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Quantitative liquid chromatographic–tandem mass spectrometric determination of orlistat in plasma with a quadrupole ion trap

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Abstract

This report evaluates the use of a quadrupolar ion trap for quantitation in a bioanalytical laboratory. The evaluation was accomplished with the cross-validation of an LC–MS–MS quantitative method previously validated on a triple quadrupole mass spectrometer. The method was a multi-level determination of the anti-obesity drug, orlistat, in human plasma. The method has been refined previously on a triple quadrupole instrument to provide rapid sample throughput with robust reproducibility at sub-nanogram detection limits. Optimization of the method on the ion trap required improved chromatographic separation of orlistat from interfering plasma matrix components coextracted during the initial liquid–liquid extraction of plasma samples. The ion trap produces full-scan collision-induced dissociation mass spectra containing characteristic orlistat fragment ions that are useful for quantitation. Data collection on the ion trap required a precursor ion isolation width of 3.0 Da and optimal quantitative results were obtained when three fragment ions were monitored with a 1.8 Da window for each ion. Although a direct cross-validation between the ion trap and the tandem triple quadrupole mass spectrometer was not possible, quantitative results for orlistat comparable to those obtained from the triple quadrupole instrument were achieved by the ion trap with the modified method. The limit of quantitation for orlistat in plasma on the ion trap was 0.3 ng ml^{-1} with a linear dynamic range of 0.3 to 10 ng ml^{-1}. Precision and accuracy varied from 4 to 15% over the quantitation range. The overall results provide an example of the utility of an ion trap in bioanalytical work.

Keywords: Orlistat

1. Introduction

In the past decade, applications using HPLC and electrospray mass spectrometry (ESI-MS) achieved great success in pharmacokinetic studies of drugs and their metabolites isolated from biological matrices. LC–MS techniques frequently provide specific, selective and sensitive quantitative results often with reduced sample preparation and analysis time relative to other commonly employed techniques. Triple quadrupole mass spectrometers (TQMSs) have contributed to a majority of the advances and applications involving atmospheric pressure ioniza-

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tion (API) methods. However, quadrupolar ion traps (QITs) designed with API interfaces for HPLC detection have many of the same tandem mass spectrometry (MS–MS) capabilities offered by TQMSs [1–4]. The recent introduction of commercial ion traps with significantly lower cost than TQMSs ensures that QITs will play an important role in the future of bioanalytical mass spectrometry. Several excellent reviews involving both GC and HPLC applications of analytical ion trap mass spectrometry are available [5–9].

In this report, we provide an evaluation of a modern, commercially available QIT system for its use in a bioanalytical application. To accomplish this, a method cross validation was performed on a Finnigan LCQ ion trap using an LC–MS–MS assay previously validated on a PE-Sciex TQMS [10]. The experiment chosen was the quantitative LC–MS–MS determination of orlistat in human plasma. Orlistat is a minimally absorbed inhibitor of gastrointestinal lipases involved in lipid metabolism [11,12]. It thus reduces dietary fat absorption and is under FDA review for control and treatment of obesity [13,14]. Orlistat acts locally in the gastrointestinal tract and because systemic absorption is not necessary for its effect, typical plasma levels found in treated patients remain low [15,16]. For this reason, as well as the strongly hydrophobic character of orlistat, its thermal instability and its lack of a strong chromophore, development of a suitable trace analytical method for pharmacokinetic studies using GC–MS or HPLC with UV detection was not practical.

The validated analytical procedure for orlistat in human plasma used in recent large scale pharmacokinetic studies employs liquid–liquid extraction followed by HPLC–MS–MS with a TQMS to achieve sub-ng ml^{-1} level determinations of the drug [10]. The method has been used to reliably analyze thousands of samples in support of pharmacokinetic studies. Our goal with the present study was to assess the performance of the established LC–MS–MS method when it was directly transferred to the Finnigan LCQ ion trap. We subsequently adapted the assay conditions to achieve optimal results with the LCQ. Particular attention was focused on achieving the established linear dynamic range (0.2 ng ml^{-1}–20 ng ml^{-1}), precision, accuracy and specificity previously obtained on the TQMS.

