Breaking Down Barriers: Can LC–MS Revolutionize the Quantitation of Drug Product Impurities?

In this article, the authors assess the precision, linearity, selectivity, accuracy, and sensitivity of three mass analyzers for liquid chromatography–mass spectrometry (LC–MS). They evaluated time-of-flight (TOF MS), single quadrupole, and triple quadrupole (MS–MS) mass analyzers for quantitation of drug product impurities. When compared with LC UV methods typically applied in these applications, the modes of LC–MS detection described within this article offer substantial improvements in selectivity and enable more-rapid method development and shorter method run times. The authors compare quantitative results obtained by MS–MS analyses with those obtained using a previously validated LC UV method. The data obtained by MS–MS in the multiple reaction monitoring mode displayed acceptable precision, accuracy, and linearity throughout 2–2.5 orders of magnitude.

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Recent advances in atmospheric-pressure ionization liquid chromatography–mass spectrometry (LC–MS) have revolutionized many of the scientific disciplines associated with drug discovery by providing improvements in efficiency, selectivity, and sensitivity for quantitative chemical analysis. The net effect has been more-accurate and more-rapidly obtained information for pharmaceutical experimentation in areas such as combinatorial chemistry, drug transport, drug metabolism, nonclinical pharmacokinetics, and metabolomics (1).

One area of drug development that also has benefited from the availability of atmospheric-pressure ionization LC–MS is clinical pharmacokinetics, in which selective and sensitive LC–MS bioanalytical assays have made drug and metabolite quantitation at the sub-nanogram-per-milliliter level readily achievable (1,2). This area of application has flourished for three reasons: LC–tandem MS (LC–MS–MS) operating in a multiple-reaction monitoring mode provides high selectivity in very complex matrices (blood plasma, serum, or urine), thus streamlining method development; the sensitivity generally is adequate for characterizing the lower concentrations in pharmacokinetic profiles, thus leading to the ability to determine biological half-lives of drugs and metabolites; and intersubject variability is the limiting source of random error in pharmacokinetic experiments (generally greater than 20% relative standard deviation [RSD]), thus analysts can tolerate somewhat greater variability (5–15% RSD) in the analytical component of the experiment.

A second area of drug development chemical analysis that has benefited from atmospheric-pressure ionization LC–MS is the identification of impurities and degradants in drug substances and drug products (3). In this area, structural information provided by direct-probe MS, which was in common use a decade ago, has been supplanted largely by atmospheric-pressure ionization LC–MS. The ability of atmospheric-pressure ionization LC–MS to separate the various related substances chromatographically before MS analysis eliminates substantial sample pretreatment required for interpretable direct-probe measurements. This qualitative approach also provides a ready means of volatilizing relatively nonvolatile analytes to obtain molecular weight information (MS) and structural

information based upon collision-induced dissociation (MS–MS and MS^n). Although the sensitivity of this approach is largely dependent upon the mass analyzer geometry (time-of-flight instrumentation and ion-trap instrumentation provide a higher duty cycle and therefore better sensitivity than scanned quadrupoles), either positive- or negative-ion full-scan spectra are readily obtainable for many ionic and neutral compounds. This information, coupled with various nuclear magnetic resonance spectrometry (NMR) techniques, allows facile determination of many types of molecular structures.

The use of atmospheric-pressure ionization LC–MS for the quantitation of drug impurities and degradation products has been a poorly represented area of investigation in the literature of pharmaceutical analysis. Several explanations are possible for this underutilization, including a perception that there is little need for additional sensitivity and selectivity in much of the quantitative chemical analysis of drug substance and drug product development, a perception that the accepted precision requirements for quantification of drug product–related substances (5–10% RSD) are tighter than those perceived achievable by atmospheric-pressure ionization LC–MS, and a conservative perspective by downstream organizations such as pharmaceutical quality-control groups or regulatory agencies that this type of approach would be inappropriate or scientifically invalid.

From our perspective as analytical chemists engaged in drug development, the ability to accurately quantify small amounts of impurities and degradation products in chemically pure samples or in formulations containing complex matrices seems entirely feasible by some form of atmospheric-pressure ionization LC–MS.

