was deposited on a disposable cartridge previously conditioned with 1 ml methanol and 1 ml deionised water. After rinsing the cartridge with 1 ml 0.1 M hydrochloric acid, elution was performed with 1 ml of a methanol–ammonium hydroxide solution (25%) (98:2, v/v) mixture. The extract was evaporated to dryness under a nitrogen stream at ambient temperature and the dry extract dissolved in 100 μl of 2 m*M* ammonium formate–acetonitrile (50:50, v/v).

The common chromatographic system consisted of a Series 200LC micro-flow-rate, high-pressure gradient pumping system (Perkin-Elmer Instruments, Les Ulis, France) including a Rheodyne Model 7725 injection valve equipped with a 5 ml internal loop. An X-TERRA MS C_{18} 3.5 mm (100 mm×1 mm I.D.) column (Waters, St.-Quentin-en-Yveline,

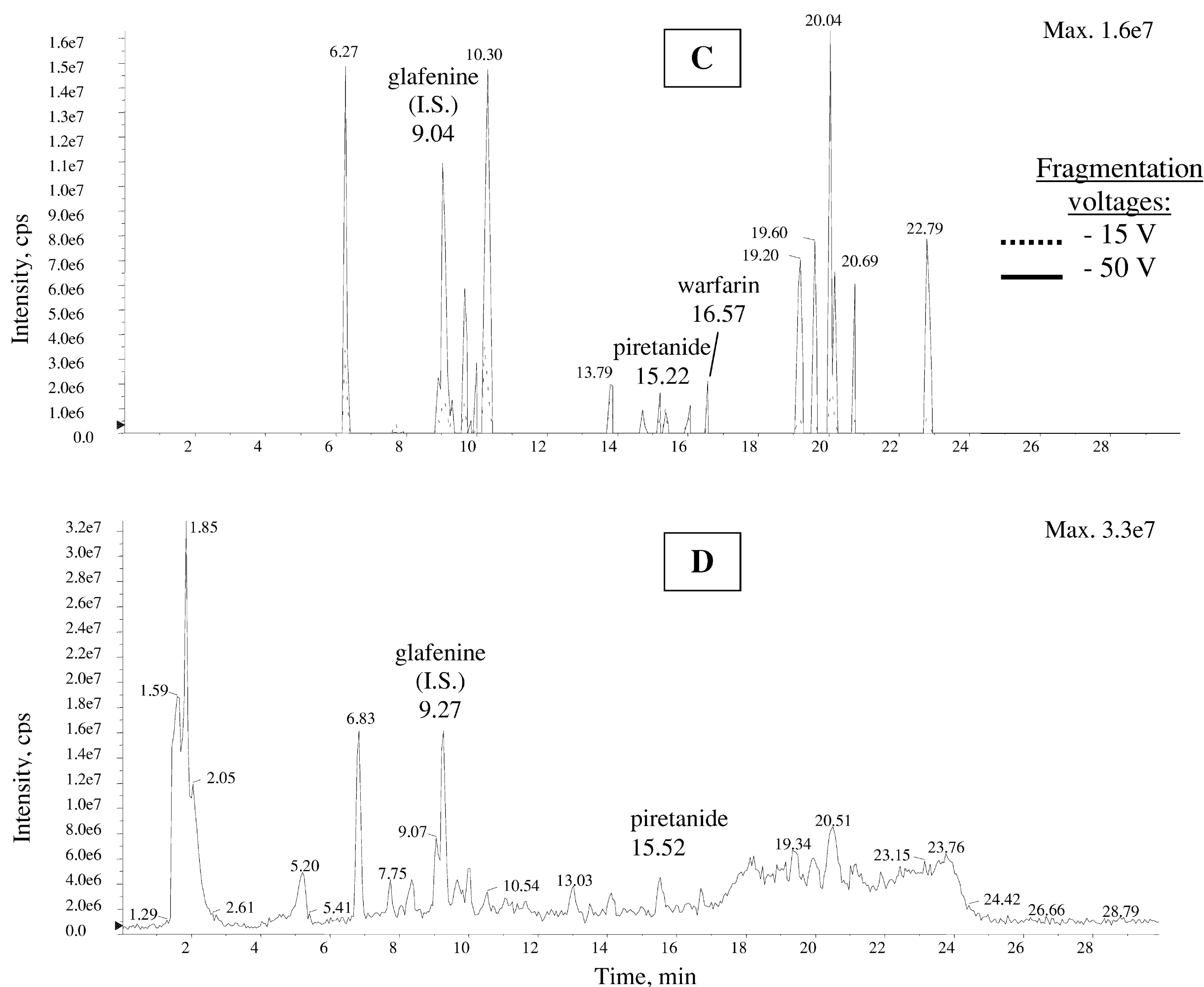

Fig. 7. Analysis of a serum sample spiked at 0.1 mg/l with eight test compounds by the LC–MS–MS technique developed (C) and the reference LC–MS technique (D) in the negative ionisation mode.