2. Experimental

2.1. Chemicals and materials

Orlistat (orlistat) and its pentadeuterated analog (orlistat-d_5) were provided by Hoffmann–La Roche (Nutley, NJ, USA). MRFA solution: the methionyl–arginyl–phenylalanyl–alanine acetate·H_2O peptide supplied as a calibration sample by Finnigan (San Jose, CA, USA) was dissolved in 50:50 methanol–water at 3 mg ml^{-1}. HPLC grade solvents (methanol and acetonitrile) were obtained from Mallinkrodt Baker (Phillipsburg, NJ USA). Deionized 18 MΩ cm^{-1} water was produced with an in-house Nanopure system (Barnstead Thermolyne, Dubuque, IA, USA). Human plasma collected in NaF and potassium oxalate was purchased from Biological Specialty Corporation (Colmar, PA USA).

2.2. Extraction of orlistat from plasma

Extraction of orlistat from plasma samples followed the previously reported procedure [10]. Briefly, a 1 ml aliquot of plasma was spiked with the deuterated internal standard, mixed with an equal portion of acetonitrile to affect precipitation, and then centrifuged. The supernatant was transferred to a new tube and combined with 5 ml of hexane. The samples were shaken and then centrifuged again to separate the layers. The upper hexane phase was transferred to a new tube and evaporated to dryness. The residue was then reconstituted in 50 μl of acetonitrile–10 m*M* ammonium acetate (70:30) for injection onto the HPLC column for LC–MS–MS analysis.

2.3. Calibration method

The samples used to evaluate quantitation on the QIT consisted of a set of calibration and quality control (QC) extracts. A duplicate set of calibration standards was prepared from plasma spiked with orlistat at 0.2, 0.3, 0.5, 0.7, 1.0, 5.0 and 10.0 ng ml^{-1} and with orlistat-d_5 at 1.0 ng ml^{-1}. These standards, blank plasma samples spiked with orlistat-d_5, and double blank (no orlistat, no orlistat-d_5) plasma samples, were run before and after the quality

control samples. The low, medium and high level quality control samples contained orlistat at 0.7, 5.0 and 8.0 ng ml^{-1} and also included the same amount of deuterated internal standard as the calibration samples.

2.4. Chromatographic conditions

Details of the chromatographic separation method used for the validated TQMS assay have been reported previously [10]. Briefly, a 2×50 mm deltabond phenyl column was used with isocratic elution at a 200 μl min^{-1} flow of 90:10 acetonitrile–2 m*M* ammonium acetate buffer. These conditions were selected to give a short run time with partial separation of the analyte and plasma components.

The chromatographic conditions were altered to improve the analytical results when the QIT was used. HPLC separations were accomplished with a 100×2 mm Spherisorb C6 analytical column (Keystone Scientific, Bellefonte, PA USA); a 2 μm frit and a 2 mm guard cartridge were placed at the column inlet. A flow-rate of 150 μl min^{-1} through the injector and analytical column to the QIT electrospray interface was provided by a Waters (Milford, MA, USA) 600 MS multi-solvent delivery system using electronic pulse dampening (Waters SILK method). A Waters WISP 717 plus autosampler was used to inject 20 μl sample aliquots.

Two different buffer systems (designated here as Methods A and B) were used in conjunction with the Spherisorb C6 column in the QIT experiments. Method A: this method employed isocratic elution with 95:5 acetonitrile–0.1% formic acid at 150 μl min^{-1} to elute orlistat at 1.1 min and give a total run time of 2 min. This eluant adequately separated orlistat from plasma components that interfered directly with the mass spectrometric detection of orlistat.

However, other hydrophobic components remained strongly adsorbed on the column using this eluant and caused a gradual increase in background chemical noise and pressure during multi-sample analyses. Method B: the second chromatographic method employed with the QIT assay further improved the separation of orlistat from plasma components and eliminated the background at the expense of a longer analysis time. This method eluted orlistat at 2.4 min with 150 μl min^{-1} flow of a 85:15 methanol–water mixture. This was followed by a rapid change to 100% methanol at 300 μl min^{-1} for 3 min and then re-equilibration with 85% methanol. Total run time was 9.5 min for this method.

