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SPECIAL FEATURE: TUTORIAL

Triple quadrupole linear ion trap mass spectrometer for the analysis of small molecules and macromolecules

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Recently, linear ion traps (LITs) have been combined with quadrupole (Q), time-of-flight (TOF) and Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometery (MS). LITs can be used either as ion accumulation devices or as commercially available, stand-alone mass spectrometers with MS^n capabilities. The combination of triple quadrupole MS with LIT technology in the form of an instrument of configuration QqLIT, using axial ejection, is particularly interesting, because this instrument retains the classical triple quadrupole scan functions such as selected reaction monitoring (SRM), product ion (PI), neutral loss (NL) and precursor ion (PC) while also providing access to sensitive ion trap experiments. For small molecules, quantitative and qualitative analysis can be performed using the same instrument. In addition, for peptide analysis, the enhanced multiply charged (EMC) scan allows an increase in selectivity, while the time-delayed fragmentation (TDF) scan provides additional structural information. Various methods of operating the hybrid instrument are described for the case of the commercial Q TRAP (AB/MDS Sciex) and applications to drug metabolism analysis, quantitative confirmatory analysis, peptides analysis and automated nanoelectrospray (ESI-chip-MS) analysis are discussed. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: linear ion trap; metabolism; peptides; nanoelectrospray

INTRODUCTION

Atmospheric pressure ionization (API) mass spectrometry (MS) and in particular electrospray ionization (ESI) allow the analysis of low molecular mass thermolabile compounds. The combination of API-MS with separation techniques, such as liquid chromatography (LC), has become essential in pharmaceutical drug development,[1] forensic science,[2] environmental analysis[3] and proteomics.[4] Currently the most widely used mass spectrometers are the quadrupole ion trap (3D-IT), the triple quadrupole (QqQ) and the quadrupole time-of-flight (QqTOF). 3D-ITs have mostly been used for qualitative applications. Compared with classical mass spectrometers such as triple quadrupoles, the 3D-IT is a physically small and relatively inexpensive instrument, which nevertheless is highly sensitive and possesses MS^n capabilities. However, it is not best suited for quantitative analysis.

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Due to their small trapping volume, 3D-ITs have a limited capacity for ion storage. Overfilling of the 3D-IT results in deterioration in the mass spectrum and loss of dynamic response range due to space charging. To avoid these effects, the number of ions introduced into the trap can be controlled automatically.[5] Trapping of ions can also be performed in linear or circular 2D ion trap devices (2D-IT),[6–8] but until recently no commercial stand-alone linear quadrupole ion trap (LIT) instrument had been developed. LITs have two major advantages over 3D-IT: a larger ion storage capacity and a higher trapping efficiency.

LITs have been successfully coupled to time-of-flight (LIT-TOF-MS)[9] and Fourier transform ion cyclotron resonance (FT-ICR-MS)[10–13] instruments. The intention in building such hybrid instruments is to combine ion accumulation and MS^n features with the superior mass analysis (accuracy and resolution) and high sensitivity of TOF-MS or FT-ICR-MS. The ions stored in the trap are ejected axially in a non-mass-dependent fashion into the mass analyzer. Very recently, LITs have emerged as commercially available, stand-alone mass analyzers rather than just serving as ion storage devices. Schwartz *et al*.[14] described a stand-alone LIT (LTQ, Thermo Electron) where mass analysis is performed by

ejecting the ions radially, through the slits of the quadrupole rods, using the mass instability mode. Detection is performed by two detectors placed axially along to the rods. The same device has been coupled with an FT-ICR mass analyzer (LTQ FT, Thermo Electron).[11] This combination allows the use of the trap for isolation and fragmentation and the use of FT-ICR-MS for accurate mass and high-resolution detection. Average mass accuracy of better than 0.4 ppm has been reported.[12]

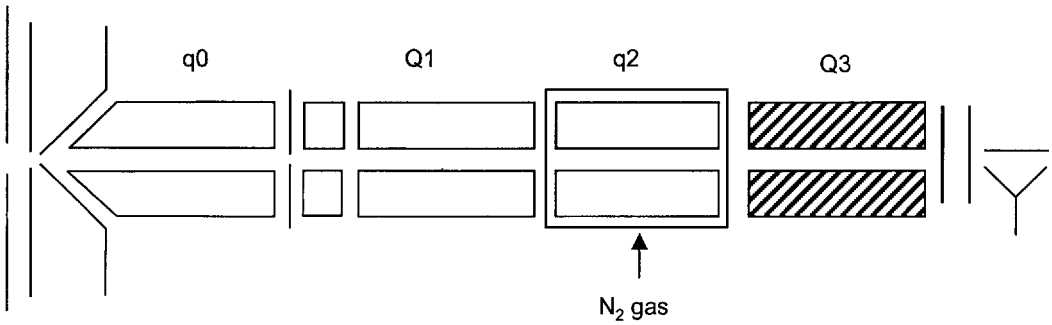
The system described in this paper (Q TRAP, AB/MDS Sciex) is based on a triple quadrupole platform where Q3 can be operated either in the normal RF/DC mode or in the LIT mode.[15] In the LIT mode, the trapped ions are ejected axially in a mass-selective fashion using fringe field effects and detected by the standard detector of the system.

Development of miniaturized analytical tools, which allow less analyte consumption and shorter analysis times, has gained interest in recent years. These systems are also called micro-total analysis systems (μ-TAS).[16] In such systems, sample preparation, chromatographic or electrophoretic separation and detection are performed on the same device. As electrospray ionization mass spectrometer behaves as a concentration-sensitive detector,[17] where the use of very low flow-rates will not jeopardize sensitivity. Efforts have been undertaken to miniaturize the MS detector but this remains a very challenging task. Nanoelectrospray[18] is used in proteomics and drug metabolism to analyze very low levels of analytes by MS/MS. Due to very low flow-rates, high-quality tandem mass spectra can be recorded by minimizing sample consumption. In addition, for each sample a new sprayer needle is used to avoid cross-contamination. Nanoelectrospray with glass capillary needles is difficult to automate and therefore the sample throughput is very limited. The alternative is to use disposable nanoelectrospray devices, which can be mass produced at relatively low costs and which are suitable for automation. Microfabricated devices, based on silicon[19,20] or plastic polymers,[21,22] for direct mass spectrometry, have been developed recently.

The aim of this paper is to illustrate the potential of the Q TRAP (AB/MDS Sciex) in combination with LC or with automated infusion nanoelectrospray (chip-MS) for the analysis of small molecules and peptides.