In our study, we examined the feasibility of using three types of atmospheric-pressure ionization LC–MS for the trace quantitative determination of degradants in a drug product. The three approaches were full-scan MS on a single quadrupole analyzer (LC–MS), extracted-ion monitoring using a time-of-flight analyzer (LC–TOF MS), and multiple-reaction monitoring using a triple quadrupole mass spectrometer (LC–MS–MS). All MS approaches used reversed-phase high performance liquid chromatography (HPLC) with positive-ion electrospray ionization. We studied the effects of using an internal standard for quantitation and compared the results with those from a traditional approach involving HPLC with ultraviolet-absorbance photometric detection (LC UV). We examined samples of a formulated drug that contained an example compound (the active pharmaceutical ingredient) and two degradation products for the usual method performance characteristics of linearity, range, precision, accuracy, and quantitation limits.

Experimental

Materials: We obtained quinapril (3-isoquinolinecarboxylic acid, 2-[2-[1-(ethoxycarbonyl)-3-phenylpropylamino]-1-oxopropyl]-1,2,3,4-tetrahydro-,monohydrochloride); quinaprilat (2-[2-(1-carboxy-3-phenylpropylamino)-propionyl]-1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid); cyclic impurity (2-(3-methyl-1,4-dioxo-1,3,4,6,11,11a-hexahydro-pyrazino[1,2-b]isoquinolin-2-yl)-4-phenyl-butyric acid ethyl ester); and d_4-quinaprilat (deuterated internal standard) from Pfizer Global R&D, Ann Arbor Laboratories (Ann Arbor, Michigan). Figure 1 shows the structures of these compounds. The formulated drug product was a 1-mg/mL suspension of quinapril in a complex, proprietary buffered syrup medium (syrup matrix). Both quinaprilat and the cyclic impurity were known impurities and metabolites of quinapril (4). We

used reagents and HPLC-grade solvents (Mallinckrodt, Phillipsburg, New Jersey) without further purification.

HPLC conditions: Chromatographic separations were performed using an Agilent 1100 HPLC system (Agilent Technologies, Wilmington, Delaware). We analyzed 3-μL injections of standards and samples using a 10 cm × 2 mm, 5-μm d_p HSB cyano column (BHK Laboratories, Naperville, Illinois). The pump was run isocratically at 0.25 mL/min with a mobile phase of 70% acetonitrile–30% water with 30 mg of ammonium acetate and 60 μL of trifluoroacetic acid added per liter.

MS conditions: The LC–MS and LC–MS–MS experiments were performed using a Quattro Ultima triple quadrupole electrospray ionization mass analyzer (Micromass, Beverly, Massachusetts). The LC–TOF MS experiments were performed using an LCT time-of-flight electrospray ionization mass analyzer (Micromass). We kept the capillary voltage, source temperature, and desolvation temperature constant in all experiments at 3.5 kV, 120 °C, and 400 °C, respectively. We adjusted the cone voltage and collision energies to optimize the instrumental response of the individual compounds.

All LC–MS–MS data were acquired using multiple-reaction monitoring, with argon as the collision gas (2.1×10^{-3} mbar). Figure 1 illustrates the cone voltages, collision energies, and multiple-reaction monitoring energy transitions monitored for each component. Each transition was monitored for 0.2 s with a 0.03-s interchannel delay.

Data for the LC–MS experiments were collected in scanning mode from 100–500 amu with a scan time of 1 s and a 100-ms interscan delay. The cone voltage was held constant at 60 V.

The LC–TOF MS data were obtained in scanning mode from 100–500 amu with a scan time of 1 s and a 100-ms interscan delay. The cone voltage was held constant at 20 V.

Standard curve preparation: We prepared stock standard solutions from neat materials and diluted them in sample solvent (50:50 [v/v] acetonitrile–50 mM ammonium acetate) to the appropriate concentrations. A 12-point calibration curve was prepared by fortifying the syrup matrix with stock solution to achieve a 90% matrix solution. We added 900 μL of 600-ng/mL deuterated quinaprilat internal standard in acetonitrile to 100-μL aliquots of the calibration curve. We made an additional 1:10 dilution with sample solvent to yield a working standard curve in the range of 0.01–500 ng/mL with an internal standard concentration of 54 ng/mL.

Sample preparation: The formulated drug product (in syrup matrix) has a nominal concentration of 1 mg/mL quinapril. To approximate the matrix concentration of the standard curve, we diluted the drug product 1:100 to achieve nominal concentrations of quinapril and internal standard of 10,000 and 54 ng/mL, respectively. At these levels, we expected the impurities to fall within the calibration curves.