2.5. Mass spectrometry

The QIT used was a Finnigan (San Jose, CA, USA) LCQ equipped with a heated capillary electrospray interface. Sprayer needle voltage was 4 kV with the nebulizer gas flow set at 60% of the maximum; temperature of the heated capillary was 250°C; capillary and tube lens potentials were 20 V and 10 V, respectively. The instrument was operated in the positive ion MS–MS mode under auto gain control with target counts set to 1×10^7. The instrument was set to store one microscan per scan and the elapsed time for each sampled chromatographic point varied from 100 ms to a maximum of 800 ms. The collision energy optimized at 20% of its maximum setting and full-scan (*m*/*z* 140 to 350) fragmentation spectra of orlistat and orlistat-d_5 were obtained. The precursor ion isolation width was 3.0 Da as described below.

The results reported here using the TQMS were performed on a Perkin–Elmer Sciex (Concord, Ont Canada) API 300 equipped with a Turbo Ion Spray LC–MS interface. The needle voltage was adjusted to 5.2 kV, the drying gas flow-rate and temperature were 4 l min^{-1} and 200°C, respectively. Nozzle–skimmer potential difference for declustering was set at 20 V. The first and third quadrupole mass analyzers were operated at unit resolution and fragmentation was accomplished with a collision energy of 17 eV. Protonated precursor ions for orlistat and orlistat-d_5 at *m*/*z* 496.5 and 501.5 were monitored alternately in the first quadrupole analyzer with 400 ms dwell times.

3. Results and discussion

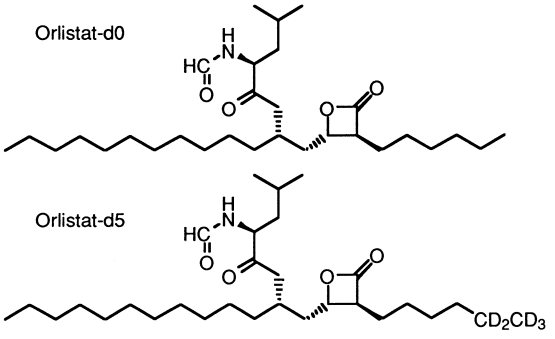
3.1. Development of an orlistat method on the QIT

Large scale clinical and pharmacokinetic studies of orlistat required an assay method that was sensitive, fast and specific. The salient features of the

validated LC–MS–MS method developed for a TQMS have been described [10]. Briefly, the procedure attained a 0.2 ng ml^{-1} limit of quantition with a per sample analysis time of 1.5 min for extracted plasma samples. Orlistat, (Scheme 1) is a hydrophobic molecule that is strongly retained on reversed-phase HPLC columns. The chromatographic conditions of the reported method used isocratic elution with a buffer containing 90% acetonitrile on a 2×50 mm phenyl column. Sample preparation employed hexane extraction (outlined in Section 2) and was used identically in the QIT assay below. For these reasons, the reconstituted samples used for LC–MS analysis contained both orlistat and other hydrophobic plasma constituents that eluted in closely overlapping bands. The plasma components completely masked the orlistat signal when UV detection was used with the fast chromatographic elution [10]. In contrast, the CID spectra of orlistat and its deuterated analog obtained with a PE-Sciex API 300 triple quadrupole spectrometer contained a set of unique fragment ions that provided highly selective MS–MS conditions that successfully differentiate orlistat from closely eluting plasma components.

3.2. Effect of isolation width

Differences in the operation of the QIT and TQMS became apparent when the validated TQMS method was applied directly to the Finnigan LCQ ion trap. First, the m/z range used for isolation during MS–MS detection of orlistat on the LCQ required a 3.5 Da width. Fig. 1 shows the effect of isolation width



Scheme 1.