GENERAL DESCRIPTION OF THE INSTRUMENT AND OPERATIONAL MODES

The hybrid quadrupole linear ion trap (QqLIT) is used in two forms, one based on an API 2000 and the other on an API 4000 triple quadrupole platform commercially named Q TRAP (AB/MDS Sciex). Figure 1 shows the various modes of operation of the instrument. All specific scan functions of the triple quadrupole[23] such as product ion (PI), constant neutral loss (NL), precursor ion scan (PC) or selected reaction monitoring (SRM) mode are maintained along with and in combination with the trap scan modes. The term enhanced is always used when Q3 is operated as an LIT. Also, the QqLIT allows two particular modes to perform MS/MS experiment in a time-delayed fashion (TDF) and the selection of multiply charged ions in the trap mode (EMC). The particularity of the system is that basically they are no new scan functions, but scan combinations of triple quadrupole mode and trap mode



Mode of operation	Q1	q2	Q3
Q1 Scan	Resolving (Scan)	RF-only	RF-only
Q3 Scan	RF-only	RF-only	Resolving (Scan)
Product Ion Scan (PI)	Resolving (Fixed)	Fragment	Resolving (Scan)
Precursor Ion Scan (PC)	Resolving (Scan)	Fragment	Resolving (Fixed)
Neutral Loss Scan (NL)	Resolving (Scan)	Fragment	Resolving (Scan Offset)
Selected Reaction Monitoring mode (SRM)	Resolving (Fixed)	Fragment	Resolving (Fixed)
Enhanced Q3 Single MS (EMS)	RF-only	No frag	Trap/scan
Enhanced Product Ion (EPI)	Resolving (Fixed)	Fragment	Trap/scan
MS^3	Resolving (Fixed)	Fragment	Isolation/frag trap/scan
Time delayed fragmentation (TDF)	Resolving (Fixed)	Trap/No frag	Frag/trap/scan
Enhanced Resolution Q3 Single MS (ER)	RF-only	No frag	Trap/scan
Enhanced Multiply Charged (EMC)	RF-only	No frag	Trap/scan

Figure 1. Schematic of QqLIT (Q TRAP, AB/MDS, Sciex) and description of the various triple quadrupole and trap operation modes.

can be performed in the same LC/MS run, which is unique and offers new possibilities for quantitative and qualitative analysis.

Q3 trap mode

The Q3 trap mode (enhanced MS) is a method of generating a conventional mass spectrum. Ions generated at atmospheric pressure are pulsed out from q0, pass through Q1 and the pressurized q2 quadrupole, and are trapped in Q3 by the RF voltage operating in the radial direction and by the DC-biased aperture plates operating in the axial direction. In Q3 the trapped ions are cooled, typically in 10–30 ms. The ion kinetic energy in q2 is set in such a way as to minimize fragmentation during the passage of the ions. Trap fill times in practice are in the range 1–500 ms. Fringe fields caused by the lenses at the end of the quadruple are exploited to eject the trapped ions, mass selectively, in an axial fashion. This produces the mass spectrum.

The LIT is calibrated for three scan rates, 250, 1000 and 4000 Th s^{-1}, and the resolution is dependent on the scan speed. Typical values are 0.1–0.2 Th (FWHM) at 250 Th s^{-1}, 0.3–0.5 Th at 1000 Th s^{-1} and 0.5–0.7 Th at 4000 Th s^{-1}. The mass ranges are 50–1700 and 70–2800 Th for the Q TRAP 2000 and the Q TRAP 4000, respectively. No helium gas is used in Q3 and the pressure monitored on the system is in the range (3–4.5) $\times 10^{-5}$ Torr (1 Torr = 133.3 Pa), resulting from nitrogen leaking from the collision cell.

The same mass spectrometer can be used to perform quadrupole or trap scans and switching from one to the other mode takes only a few milliseconds. For quadrupole scans Q3 is operated in the RF/DC mode whereas in the trap mode Q3 is operated as an ion trap by applying an RF potential to the quadrupole. This means that it is possible to perform one scan using a typical quadrupole mode (SRM, PI, NL, PC) and the next scan using a trap scan. One may argue that with a hybrid instrument the performance in one or the other mode may be jeopardized. No difference in performance (resolution or sensitivity) between the Q1 and Q3 quadrupoles was observed when operating the Q3 in the RF/DC mode (m/z 74–1000), suggesting that, for example, in the SRM mode an API 4000 and an API 4000 Q Trap have the same performance.

Enhanced resolution mode

The enhanced resolution (ER) mode also produces a conventional mass spectrum but with increased resolution as a result of slow scans of the LIT ion trap component. In a typical experiment, ions within a 30 Th region are collected in Q3 for a specified time and scanned slowly at 250 Th s^{-1}. Only a 10 Th window is displayed. Resolution of about 6000 (FWHM) can be achieved, allowing unambiguous determination of the charge state of doubly, triply and quadruply charged ions. The ER scan is particularly interesting for 2D-LC/MS analysis of peptides using information-dependent data acquisition and it is often performed after the enhanced MS mode and before the enhanced product ion scan.

Enhanced product ion mode

There is a fundamental difference in the way product ion scan MS/MS experiments are performed using a 3D-IT and on the QqLIT. On the QqLIT, in enhanced product ion (EPI) mode, the selection of the precursor ion is performed in Q1 utilizing RF/DC isolation at any resolution. Collision-induced dissociation (CID) occurs in the collision cell q2, and fragment ions are trapped in Q3 operated in LIT mode. RF/DC isolation has a significant advantage over isolation waveform, where for isolation of fragile ions elimination of the precursor ion can be observed.[24] However, it still has disadvantages of not easily allowing high selection resolution without loss of signal.

In a quadrupole collision cell, the ions undergo multiple collisions. As soon as the fragment ions are formed they become reactivated and undergo further fragmentation. However, fragmentation within a 3D-IT occurs solely by excitation of the precursor ion. In most cases product ions are too cool to fragment further, and therefore require specific excitation which is done in MS^3 and MS^4 experiments. Typically, ion traps have a low mass cut-off which usually corresponds to about one-third of the precursor ion mass. With the QqLIT in the enhanced product ion mode, the precursor ion selected in Q1 is fragmented in the quadrupole collision cell q2 and mass segments have to be used to obtain a complete CID spectrum down to m/z 50 or 70. The number of segments is dependent of the mass range and the mass of the precursor ion. The drawback is that each mass segment requires a full cycle (injection, trapping and mass analysis), which can significantly increase the duty cycle of the EPI scan. In fact, the time-limiting step is the injection time. For a mass range of 1000 Th a scanning speed of 4000 Th s^{-1} and an injection time of 250 ms, the complete cycle time is typically ~0.55 s. If the scan range of 1000 Th has to be split in two segments, the cycle time increases to 0.85 s. On the other hand, for an injection time of 10 ms no significant difference is observed in duty cycle time when the scan range has to be split in two segments.