Data treatment: All the data were processed using MassLynx software with QuanLynx data quantitation package, version 3.5 (Micromass). Multiple-reaction monitoring chromatograms were collected with 1.0-Da windows. The single-quadrupole (LC–MS) and LC–TOF MS chromatograms were extracted from scanned data with 1.0-Da windows. All data were mean smoothed twice. The data system was configured to automatically calcu-

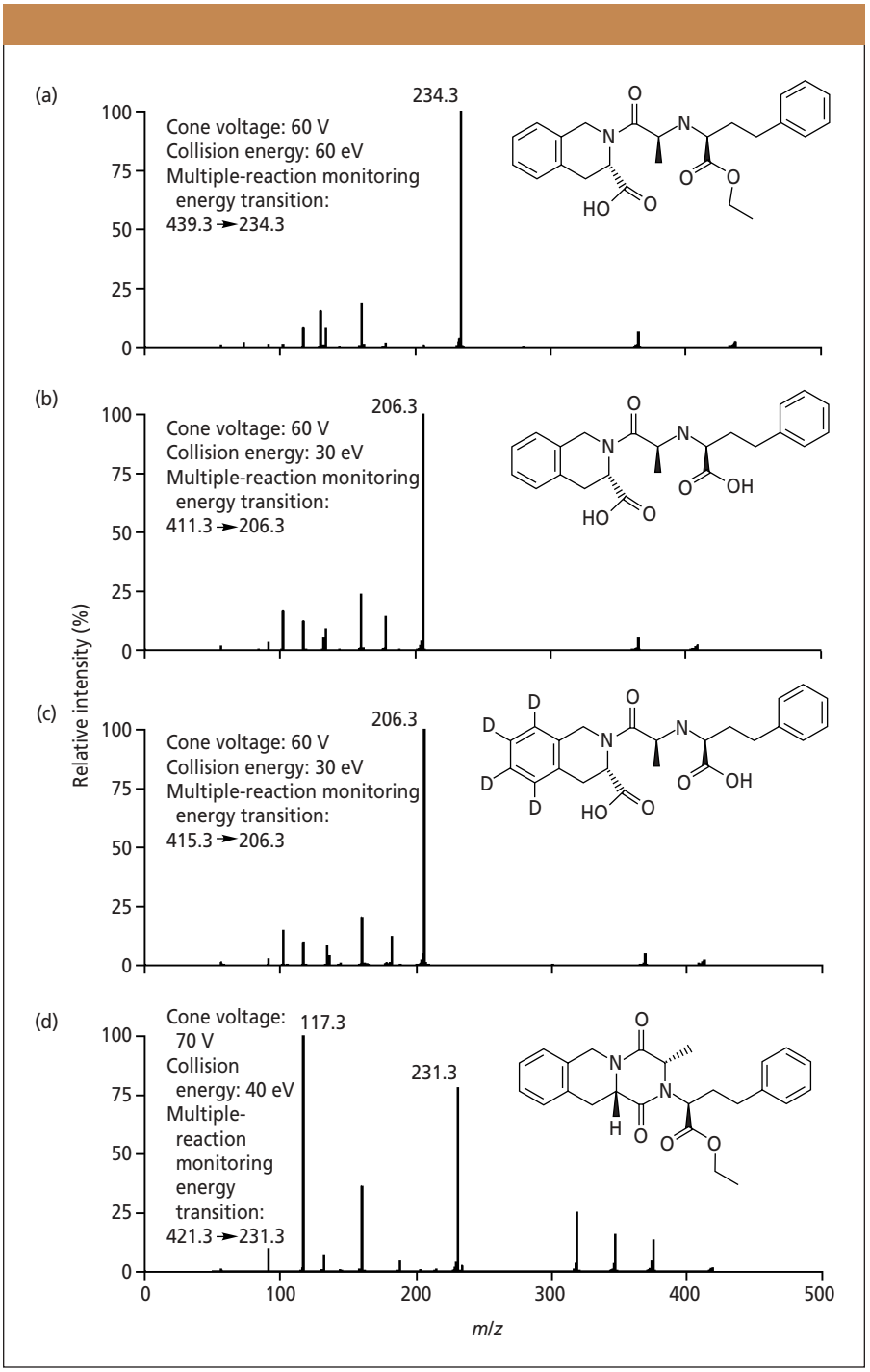

Figure 1: Multiple-reaction monitoring daughter ion scans with chemical structures: (a) quinapril; (b) quinaprilat; (c) d_4-quinaprilat; and (d) cyclic impurity.

late and annotate the areas of the product ions of quinapril, the cyclic impurity, quinaprilat, and its corresponding internal standard. We constructed a calibration curve using peak-area ratios of the calibration samples by applying the appropriate regression analysis. All concentrations then were calculated from their peak-area ratios against the calibration curve.