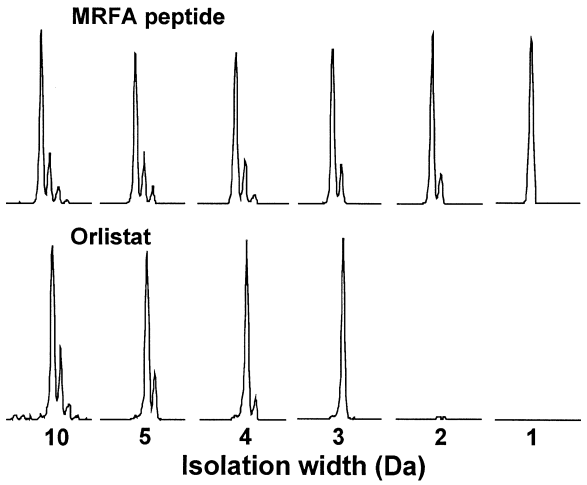


Fig. 1. Effect of QIT isolation width on precursor ion intensity. The signal intensities of the MRFA peptide (top) and orlistat (bottom) obtained in MS–MS mode on the QIT with collision energy set to zero were monitored. The numbers below the lower traces indicate the set isolation width for each measurement.

in the first stage of mass selection with the QIT on a reference compound and on orlistat. Separate solutions of orlistat (2 ng ml^{-1} in 90% methanol, 10% 23 m*M* formic acid, pH 3.1) and MRFA (Section 2) were infused into the QIT electrospray interface at 5 μl min^{-1}. The instrument was set up in the MS–MS mode with a target count value of 1×10^{7} and a 0.1% relative collision energy. The variable isolation width and the 10 Da scan range were centered on the protonated precursor ions for MRFA and orlistat (m/z 525 and m/z 496, respectively). These conditions isolated the precursor ions in the QIT by ejecting all other masses and then scanning out the remaining selected ions with no intervening fragmentation. In the traces shown in Fig. 1, only the isolation width was varied for each series of spectra.

Typical minimum isolation widths in the MS–MS mode on the Finnigan LCQ are 1.2 Da. The minimum acceptable ion isolation width is defined as the lowest range providing no appreciable signal attenuation when compared to a wider setting. The MRFA peptide signal remained unaffected when the isolation width was lowered from 10 to 1 Da. The orlistat signal, however, diminished when the isolation width was below 3.5 Da. It is possible that the resonance

ejection step used to remove masses above m/z 496 for orlistat also caused some excitation and collision-induced dissociation of orlistat. The test was repeated with different QIT conditions to confirm the results. No difference in this isolation width effect was found when the experiment was performed before and immediately after full calibration of the QIT by the automatic procedure specified by Finnigan. In addition, careful optimization of the setting of the center of the isolation range to m/z 497.2 permitted reduction of the isolation width to 3.0 Da with retention of the maximum signal. Apparently, adjusting the lowest resonance ejection frequency to a slightly higher setting improves the isolation of the orlistat precursor ion. The consequence of a wider isolation width for orlistat and other similarly labile molecules analyzed with this QIT is that the potential for matrix component interference is increased and analytical specificity may be reduced.

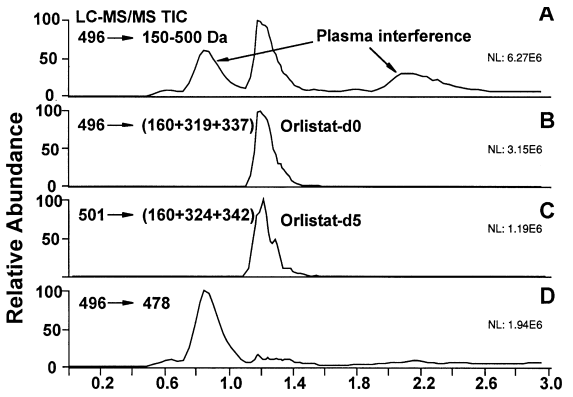


Fig. 2. Full-scan TIC and extracted ion current chromatograms of orlistat and plasma constituents. MS–MS detection was used on the QIT with isolation of m/z 496 precursor ions and acquisition of fragments over 140 to 500 Da. (A) is the summed ion current profile of all fragments in the full-scan range. (B) and (C) show the summed ion current profiles of the three most prominent fragments of orlistat and orlistat-d_5. (MS–MS selection of the precursors for the orlistat and its deuterated analog were collected on alternate scans). (D) is the extracted ion current for the m/z 496 to 478 transition of an early eluting plasma component.