Figure 2A shows the EPI spectrum of trocade (M_r = 436 Da) where many fragments are observed down to m/z 86. In contrast to stand-alone ion traps, it is possible to obtain a product ion spectrum below the low mass cut-off. An ion trap-like tandem mass spectrum can also be generated using the QqLIT when the collision energy in q2 is set (typically 5–10 eV) so that no fragmentation occurs in this region of the instrument. In this case, a low mass cut-off (about one-third of the precursor ion mass) is also observed. Figure 2(B)–2(D) show the MS^2, MS^3, MS^4 product ion spectra of trocade obtained on a 3D ion trap (LCQ, Thermo Finnigan). As expected, the fragmentation pathway can be simply followed. However, sensitivity is lost at each MS step. The QqLIT does not have MS^4 capabilities. This is not often necessary because quadrupole CID spectra are in general very informative.

MS^3 mode

The QqLIT also has MS^3 capabilities. A typical experiment is performed in the following manner: the first stage of fragmentation is accomplished by accelerating the precursor ions chosen by Q1 into the pressurized collision cell, q2. The fragments and residual precursor ions are transmitted into the Q3 linear ion trap quadrupole and are cooled for

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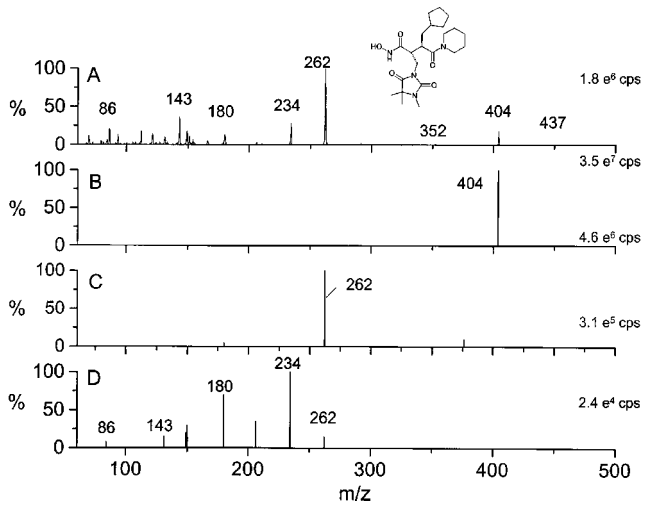

Figure 2. Mass spectra of trocade ($M_r = 436$ Da); (A) Q TRAP enhanced product ion, CE = 50 eV; (B) LCQ MS^2; (C) LCQ MS^3; (D) LCQ MS^4. For the LCQ the isolation width was 1 and the CE 35% (adapted from *J. Mass Spectrom.*[37]).

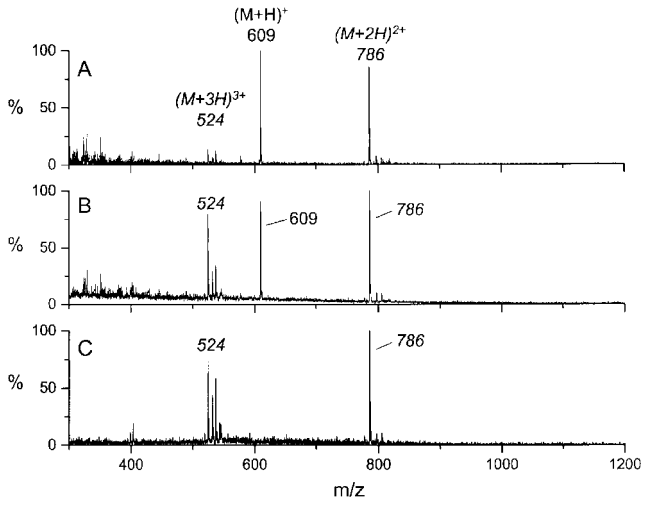

Figure 3. Infusion of a reserpine $[M + H]^+$ *m/z* 609 and glufibrinopeptide mixture, $[M + 2H]^{2+}$ *m/z* 786, $[M + 3H]^{3+}$ *m/z* 524. (A) Enhanced mass spectrum; (B) enhanced multiply charged ion spectrum with an empty time of 10 ms; (C) enhanced multiply charged ion spectrum with an empty time of 40 ms.

~10 ms. The next-generation precursor ion is isolated within the linear ion trap by application of a resolving DC voltage near the apex of the stability diagram at $q \approx 0.706$. The RF voltage of the linear ion trap is adjusted such that the isolated ions are at a q value of 0.238, where they are excited by a single frequency 85 kHz auxiliary signal and fragmented to give the sequential product ion spectrum. This auxiliary signal is user controllable up to 200 mV_{p-p} for durations up to 200 ms. MS/MS trap-like spectra can also be obtained by setting the value of the collision energy to 10 eV and causing the precursor ion, selected in Q1, to pass through the collision cell and become trapped in Q3, before undergoing fragmentation in the linear ion trap.

Enhanced multiply charged mode

The enhanced multiply charged mode (EMC) allows the removal of singly charged ions from the LIT. EMC is basically an ion processing mode and works on the principle that once ions have been trapped and cooled for a sufficient time they have the same kinetic energy. After thermalization, the effective DC trapping barriers depend only on the charge state of the ions and not on their masses. Appropriate settings of the DC voltage and trap emptying time will result in preferential release, starting with ions with the lowest charge. Typically, the emptying time is in the range 20–40 ms, allowing the use of EMC as a survey scan with LC/MS analysis.

Separation of ions according to their charge state using a quadrupole TOF mode has also been reported.[25,26] With the QqTOF configuration, multiple charge separation (MCS) is performed in the collision cell q2 filled with argon.

The effect of an EMC scan for a solution containing glufribinopeptide and reserpine infused by nanoelectrospray is illustrated in Fig. 3. Figure 3(A) presents the EMS spectrum in which glufibrinopeptide doubly and triply charged ions are found at m/z 786 and 524, respectively, whereas reserpine singly charged ion is found at m/z 609. By varying the trap emptying time, one can selectively remove the singly charged pseudomolecular ion of reserpine (Fig. 3(B) and (C)).

Time-delayed fragmentation mode

Time delayed fragmentation (TDF) is particularly interesting for spectra interpretation because it reduces multiple sequential fragmentations and so leads to simpler tandem mass spectra.[27] CID is the consequence of the conversion of translational energy of the precursor ion, generated by its collision with a neutral gas, into internal energy. If the internal energy is high, multiple generation fragments will be observed. Once a precursor ion has been activated, the time window can be defined to allow ion relaxation. It is a three-step process including ion activation, ion relaxation and fragment collection. Ion relaxation occurs via fragmentation and cooling with residual gas. In contrast to the classical triple quadrupole, ion activation occurs via q2-to-Q3 acceleration rather than via Q1-to-q2 acceleration. Hence the product ion spectrum originates from a precursor ion which has a modified internal energy based on a time delay. This is achieved by first collecting the precursor ions in the trap, while fragment ions outside a given mass range are not trapped. After a cooling period, typically in the range of milliseconds, the trap is adjusted such that it can trap the fragments originating from the cooled precursor ion (Q3 fill mass). TDF can be applied to determine the origin of secondary fragment ions by changing the Q3 fill mass. Hager[27] investigated the fragmentation of bosentan ($M_r = 551$ Da) using TDF. Bosentan is a sulfonamide compound and its fragmentation pathway has been extensively investigated.[28] CID of bosentan generates four major fragments at m/z 508, 311, 280 and 202 (data not shown). The TDF spectrum of bosentan with a Q3 fill mass of 200 Th is closely comparable to that of the product ion spectrum whereas with the TDF spectrum using a fill mass of 400 Th a strong decrease of the

signal at m/z 280 was observed. This implies that the fragment at m/z 280 can only originate from one ion between 281 and 400 Th. It was shown previously[28] that the fragment at m/z 280 originates from the ion at m/z 311 Th through the loss of a CH_3O radical, which is also confirmed in the TDF experiment.