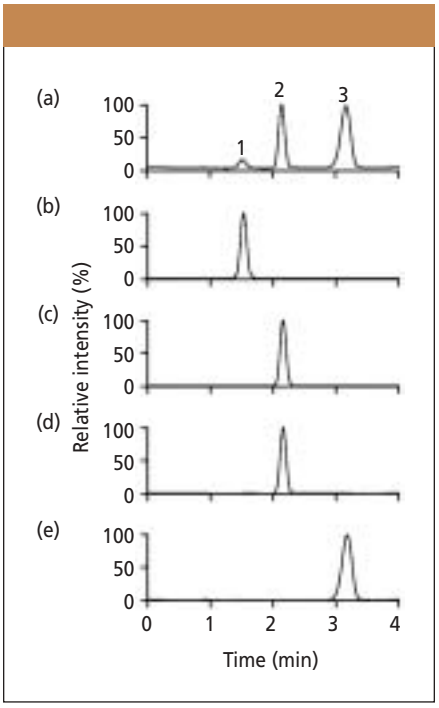

Figure 2: Sample chromatography of a 10-ng/mL solution of cyclic impurity, quinaprilat, d_4-quinaprilat, and quinapril dissolved in 50% acetonitrile and 50% 50 mM ammonium acetate. Shown are (a) the sum of four multiple-reaction monitoring transitions and individual multiple-reaction monitoring transitions of (b) cyclic impurity, (c) quinaprilat, (d) d_4-quinaprilat, and (e) quinapril. Peaks: 1 = cyclic impurity, 2 = quinaprilat and d_4-quinaprilat, 3 = quinapril.

Results and Discussion

Figure 1 shows the product ion mass spectra of the $[M + H]^+$ molecular ion proton adducts of quinapril, quinaprilat, d_4-quinaprilat, and the cyclic impurity, as well as their chemical structures. The listed cone voltages and collision energies were optimized to yield a maximum signal for each MS mode. The most intense and characteristic product ions were selected for the multiple-reaction monitoring.

The upper trace of Figure 2 shows the total-ion current chromatogram (in multiple-reaction monitoring mode) of a standard mixture of 10 ng/mL quinapril, quinaprilat, d_4-quinaprilat, and cyclic impurity dissolved in 50:50 (v/v) acetonitrile–50 mM ammonium acetate. All four compounds were well retained (retention factor $[k] \geqslant 3$) and well separated (resolution $[R_s] >> 2$); as expected, the deuterated-quinaprilat internal standard was coeluted with the quinaprilat, and we observed three peaks. The lower traces of Figure 2 show multiple-reaction monitoring chromatograms for each of the four compounds, which were clearly distinguished by their characteristic precursor to product transitions. It was the tremendous selectivity of these transitions that enabled the differentiation of even structurally similar compounds.

Generally, the retention of dicarboxylic acids with low pK_as using MS-compatible mobile phases is difficult, and we screened numerous columns and mobile phases to achieve retention of the dicarboxylic acid. To retain quinaprilat, we had to add small amounts of trifluoroacetic acid to protonate the compound. We also added ammonium acetate to improve the peak shape of quinapril. Because trifluoroacetic acid was necessary for chromatography and the cyclic impurity did not have a deprotonation site, we used positive-ion electrospray ionization. Zhang and co-workers used a similar mobile phase for the LC–MS–MS determination of enalapril and enalaprilat (5).

Tables I and II list the results for the standard curves for quinaprilat and the cyclic impurity, respectively. These data were collected throughout several orders of magnitude from 0.01 ng/mL to 500 ng/mL, equivalent to 0.001–5.0% impurity (w/w) in the drug product. We assessed the method linearity and accuracy by measuring the response factor at each concentration. The response factors for the mass spectral data were calculated as

$$RF = \left(\frac{A}{A_{IS}}\right)\left(\frac{1}{c}\right) \qquad [1]$$

where RF is the response factor, A is the analyte peak area, A_{IS} is the internal standard peak area, and c is the analyte concentration.