3.3. Effects of coeluting isobaric plasma components

The original chromatographic conditions of the validated TQMS method caused coelution of isobaric plasma components with orlistat. On the QIT, these isobaric interfering components prevented efficient isolation of the m/z 496 $(M+H)^+$ orlistat precursor ion and significantly reduced the detection sensitivity for orlistat in the MS–MS mode. Adequate separation of the interfering components was achieved by changing the chromatographic conditions to an isocratic elution with 95:5 acetonitrile–0.1% formic acid on a Keystone Spherisorb C6 2 mm × 50 mm column (Method A in the Section 2). Fig. 2 shows an LC–MS–MS trace produced by an extracted plasma sample analyzed on the Finnigan LCQ. The sample was prepared as described in the Experimental section using a plasma sample spiked with orlistat at 1 ng ml^{-1}. Full-scan CID spectra were generated with a 3.5 Da isolation window centered on 496.4 Da. Fig. 2A shows the summed ion current of all fragments produced from precursors in the isolation window over the fragment mass range of 140 to 500 Da. Orlistat and orlistat-d_5 (which was spiked into plasma at 1 ng ml^{-1}) eluted at 1.1 min as shown with surnamed ion current profiles of their three most prominent fragment ions (Fig. 2B,C). Finally, the early eluting plasma component having a precursor ion at m/z 496 is shown in Fig. 2D by an extracted ion current profile for its m/z 478 fragment that arises from the m/z 496 to 478 transition. This interfering m/z 496 plasma component with its m/z 478 fragment ion was associated only with plasma extracts and did not appear in samples containing only orlistat or orlistat-d_5.

CID spectra for orlistat as well as the early and late eluting plasma components are shown in Fig. 3. Spectra on the two instruments are frequently qualitatively different since collision-induced dissociation on the QIT is a low energy, multiple collision process using He as the collision gas, whereas fragmentation on the TQMS occurs via a small number of collisions with molecular nitrogen. In these experiments however, the spectrum of orlistat on the Finnigan LCQ was similar to that produced on the PE-Sciex API 300 TQMS with the exception that the m/z 160 fragment was less abundant on the QIT. In the orlistat assay validated on the TQMS [10], the isobaric plasma constituents do not interfere with quantitation because only the ion current for the m/z