In quadrupole CID spectra of peptides, the y-ion series is in general predominant; however, in the low-mass range they are sometimes difficult to assign owing to interferences with other fragments. The y-ion series are primary fragments and the TDF mode is a particularly interesting method for simplifying tandem mass spectra and therefore facilitating sequencing. This is demonstrated in Fig. 4, which compares (A) the enhanced product ion scan with (B) the TDF scan of an infused solution of glufibrinopeptide. The Q3 fill mass was set at 700 Th at a lower m/z value of the doubly charged precursor ion. The simplification of the spectra in particularly efficient in the low-mass region. In Fig. 4(B), the ions y_1 (m/z 175) and y_2 (m/z 246) can be easily identified. The TDF mode is therefore particularly powerful for *de novo* sequencing of peptides.

Information-dependent acquisition

To increase throughput, the use of information-dependent data acquisition (IDA) becomes very important. IDA is a procedure that combines two or more different scan modes in a sequential fashion for the same LC/MS run. The first scan is defined as the survey scan, where data are processed 'on-the-fly' to determine the candidates of interest based on predefined selection criteria. If the selection criteria are met, a second scan (data dependent) is then performed. A typical IDA experiment is to perform a full-scan single MS run as a survey scan and an MS/MS experiment as the dependent scan. This type of experiment can also be performed on most tandem MS instruments.[29] Unlike the 3D ion trap, the QqLIT retains the traditional triple quadrupole scan modes such as SRM, NL or PC. The uses of these scan functions as survey scans, with EPI as a dependent scan, is particularly interesting for achieving better selectivity in the selection of the precursor ion. Less conventional combinations such as SRM/EPI, SRM/MS^3 or EMC/EPI offer interesting possibilities for the analysis of metabolites or peptides. The various possible combinations of operation modes for the analysis of small molecules or peptides with the QqLIT instrument are illustrated in Table 1.

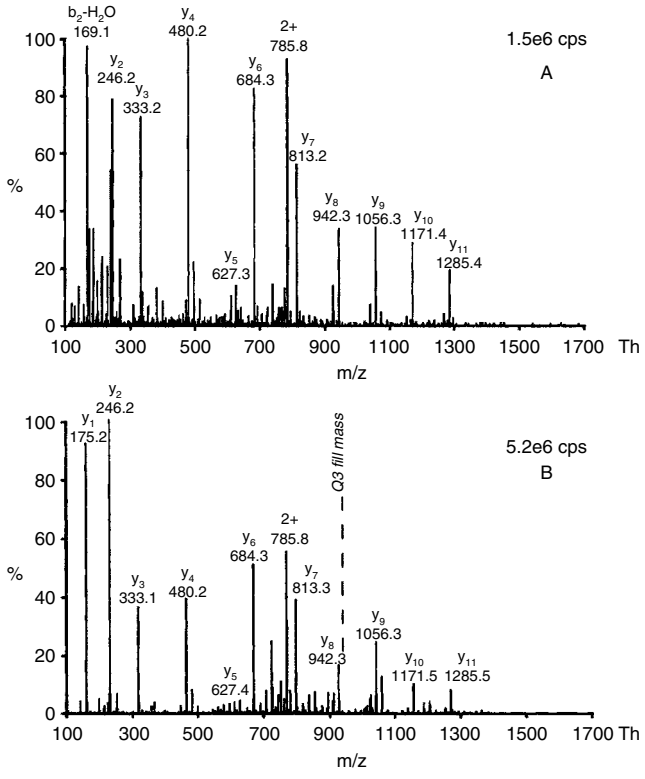

Figure 4. (A) Enhanced product ion spectrum of glufribinopeptide, precursor at *m/z* 785.8 (EGVNDNEEGFFSAR, M_r = 1570.6 Da), CE = 38 eV (Q1 to q2 precursor ion activation). (B) Time-delayed fragmentation spectrum, CE = 25 eV (q2 to Q3 activation). Cooling delay, 10 ms; LIT fill mass, 708 Th.

APPLICATIONS

MS analysis of remikiren

With quadrupole CID, the product ion spectrum of a given compound is strongly dependent on the collision energy (CE). If the CE is set too low, only the precursor ion and some high-mass fragments can be observed. On the other hand, if the CE is set too high, only low-mass fragments are observed. Both situations are of interest so it is sometimes of important to perform MS/MS experiments under several conditions simultaneously. In the QqLIT, CID is performed in the collision cell of the triple quadrupole while fragments are trapped into the Q3 LIT. This configuration allows the CE to be changed during the fill time of the trapped ions.[30] In this way, tandem mass spectra including low- and high-mass fragments can be obtained in one experiment. Figure 5 compares the product ion spectra of remikiren (M_r = 630 Da) obtained at (A) 30 eV and (B) 70 eV with that obtained at (C) 50 eV with a collision energy spread of 20 eV. The last CID spectrum represents the sum of the fragments obtained at 30, 50 and 70 eV. In this way, informative product ion spectra can be obtained for most compounds using a single MS/MS experiment and a standard CE setting. The fragmentation pathway of remikiren has been extensively investigated and reported elsewhere.[31] The principle of multi-level CID on the 3D ion trap has also been described where the trapped ions are subjected to, typically, three separated resonance excitation voltages.[32]

MS^n experiments are very useful when following the fragmentation cascade of an analyte. With the QqLIT, MS^2 ion trap spectra can be recorded when a very low CE (5–10 eV) is set in the collision cell as depicted in Fig. 6(A). MS^3 of m/z 631 → 404 and m/z 631 → 376 is illustrated in Fig. 6(B) and (C). There is no difference when comparisons are made with the spectra obtained on a 3D ion trap (data not shown). The ion at m/z 376 results from the loss of carbonyl moiety (28 Da) from the ion at m/z 404. It is noteworthy that the ion at m/z 282 is generated exclusively from its precursor at m/z 404.