The response factor was plotted against the analyte concentration, which enabled linearity assessment independent of concentration (6).

Figure 3 displays a linearity plot using RF versus c for the cyclic impurity by LC UV, LC–MS, LC–TOF MS, and LC–MS–MS; minimally, each data point met the precision criterion of not greater than 10% RSD.

Table I: Calibration curve data for quinaprilat in multiple-reaction monitoring (LC–MS–MS) mode

Nominal Standard Concentration (ng/mL)	N	Average Peak Area	Average Peak Area Ratio	Average Response Factor	RSD (%)	High-Range* Relative Error (%)	Low-Range† Relative Error (%)
0.01	6	37.6	0.0007	0.07384	33.9	—	—
0.02	6	75.1	0.0009	0.04787	12.4	—	—
0.05	6	277.3	0.0021	0.04349	8.7	—	−7.9
0.1	6	578.4	0.0036	0.03824	9.0	—	−4.9
0.2	6	1274.0	0.0073	0.03848	7.1	—	5.2
0.5	6	3080.5	0.0175	0.03678	6.0	—	6.0
1	6	5844.1	0.0341	0.03590	3.8	—	5.7
2	6	11059.6	0.0601	0.03164	2.3	—	−5.5
5	6	29762.3	0.1587	0.03343	2.7	−3.6	3.2
10	3	51953.0	0.2826	0.02975	2.6	−1.1	−4.6
20	3	96978.9	0.5428	0.02857	3.3	2.7	1.0
50	3	254255.3	1.3807	0.02907	1.4	10.0	—
100	3	420393.4	2.4040	0.02531	3.6	−2.7	—
200	3	749519.0	4.7623	0.02506	0.3	−2.1	—
500	3	1492595.2	11.8554	0.02496	2.5	0.4	—

*High range: Quadratic fit with 1/x weighting, $y = -0.0000020 \times x^2 + 0.0257506 \times x + 0.04091110$, $r^2 = 0.999155$.
†Low range: Quadratic fit with 1/x weighting, $y = -0.0002815 \times x^2 + 0.0336616 \times x + 0.00059226$, $r^2 = 0.998314$.

Table II: Calibration curve data for cyclic impurity in multiple-reaction monitoring (LC–MS–MS) mode

Nominal Standard Concentration (ng/mL)	*N*	Average Peak Area	Average Peak Area Ratio	Average Response Factor	RSD (%)	High-Range* Relative Error (%)	Low-Range† Relative Error (%)
0.01	6	24.2	0.0005	0.04452	42.3	—	—
0.02	6	54.4	0.0007	0.03625	41.7	—	—
0.05	6	109.4	0.0008	0.01643	24.4	—	—
0.1	6	186.7	0.0012	0.01182	24.4	—	—
0.2	6	347.0	0.0020	0.00984	8.1	—	−10.9
0.5	6	859.7	0.0049	0.00963	9.5	—	10.0
1	6	1551.9	0.0086	0.00923	2.6	—	2.8
2	6	2869.1	0.0156	0.00771	2.4	—	−3.1
5	6	7653.7	0.0409	0.00808	3.8	−3.2	4.7
10	3	13670.0	0.0743	0.00734	2.1	−0.3	−3.4
20	3	25681.0	0.1437	0.00710	2.6	3.0	−4.7
50	3	67215.8	0.3650	0.00722	2.5	9.0	2.8
100	3	112503.4	0.6433	0.00636	3.0	−2.9	−0.6
200	3	200377.8	1.2731	0.00629	1.6	−3.2	—
500	3	415589.1	3.3018	0.00653	4.4	0.9	—

*High range: Linear fit with 1/*x* weighting, $y = 0.00645166 \times x + 0.00928133$, $r^2 = 0.997864$.
†Low range: Quadratic fit with 1/*x* weighting, $y = -0.0000127 \times x^2 + 0.0076688 \times x + 0.000610658$, $r^2 = 0.998946$.