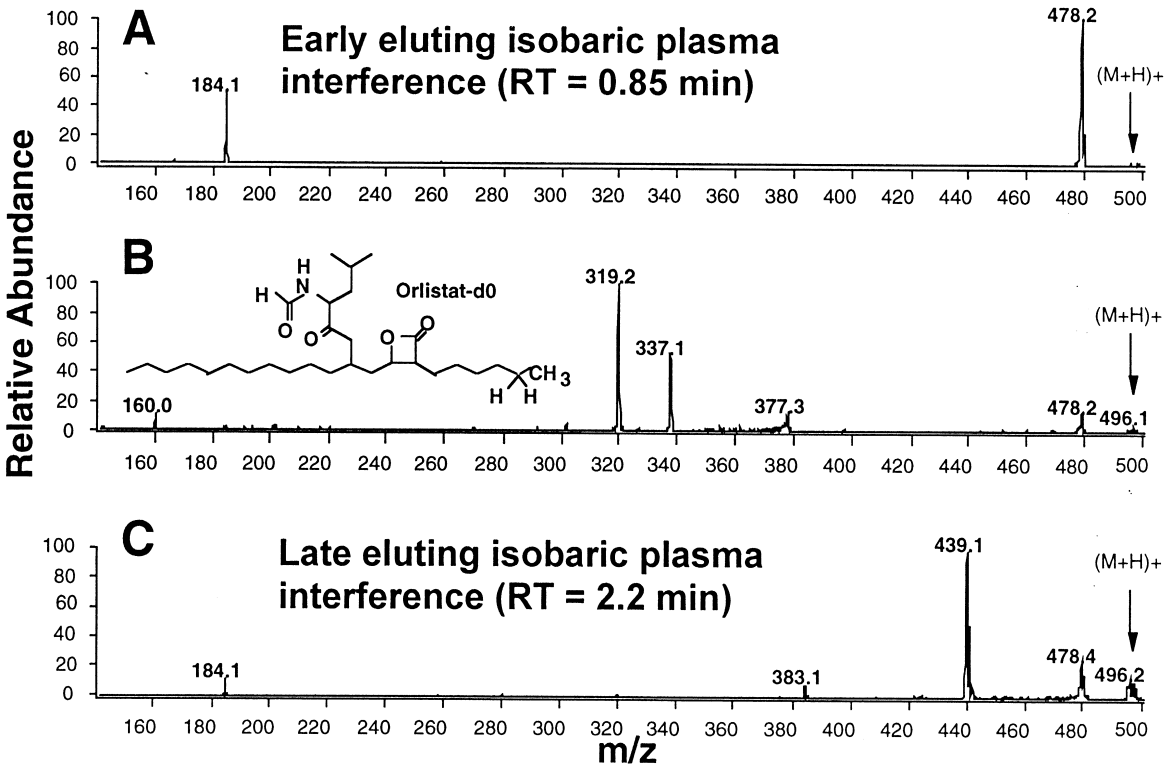


Fig. 3. CID mass spectra of orlistat (trace (B)) and isobaric m/z 496 plasma components. The spectra are reconstructed from the LC–MS–MS traces shown in Fig. 2. Traces (A) and (C) are the CID spectra of the plasma components eluting at 0.8 and 2.2 min in the chromatogram shown in Fig. 2.

496 to 160 transition specific for orlistat is monitored. On the QIT, when the orlistat and plasma components at m/z 496 coelute, the ion trap fills with isolated m/z 496 ions in proportion to the concentration of each species present having that mass-to-charge ratio. The total number of ions that the QIT can retain is limited and therefore, an increase in plasma components at m/z 496 decreases the abundance of the orlistat precursor ions. A further effect of coeluting plasma components is that they can suppress ionization in the electrospray sources for both the QIT and TQMS. Residual salts and other components from extracted plasma can act as ion pairing agents to neutralize the analyte ions and reduce the detected signal. For these reasons, optimal, high sensitivity quantitative detection with the QIT requires chromatographic separation of the target analyte from all isobaric interfering components.

3.4. Optimal chromatographic conditions for orlistat quantitation on the QIT

When analytical runs consisting of more than 30 extracted plasma samples were carried out on the Finnigan LCQ, the quantitation results using chromatographic Method A showed a progressive degradation in sensitivity and reproducibility. This was attributed to gradual column contamination from endogenous plasma components and to tailing by strongly retained plasma constituents which included components having m/z 496. For this reason a second set of chromatographic conditions (Method B in Section 2) was developed that greatly improved the quantitation result in two ways. First, isocratic elution with an 85:15 methanol:water eluant improved the separation of orlistat from the isobaric plasma components. Following this, the Spherisorb C6 column was stripped with a post elution treatment

of 100% methanol that effectively removed the strongly adsorbed plasma components and provided consistent results for repetitive sample analysis. Second, these chromatographic conditions broadened the orlistat peak to approximately 30 s (Fig. 4). Peaks broader than 20 s increase the scan count across each peak and improve the quantitative peak integrations on the QIT. This is especially important since the time per scan depends on the ion current. When the analyte concentration is low, scan time is long and the number of sampled points across weaker peaks is reduced. The adaptations of the chromatographic conditions had a number of beneficial effects useful for quantitation. Interference from the adsorbed components was eliminated. Chromatographic peak shapes were improved and typically 20 sampled points in the chromatogram across the peaks of orlistat and orlistat-d_5 were possible even for lower sample levels. These changes lead to improvements in the quantitative results for sequential analysis of up to 96 samples in one run.

3.5. Quantitation results

Chromatographic Method B was used to evaluate the quantitation of orlistat on the Finnigan LCQ. A series of standards and quality control samples were prepared and run sequentially as described in the Experimental section. Data collection with the QIT was performed with centroided mass peaks. When the mass spectra associated with each chromatographic time point from different sample injections were examined, the apparent spread of m/z values recorded for specific fragment ions during elution of the orlistat peak occasionally was as wide as 1.4 Da with a skew to higher mass values. Therefore, the extraction range of each reconstructed ion current chromatogram was adjusted to 1.8 Da to take this into account (see below).