Table 1. Summary of combinations of operation modes of the QqLIT

Combination	Analysis type	Specificity	Comments
EMS–EPI(n)–MS^3	Screening of metabolites Proteomics	High sensitivity but poor selectivity	With dirty samples requires inclusion and exclusion lists $n = 2$
EMC–EPI(n)–MS^3	Proteomics	Multiply charged charged precursors	Allows one to eliminate singly charged ions
NL–ER–EPI(n)–MS^3	Screening of structural analogues Proteomics	High selectivity, moderate sensitivity with NL	Requires the understanding of the fragmentation process Limited to $n = 2$ Phosphopeptides
PC–ER–EPI(n)–MS^3	Screening of structural analogues Proteomics	High selectivity, moderate sensitivity with NL	Requires the understanding of the fragmentation process Limited to $n = 2$ Glycopeptides
EPI(n)	Target analysis	Up to up 8 simultaneous EPI experiments are possible	The mass of the precursor are predicted on known phase I and phase II metabolism
SRM–EPI(n)	Target analysis and confirmatory analysis	High sensitivity and selectivity	Up to 50 SRM transitions can be defined $n = 2$ is case of overlapping peaks
SRM–EMS	Target analysis Screening Quantitative analysis	High sensitivity and selectivity	Search for predicted and unexpected metabolites Monitoring of endogenous compounds for suppression
SRM–MS^3	Quantitative and confirmatory analysis	Quantitative and qualitative analysis	Quantitation

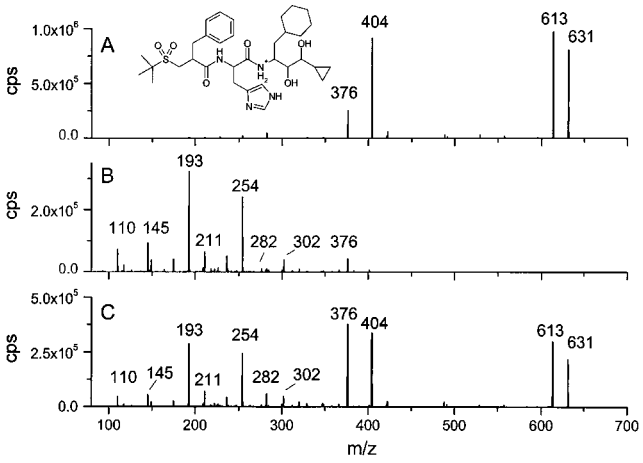

Figure 5. Enhanced MS/MS of remikiren on Q TRAP: CE = (A) 30, (B) 70 and (C) eV with a collision energy spread of 20 eV (30, 50, 70 eV).

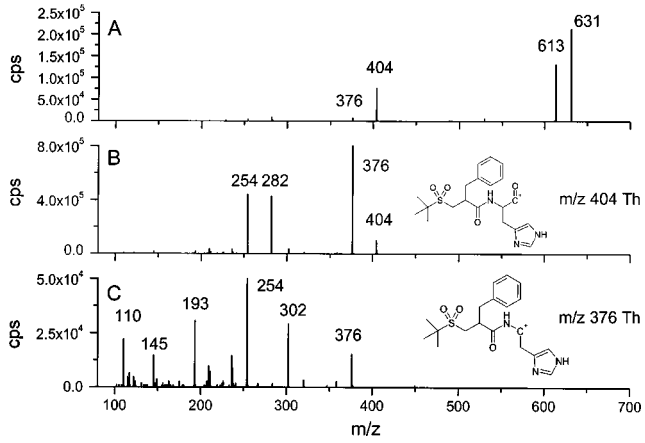

Figure 6. MS^2 and MS^3 of remikiren: (A) *m/z* 631 → 631; (B) *m/z* 631 → 404; (C) *m/z* 631 → 376.

TDF is complementary to MS^3 and provides information on fragmentation kinetics. For small molecules, it is interesting to perform TDF on any fragment. In the current setup of the instrument, in order to perform TDF experiments on fragments they have to be generated by up-front CID in the interface of the mass spectrometer by increasing the orifice potential. The enhanced product ion spectrum of the fragment at m/z 376 is shown in Fig. 7(A) and the TDF spectra of the same fragment with different Q3 fill mass values are displayed in Fig. 7(B) and (C). When the Q3 fill mass value is set at m/z 340 (Fig. 7(C)), only four fragments are observed at m/z 110, 254, 302 and 320. Theses ions are directly generated from the precursor ion at m/z 376. When comparing this spectrum with the MS^3 result (Fig. 6(C)), it is noteworthy that three major fragments are missing, at m/z 145, 193 and 211. However, they are observed in the TDF spectrum where the Q3 fill mass value is set at 150 Th (Fig. 7(A)). These results suggest that these fragments are second-generation fragments from an unstable precursor and can also be observed in the trap mode. This example illustrates very well the strength of both MS^3 and TDF to follow the fragmentation cascade of a given analyte.

Application to peptide analysis

As any trap system, the QqLIT allows the generation of good tandem mass spectra in the trap mode with adequate sensitivity for data-dependent LC/MS analysis of peptides. The enhanced multiply charged scan is particularly

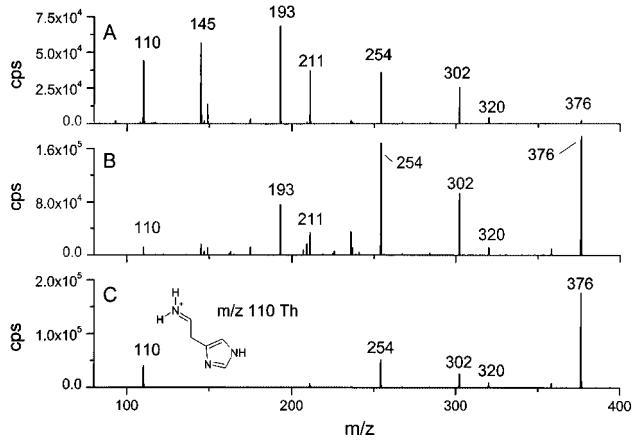

Figure 7. Remikiren: (A) enhanced product ion spectrum (CE = 30 eV, DP = 110 V) of precursor of *m/z* 376 generated by up-front CID; (B) TDF with Q3 fill mass value of 150 Th; (C) TDF with Q3 fill mass value of 340 Th. For TDF CE = 20 eV, the trap fill time was 50 ms and the cool time was 5 ms.

interesting for peptide analysis and it is fast enough to be used as a survey scan in LC/MS analysis. The precursor ion scan on a triple quadrupole instrument is particularly effective for screening phosphopeptides, but suffers from insufficient sensitivity when recording the product ion scan. Le Blanc *et al*.[33] recently showed the potential for screening phosphopeptides using the QqLIT. Precursor ion scanning was performed in negative mode, then an enhanced resolution scan was performed to determine the peptide charge state and finally an enhanced product ion scan was performed in the positive mode. Similar approaches can also be used with the NL scan function.