This result is equivalent to a single-point calibration. To evaluate the suitability of a single-point calibration, we plotted response factors as a percentage of the response factor for the 0.5% impurity standard. Data points that fell within the control lines exhibited acceptable accuracy (± 15%). Observe that the UV analysis showed excellent accuracy throughout the full range (0.05–5%) with the use of a one-point calibration. Detection by LC–MS–MS also provided good range, but with somewhat less accuracy than UV. Scanning MS detection methods will not allow the use of a single-point calibration because of their lack of accuracy.

The accuracy and working range of the method can be improved by applying weighting or a quadratic fit to a multiple-point calibration curve approach. Tables I and II list the fit and percent relative error for each impurity. If the percent relative error at any standard level was outside of the 90–110% range (or 85–115% at the lowest concentration level), then data points were removed from either the high or the low end, the line was refit, and the accuracy was re-evaluated. This process was repeated until the percent relative error was within 90–100% (85–115% at the lowest concentration level) for all standard levels. We defined the lowest concentration on this curve as the lower limit of quantitation, and the range is from the lower limit of quantitation to the highest concentration on this curve.

The internal standard is important in LC–MS quantitation (7). Ideally, in quantitative LC–MS, a stable, isotopically labeled analog of the component of interest is used as an internal standard to account for variations in the ionization efficiency of the source. When a stable isotopic label is unavailable, a structural analog, which would be eluted closely with the analyte of interest and ionize similarly, can be used as an internal standard. The advantages of the stable isotopic label internal standard can be seen when comparing precision data in Tables I and II, which show improved precision for quinaprilat when compared with the cyclic impurity.

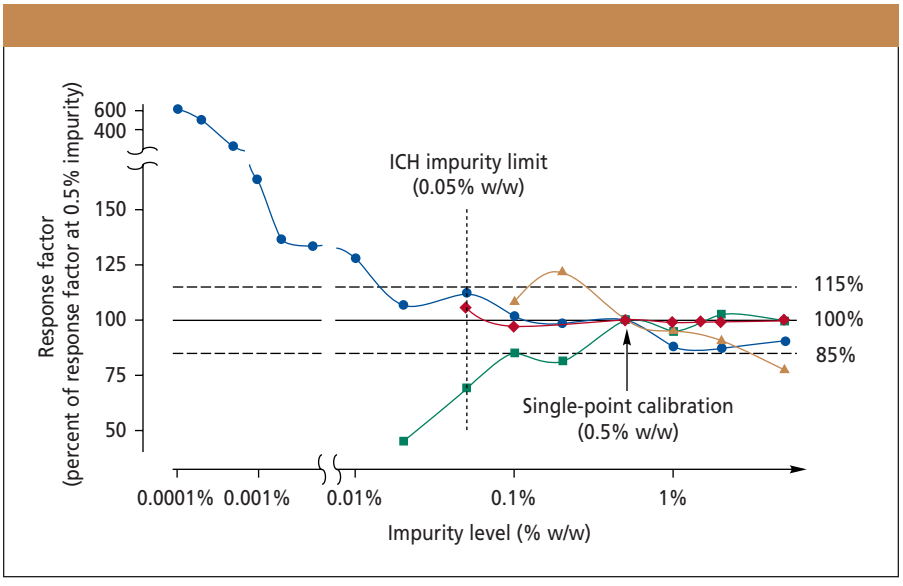

Figure 3: Linearity plot (response factor vs. concentration) for a single-point calibration of the cyclic impurity as determined by LC–MS–MS (●), LC–TOF MS (■), LC–MS (▲), and LC UV (◆). Each data point included in the plot has a precision of not less than 10% relative standard deviation. The hash marks on the *x* and *y* axes represent breaks to show the expanded response range available to the LC–MS–MS technique. The horizontal control lines represent the required response factor range to allow the use of a single-point calibration. The use of calibration curves for the LC–MS–MS data allows accurate impurity determinations, even at the lowest concentrations.