The quantitation results for orlistat in plasma using the QIT are shown in Table 1. The Finnigan LCQuan (version 1.0) quantitation software was used to analyze chromatographic data. Calculation of concentrations relied on the ratio of the integrated MS–MS chromatographic peak areas for precursor ion to product ion transitions for orlistat and orlistat-d_5. Extracted ion current chromatograms for these analytes were constructed by summation of the ion currents for orlistat (m/z 496→160+319+337) and orlistat-d_5 (m/z 501→160+324+342) using a 1.8 Da window for each fragment ion mass. Fig. 5 shows the calibration curve derived from fitting the data

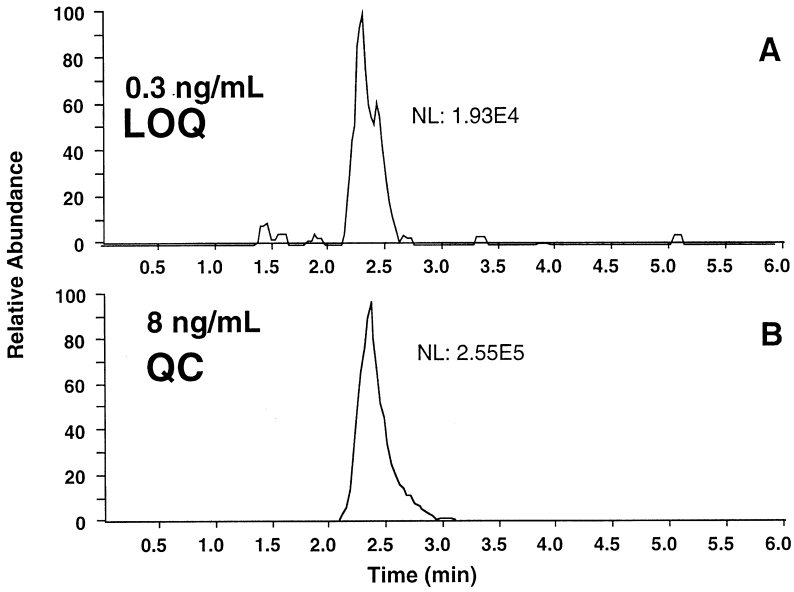


Fig. 4. Chromatographic peak shape obtained with the QIT using Method B (Section 2) at the lower limit of quantitation and at the high QC level.

Table 1
Within-run accuracy and precision for the QIT determination of orlistat

Nominal conc. (ng ml^{-1})	Mean calculated conc.(ng ml^{-1})	% Accuracy (calculated/actual)	Precision (R.S.D. %)
LLQ (0.3)	0.33	109.1 (n=2)	–
QC Low (0.7)	0.64	92.3 (n=4)	15.0
QC Med (5.0)	4.8	96.1 (n=6)	10.0
QC High (8.0)	7.1	88.0 (n=5)	6.4

Linearity: 0.3 to 10 ng ml^{-1}.

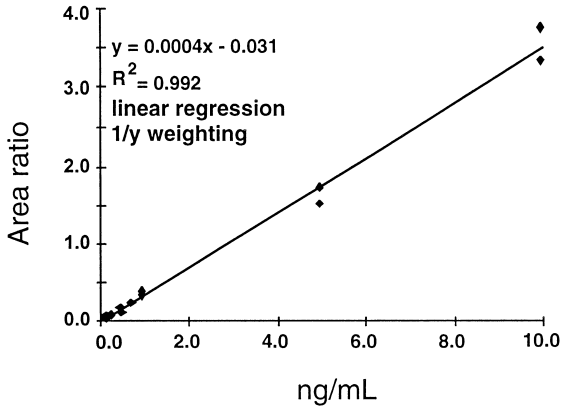


Fig. 5. Calibration data and least squares fit for a set of extracted plasma samples using the QIT. The ordinate is the calculated area ratio of orlistat to the orlistat-d_5 internal standard derived from extracted ion current profiles.

with a $1/y$ weighted, linear least squares model without forcing the fit through the origin. The linear calibration range obtained for orlistat quantitation on the QIT was 0.3 ng ml^{-1} to 10 ng ml^{-1}. Concentrations higher than 10 ng ml^{-1} were not tested in these trials so this range represents the minimum linear range. The accuracy and precision are given in the table.