Application to drug metabolism

One of the first steps in the investigation of the biotransformation of a new potential drug is the characterization of its metabolites in *in vitro* systems. To cope with new demands on the pharmaceutical industry, such as shorter discovery and development times for new drugs, powerful tools are needed to complete the various tasks. This is particularly true for the bioanalytical support for drug metabolism and pharmacokinetic studies. Numerous *in vitro* samples can be generated using hepatocytes and microsomes from different species and LC/MS already plays a very important role in this field.[34–36] Once these metabolites have been characterized *in vitro*, it is important to monitor their occurrence *in vivo* and to follow their pharmacokinetic profile. The goal is to obtain sufficient information regarding the quantity and structure of the metabolites circulating in plasma, in a very short period of time. Often in *in vivo* samples, the concentrations of the drug and its metabolites are very low and only a limited sample volume is available. Sensitivity in the low nanogram range is required and this aspect is very challenging for qualitative analysis. Currently there is no single mass spectrometer which has all the desired features required for this type of work and most laboratories use combinations of various types of mass spectrometers including triple quadrupole, ion trap and quadrupole TOF instruments. The analysis times are relatively long because good chromatographic separations are required.

Selective MS/MS scan modes such as neutral loss scan and precursor scan experiments which can be performed on triple quadrupole instruments are very useful for identifying the most relevant biotransformation products from complex matrices. Phase I metabolites such as oxidative products and phase II metabolites such as glucuronides, sulfates and glutathiones, can be identified rapidly.[36,37] On triple quadrupole instruments the sensitivity in the product ion scan mode is often not sufficient to obtain good spectral quality. On the other hand, QqTOF or LIT-FT-ICR instruments can overcome this lack of sensitivity with additional accurate mass information. However, the limitation is that true precursor and neutral scan experiments and reliable quantitative analysis are not possible with these instruments. The same issue applies to the ion trap, where sensitive MS^n experiments allow clarification of the fragmentation process, which simplifies interpretation of the spectra.

Ideally, one would like to perform many different experiments in one LC/MS analysis. This can be done with the use of IDA. IDA allows to one to reduce the analysis time or enhance the information to be obtained in a single LC/MS run, but the slow scan speed of triple quadrupole MS and the moderate sensitivity when performing IDA LC/MS analysis are issues.

As shown previously, the QqLIT system can be operated either in the classical triple quadrupole mode with its particular strength of accurate and precise quantitation in the SRM mode, or in the ion trap mode for full-scan spectra. The different scan modes can be combined during the same LC/MS run without compromising the chromatographic performance. It also allows the generation of either quadrupole or ion trap CID spectra and also MS^3. Various combinations of quadrupole and trap modes are possible and have been described (Table 1). NL or PC can be used for a survey scan for IDA to extract the relevant information to obtain a sensitive EPI spectrum. NL and PC still suffer from moderate sensitivity and scan speed; however, they are only used to trigger the precursor ion selection. An alternative to NL and PC to gain sensitivity is to use SRM for a survey scan. SRM is much more sensitive than NL or PC. The drawback is that the SRM transitions have to be predicted. The duty cycle in SRM can be has low has 5–10 ms, allowing the setup of 50–100 experiments to screen various possibilities. Various possible scan combination approaches have already been applied to support drug quantitation and metabolism investigations[37–39] and for the general screening of unknown drugs and toxic compounds.[40] When searching for metabolites in biological matrices, selectivity becomes even more important than sensitivity and therefore the neutral loss or precursor scan mode becomes essential. Figure 8 shows the LC/MS analysis of remikiren metabolites in rat hepatocytes using a precursor scan as a survey scan and an enhanced product ion scan as a dependent scan. In this way, it is possible to identify a minor metabolite corresponding to the hydroxylation of the *tert*-butyl moiety of remikiren (Fig. 9). When analyzing the sample using EMS

JMS

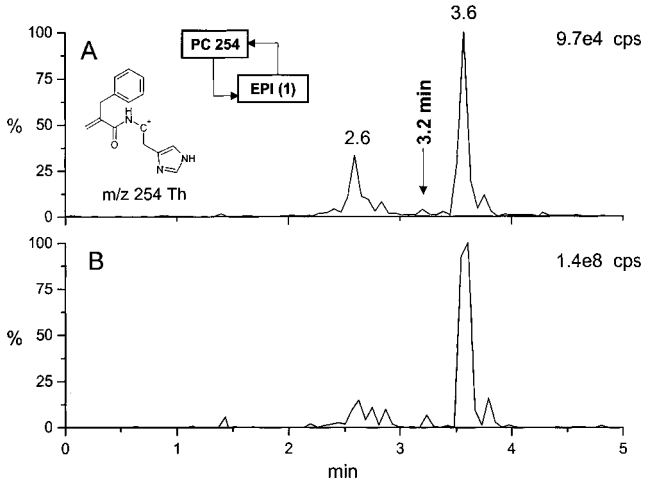

Figure 8. LC/MS/MS analysis of remikiren: (A) precursor ion scan trace of *m/z* 254; (B) enhanced product ion trace (adapted from *J. Mass Spectrom.*).

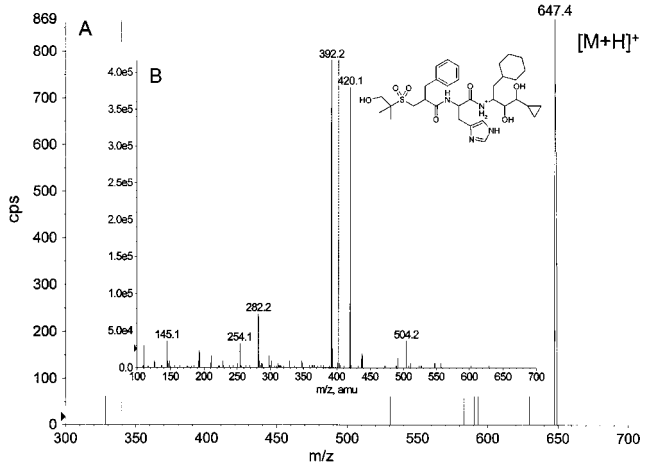

Figure 9. (A) Precursor ion spectrum of the peak at 3.2 min in Fig. 8. (B) Enhanced product ion spectrum of the precursor ion at *m/z* 647 (CE = 40 eV, trap fill time = 50 ms) (adapted from *J. Mass Spectrom.*).

for a survey scan the system was not able to select the right precursor ion without an inclusion list.[37]