France) was used together with a linear gradient of acetonitrile (ACN) in 2 m*M*, pH 3.0 ammonium formate as mobile phase (constant flow-rate 50 μl/min), programmed as follows: 0–1 min, 5% ACN; 1–20 min, 10 to 90% ACN; 21–22 min, 90% ACN; 22–22.5 min, decrease from 90 to 5% ACN; 22.5–25.5 min, column equilibration with 5% ACN.

3. Results and discussion

The Q3 EMS option was chosen for the survey scan, because it gave much higher signal intensity than the normal Q3 scan mode, due to ion accumulation prior to filtration (both are single MS scan modes which use the second quadrupole as filter, the first one being used as an ionic lens to transmit the ions to the following without any filtration). Similarly, in the product-ion scan mode, the EPI setting gave much higher signal intensity than the regular PIS mode (Fig. 2). Another possibility offered by this instrument, called Q0-trapping, is to accumulate ions in the focalising quadrupole (Q0) upstream to the first mass filter during the time when ions previously accumulated in the trap are being sequentially analysed. It was not used in this study because

Table 1
Detection limits with the present LC–MS–MS GUS procedure, as compared to the therapeutic and toxic serum concentration levels of eight test compounds

Compound (class)	Highest therapeutic concentration [7,8] (mg/l)	Lowest toxic concentration [7,8] (mg/l)	Detection limit (mg/l)
Clindamycin phosphate (antibiotic)	26	–	1
Fluometuron (pesticide)	–	–	0.1
Lorazepam (benzodiazepine)	0.3	1	0.1
Milrinone (cardiotonic)	0.25	0.3	0.1
Phenobarbital (anti-epileptic)	40	100	1
Piretanide (diuretic)	0.8	–	0.1
Simvastatin (anti-cholesterol)	–	–	1
Warfarin (anticoagulant)	7	10	0.1

it resulted in too high intensity for several chromatographic peaks, leading to saturation of the corresponding mass spectra.

By ramping the fragmentation energy in the collision cell of the instrument, we found that no single fragmentation energy was able to provide both molecular or pseudo-molecular ions and fragments with high enough intensity for each of the eight test compounds. Therefore, in order to obtain richer mass spectra we chose to combine the information contained in two spectra, one acquired at low and one at high fragmentation energy, respectively, by adding them in the positive mode on the one hand and in the negative mode on the other. The same principle was previously applied for the LC–MS technique used as reference in the present study. The combination of fragmentation voltages giving the most informative spectra for most of the test compounds was found to be +20 and+50 eV in the positive mode and −15 and −40 eV in the negative mode (Fig. 3). The reconstructed spectra thus obtained were easier to record in libraries and to compare with ''unknown'' spectra than the respective couples of positive or negative spectra, that other authors chose to use.

Better signal-to-noise ratio was obtained with the LC-QqQlinear ion-trap instrument than with the reference in-source CID-MS technique, with even slightly higher signal intensity. The reconstructed mass spectra obtained using IDA and the EPI mode were devoid of contaminant ions (adducts or oligomers of the molecules, or even background noise) and as informative as, or even more than, the corresponding reconstructed CID-MS spectra (Fig. 4).

The critical parameter was the IDA intensity threshold, influencing the detection of tiny chromatographic peaks in noise, mainly in the negative mode (Fig. 5). Indeed, a total ion chromatogram in the scan mode as obtained in the survey scan mode can be considered as a three dimension space (time, m/z and intensity) where background noise and signal may vary from valleys to peaks. The IDA intensity threshold available on the instrument used herein, as well as on those used by other authors [3,4], can be represented by a horizontal plane supposed to pick up the signal peaks in this mountain relief, meaning that too low a threshold will not be selective enough and that too high values will result in the loss of informative peaks. In that respect, a threshold that could be adjusted at different levels as a function of the m/z ratio (the higher the m/z, the lower the threshold) and chromatographic time (mainly when a solvent gradient is used) should be better suited to a GUS procedure.

However, after optimising the only intensity threshold available, extracts of serum samples spiked with the eight test compounds at 0.1 and 1 mg/l were analysed in parallel by this LC-QqQ-linear ion trap technique and by the (reference) in-source CID-MS technique. At the concentration of 0.1 mg/l, the reconstructed MS–MS spectra of five compounds (milrinone, lorazepam, fluometuron, piretanide and warfarin) were unambiguously identified, versus only two reconstructed MS spectra, in the positive or negative mode, or in both (Figs. 6 and 7). At the higher concentration level (1 mg/l), all eight compounds could be identified by both techniques. When compared with the therapeutic and/or toxic concentration thresholds of these molecules, where available [7,8], these preliminary results are in favour of the suitability of LC–MS–MS with IDA for the GUS of drugs and toxic compounds using this new type of MS–MS instrument (Table 1). However, the test compounds selected, if representative of a large range of lipid solubility, polarity and ionisation modes, were not all of toxicological interest and most of them were only detected at much higher concentrations than the best detection limits achieved with the aforementioned LC–MS technique (down to 0.01 mg/l). The present results should thus be confirmed with a larger number of molecules, including toxic compounds more frequently encountered in clinical toxicology.

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