In contrast, the MS–MS method for the TQMS described by Bennett et al., Ref. [10], provided the calibration results in Table 2 for a separately prepared set of extracted plasma samples. The reported method was performed on a PE-Sciex API 300 mass spectrometer as described in Section 2. The results show somewhat better sensitivity than obtained with the QIT and increased precision at each of the quality control levels.

4. Conclusions

The differences in the results between the TQMS and the QIT underscore the need to optimize sample preparation and chromatographic separation when the ion trap is used for bioanalytical applications. Robust quantitation on the QIT requires sample cleanup and chromatographic procedures that fully resolve isobaric components that interfere with reproducible accumulation of analyte ions in the ion trap. This may, however, increase analysis times on the QIT relative to a TQMS. One important advantage of the ion trap is its ability to rapidly and efficiently collect product ions in the MS–MS mode. The resulting full-scan CID mass spectra provide flexibility for post acquisition data processing. Summation of the chromatographic signals is performed on ion currents of single or multiple fragment ions selectively chosen to give the optimal quantitation

Table 2
Within-run accuracy and precision for the TQMS determination of orlistat

Nominal conc. (ng ml^{-1})	Mean calculated conc.(ng ml^{-1})	% Accuracy (calculated/actual)	Precision (R.S.D. %)
LLQ (0.2)	–	– (n=2)	–
QC low (0.7)	0.67	96.0 (n=6)	2.2
QC med (5.0)	4.89	97.8 (n=6)	2.2
QC high (8.0)	8.28	96.5 (n=6)	2.0

Linearity: 0.2 to 20 ng ml^{-1}.

results. In contrast, on the TQMS, acquisition of single precursor/product ion transitions is usually preferred.

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References

[1] G.J. van Berkel, G.L. Glish, S.A. McLuckey, Anal. Chem. 62 (1990) 1285–1295.
[2] S.A. McLuckey, G.J. van Berkel, G.L. Glish, E.C. Huang, J.D. Henion, Anal. Chem. 63 (1991) 375–383.
[3] J. Henion, T. Wachs, A. Mordehai, J. Pharm. Biomed. Anal. 11 (1993) 1049–1061.
[4] J.D. Henion, A.V. Mordehal, J. Cai, Anal. Chem. 66 (1994) 2103–2109.
[5] R.G. Cooks, G.L. Glish, S.A. McLuckey, R.E. Kaiser, Chem. Eng. News 25 (1991) 26–41.
[6] J.F.J. Todd, Mass Spectrom. Rev. 10 (1991) 3–52.
[7] J.F.J. Todd, A.D. Penman, Int. J. Mass Spectrom. Ion Proc. 106 (1991) 1–20.
[8] L.D. Bowers, D.J. Borts, Clin. Chem. 43 (1997) 1033–1039.
[9] K.R. Jonscher, J.R. Yates III, Anal. Biochem. 244 (1997) 1–15.
[10] P.K. Bennett, Y.-T. Li, R. Edom, J. Henion, J. Mass Spectrom. 32 (1997) 739–749.
[11] B. Borgstrom, Biochim. Biophys. Acta 962 (1988) 308–316.
[12] P. Hadvary, H. Lengsfeld, H. Wolfer, Biochem. J. 256 (1988) 357–361.
[13] M.L. Drent, E.A. van der Veen, Int. J. Obes. Relat. Metab. Disord. 17 (1993) 241–244.
[14] A.M. Wolf, G.A. Colditz, Am. J. Clin. Nutr. 63 (1996) 466S–469S.
[15] J. Zhi, A.T. Melia, H. Eggers, R. Joly, I.H. Patel, J. Clin. Pharmacol. 35 (1995) 1103–1108.
[16] J. Zhi, A.T. Melia, C. Funk, A. Viger-Chougnet, G. Hopfgartner, B. Lausecker, K. Wang, J.S. Fulton, L. Gabriel, T.E. Mulligan, J. Clin. Pharmacol. 36 (1996) 1006–1011.