LC/MS has an intrinsic problem: on the one hand, on-line coupling is highly desirable and on the other most LC peaks elute too fast to perform all possible MS experiments at the same time. The situation becomes even worse with monolithic LC columns where peak widths of 1 s have been reported. To overcome this problem, the chromatographic separation may be slowed during peak elution. For complex samples, this approach can only be followed with target analysis and sophisticated software which is able to trigger the right peak. Another solution is to perform fraction collection. One part of the LC effluent goes to the mass spectrometer and the remaining part goes to a 96-well collection plate. After a preliminary evaluation of the MS data, selected fractions can be re-infused to perform further experiments. Fraction collection with radiolabeled compounds to enhanced the sensitivity of metabolite detection using a microplate scintillation counter

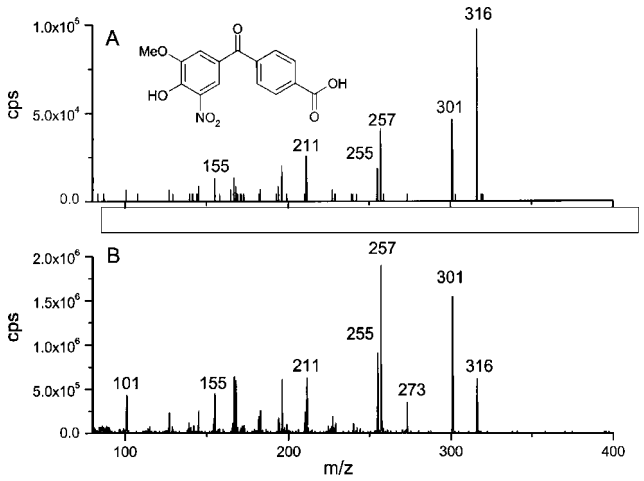

Figure 10. (A) LC/MS enhanced product ion spectrum of the metabolite of tolcapone eluting at 10 min. (B) Infusion EPI spectrum of fraction 30 infused for 2 min (multi-channel acquisition), negative mode ion detection.

has been demonstrated.[41] At first glance, fraction collection looks to be a very undesirable solution. On the other hand, analysis time and sample throughput have always been an issue. In drug metabolism, good chromatography is almost mandatory and reinjection of the sample to analyze a specific peak is time consuming. The feasibility of fraction collection followed by chip-MS infusion has been investigated for the analysis of a nitrocatechol compound, tolcapone (M_r = 272 Da) incubated in rat hepatocytes. LC separation was achieved on an ODS-3 Inertsil column (1 × 150 mm) at a flow-rate of 80 µl min^{-1} using a generic 1% HCOOH–MeOH gradient. The effluent was split prior to reaching the mass spectrometer, with 8 µl min^{-1} directed to the mass spectrometer where detection was performed in the negative mode using enhanced MS for a survey scan and an enhanced product ion scan for a dependent scan. Fraction collection was performed using a Probot (LC-Packings) at a flow-rate of 72 µl min^{-1}. A fraction was collected every 20 s with the addition of 25 µl of 1% HCOOH–MeOH to the well to minimize sample evaporation. The LC/MS enhanced product ion spectrum of the precursor ion at *m/z* 316 is depicted in Fig. 10(A). The peak eluted at 10 min corresponds to fraction 30. A volume 10 µl of this fraction was infused into the mass spectrometer using a NanoMate, a silicon chip-based device (Advion BioSciences). With infusion and multi-channel acquisition the quality of the tandem mass spectra (signal-to-noise ratio) improves with the number of scans which are summed.[42] The NanoMate typically operates at flow-rates of ~100–250 nl min^{-1}, allowing plenty of time to perform MS/MS optimization and acquisition. Figure 10(B) shows the sum of the enhanced product ion spectrum over 2 min (MCA) of the precursor ion at *m/z* 316. Fraction collection followed by infusion results in an ~20-fold gain of sensitivity compared with the LC/MS data (Fig. 10(A)) and increased structural information. The time available due to very low flow-rates of nanoelectrospray also allows one to perform many different types of experiments using EPI, MS^3 or TDF in positive or negative mode. This would have required many injections when using only LC/MS.

Confirmatory analysis for quantitative LC/SRM-MS

To support pharmacokinetic studies in drug discovery and development, precise and accurate determination of pharmaceutical compounds in biological fluids is required. Quantitative LC/MS analysis using the SRM mode has been demonstrated to be a very selective and powerful approach.[43] During method development in most cases only the parent drug is available and possible interferences due to metabolites cannot always be investigated. Inaccurate quantitation has been reported in a case where an isobaric interfering metabolite was co-eluting with the internal standard.[44] An important aspect is that method development is performed with spiked samples whereas for the analysis of study samples the identity of the quantified compound has to be proved. A second SRM transition can be used as a diagnostic transition but this approach often suffers from insufficient sensitivity for the second transition. With the QqLIT it is possible to perform an SRM and an EPI experiment simultaneously, with similar sensitivities. It is therefore possible to obtain a product ion spectrum of the analyte at the limit of quantitation (LOQ) of the assay. This concept has been evaluated for the quantitation of talinolol ($M_r = 363$ Da) in human plasma (calibration range 2.5–200 ng ml^{-1}). The plasma proteins were precipitated with perchloric acid (0.5 M) and after adjusting the pH with ammonium formate the supernatant was directly injected into an automated column-switching HPLC device (Prospekt II, Spark Holland). LC/MS analysis was performed using atmospheric pressure chemical ionization in the positive mode. Detection of the analytes in the positive mode was performed in the SRM mode (m/z 364 → 308 for talinolol and m/z 260 → 116 Th for propranolol (internal standard) for quantitation and in the EPI mode for confirmatory analysis. Figure 11 shows the enhanced product ion spectrum of a representative clinical sample, 15 h after talinolol administration. Typical MS/MS fragments can be clearly identified, which confirmed that the 3.64 ng ml^{-1}, quantified in SRM, is talinolol. This confirmatory approach may be particularly useful in method development or discovery analytics because it significantly improves data quality. No significant drawback in SRM quantitation was observed when the instrument was operated simultaneously in quadrupole and LIT modes. The higher pressure required in the collision cell for the LIT mode slightly reduces the detection limits in SRM.

Quantitation of pharmaceutical compounds in human plasma without chromatography

One way to increase analytical confidence for quantitative analysis is to use a second (diagnostic) SRM transition, but ideally it would be useful to have a full-scan product ion spectrum at the LOQ. However, this is not possible on classical triple quadrupole instruments. These issues may become even more serious with quantitative bioanalysis on a disposable ESI chip, which has been demonstrated recently, since there is no chromatographic separation.[45] To perform plasma samples analysis of an oxadiazole derivative and its depropyl metabolite (Fig. 12), a consecutive SRM, enhanced product ion and MS^3 acquisition was performed.

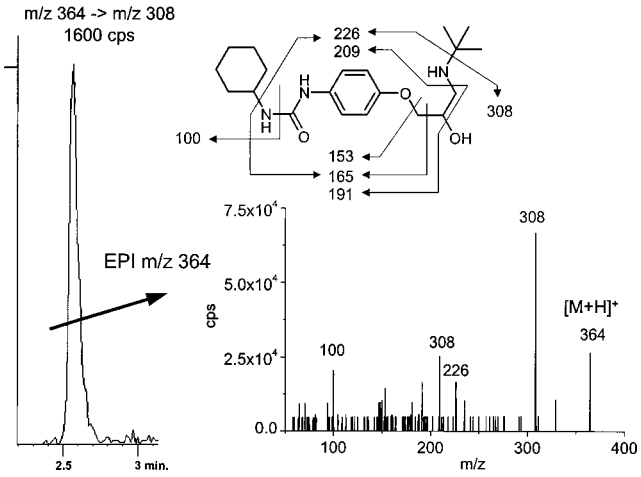

Figure 11. SRM (m/z 364 → 308, dwell time 100 ms, CE = 30 eV) and enhanced product ion (trap fill time = 50 ms, CE = 30 eV) LC/MS analysis of a human plasma sample 15 h after oral administration of 25 mg of talinolol.

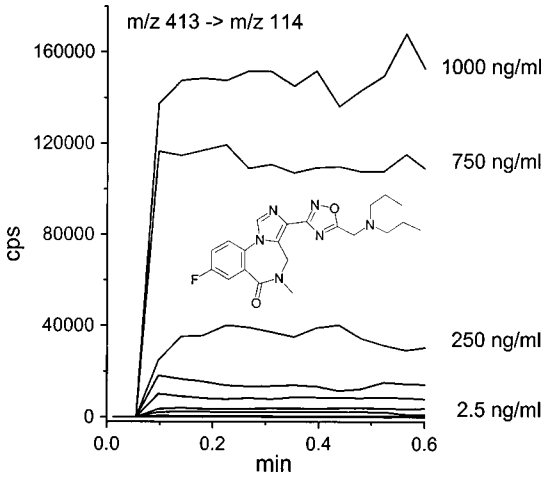

Figure 12. Infusion SRM traces (dwell time = 100 ms, CE = 20 eV) of human plasma calibration samples obtained with the NanoMate, sprayer voltage 1.6 kV.

One advantage with infusion one LC analysis is that sufficient time is available to perform a large number of MS experiments. The sensitivity of the SRM is dependent on the selected transition while in the ion trap mode (EPI, MS^3); the injection time can be set in such a way that the required sensitivity should be achieved. In the EPI mode, chemical noise could an issue, as demonstrated later. After addition of the deuterated internal standard, sample preparation consisting of liquid–liquid extraction was used for sample preparation followed by evaporation of the organic phase. Reconstituted samples were directly infused into the mass spectrometer using an automated, chip-based nanoelectrospray device (NanoMate 100, Advion BioSciences). The NanoMate 100 holds a 96-well plate, a rack of 96 disposable, conductive pipette tips and an ESI chip with a 10 × 10 array of nozzles, etched from the surface of a silicon wafer. The system can automatically infuse 96 samples in ~70 min. For each analysis a new tip and a new nozzle is used, eliminating the carry-over that is often

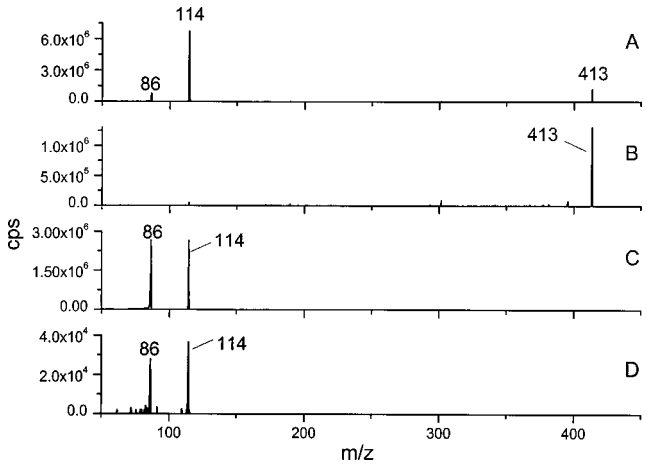

Figure 13. (A) Enhanced product ion (EPI) spectrum of reference solution at 1 ng μl^{-1}, precursor ion *m/z* 413 (ion trap fill time 200 ms, CE = 20 eV). (B) EPI spectrum of human calibration sample at 2.5 ng ml^{-1} (C) MS^3 (*m/z* 413 → 114) of reference solution (ion trap fill time 350 ms). (D) MS^3 of human plasma calibration sample at 2.5 ng ml^{-1}.

encountered with LC autosamplers. The infusion area ratio of the analyte and its deuterated internal standard was used for quantitation. The calibration was found to be linear from 2.5 to 1000 ng ml^{-1} using a 100 µl plasma aliquot. Precision and accuracy were found to be in an acceptable range for quantitative analysis (±15% acceptance criteria).

Figure 13(A) illustrates the enhanced product ion spectrum of a reference solution and Fig. 13(B) shows the enhanced product ion spectrum of a plasma sample at the LOQ of 2.5 ng ml^{-1}. Unfortunately, in the spectrum the ratio of the ions at m/z 413 and 114 is significantly changed. This is due to isobaric interfering ions of m/z 413 which pass through Q1 and are not fragmented. In the normal quadrupole mode, selection of the precursor ion is performed at unit mass resolution (0.7 Th FWHM). The quality of this spectrum is certainly not sufficient for confirmatory analysis at the low concentration range of the assay. One way to reduce the interferences would be to increase the resolution of the Q1 quadrupole from 0.7 to 0.2 Th (FWHM). This will cost sensitivity and may even not be sufficient. Another approach, which is even more effective, is to perform an MS^3 experiment on a fragment ion. Figure 13(C) and (D) show the MS^3 trace of the precursor ion at m/z 114 for the reference solution and for the plasma sample, respectively, at the LOQ of 2.5 ng ml^{-1}. Here, the quality of MS^3 is good enough to identify compound A.

CONCLUSIONS

Hybrid mass spectrometers using linear ion trap technology, such as QqLIT, provide new capabilities for solving analytical problems. In the case of the QqLIT, the uniqueness of the instrument is that the same mass analyzer Q3 can be run in two different modes. This allows very powerful scan combinations when performing information-dependent data acquisition. In the case of small molecules, qualitative and quantitative work can be performed concomitantly on the same instrument. For quantitation, confirmatory analysis can be performed either with the help EPI or MS^3 experiments. The application of two particular modes of operation was described: enhanced multiply charged (EMC) and time-delayed fragmentation (TDF). EMC selectively filters out singly charged ions whereas TDF uses time-dependent data to assist in *de novo* sequencing and to follow the fragmentation cascade. With data-dependent acquisition the selection of multiply charged precursor ions can be performed using smart software features. On the other hand, EMC has the same function using hardware, which offers obvious advantages, in particular for samples containing very low peptide levels. For many analytical challenges, selectivity often becomes more important than sensitivity.

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