Table III is a comparison of all four methods of quantitation, outlining overall range, correlation coefficient (r^2), limit of detection, limit of quantitation, and selectivity. Each detection mode was capable of meeting the requirement for correlation coefficient ($r^2 \geq 0.98$), with r^2 values increasing in the order of LC–MS < LC–TOF MS ~ LC–MS–MS < LC UV. Detection in the multiple-reaction monitoring mode enabled sufficient range to allow for testing throughout the full range of impurity con-

Table III: Comparison of quantitative performance and selectivity for the cyclic impurity

Detection Mode	Range (ng/mL)	Limit of Detection (ng/mL)	Limit of Quantitation (ng/mL)	Best-Fit Correlation	Selectivity
MS	20–200	10	20	0.979	Good
TOF MS	10–500	5	10	0.999	Very good
MS–MS (high range)	5–500	0.01	5	0.998	Excellent
MS–MS (low range)	0.2–100	0.01	0.2	0.999	Excellent
UV	50–5000	10	50	1.000	Poor

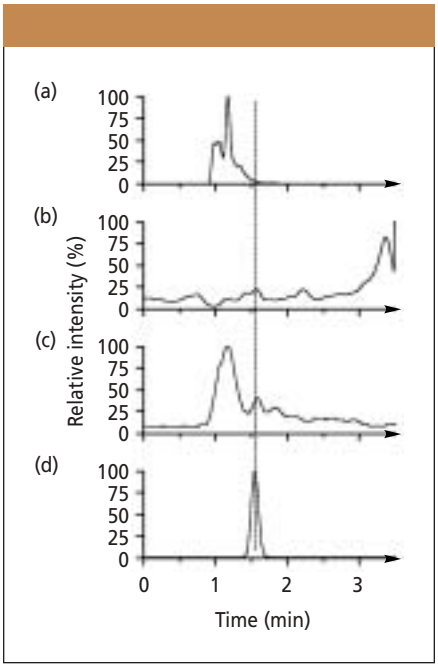

Figure 4: Chromatograms showing 5-ng/mL (0.05% in drug product) cyclic impurity (t_R = 1.55 min) spiked in matrix. Shown are (a) the UV chromatogram at 235 nm; (b) the MS extracted chromatogram at 421.3 Da; (c) the TOF MS extracted chromatogram at 421.3 Da; and (d) the MS–MS multiple-reaction monitoring transition 421.3 → 231.3.

Table IV: Comparison of results obtained by an LC–MS–MS method to those obtained by a validated LC UV method

Detection Mode	Quinaprilat (% w/w)	Cyclic Impurity (% w/w)
Multiple-reaction monitoring	4.88 (2.6% RSD)	1.44 (3.7% RSD)
UV	4.83 (0.4% RSD)	1.25 (1.7% RSD)
Difference (%)	1	15

centrations typically monitored in a pharmaceutical drug product. For multiple-reaction monitoring detection, the range of the detector restricted the limit of quantitation; the detector was able to maintain a linear response only through 2–2.5 orders of magnitude. If analysts require limits of quantitation lower than 0.05%, these limits can be achieved at the expense of a decreased upper limit of quantitation. This information is given by the LC–MS–MS low-range data in Table III.

We determined the limit of detection as the lowest concentration standard level that provided peaks with a signal-to-noise ratio greater than three. The limit of quantitation requires a signal-to-noise ratio of not less than 10, with imprecision of not more than 10% RSD and accuracy of ±15%. With the selectivity advantages of multiple-reaction monitoring detection, virtually no background is present, which allowed extremely sensitive detection of trace impurities. Detection and quantitation limits for multiple-reaction monitoring detection were at least two orders of magnitude better than the other detection modes we explored, including LC UV.

The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH, Geneva, Switzerland) guidelines require that impurities present at 0.05% of the active pharmaceutical ingredient can be quantified, and this amount often is denoted as the target limit of quantitation (8).

Figure 4 shows the dramatic differences in selectivity of the different detection modes using a 5-ng/mL standard matrix solution, which corresponded to the 0.05% impurity limit. Figure 4a corresponds to LC UV detection, and we were unable to observe any impurities because they are buried underneath the excipient peaks. Figure 4b corresponds to an extracted chromatogram for 421.3 (MH^+) corresponding to the cyclic impurity from a full scan (100 to 500 amu) single quadrupole (LC–MS); the impurity peak is unobservable, obscured by noise. Figure 4c corresponds to the extracted chromatogram (*m/z* 421.3) from the LC–TOF MS data, and although a small peak was observed, it still is difficult to distinguish from the chemical noise. With MS–MS detection, we observed a dramatic improvement in selectivity with the use of multiple-reaction monitoring. At 5 ng/mL (0.05% of atmospheric-pressure ionization concentration), the cyclic impurity is easily distinguishable from noise, giving a signal-to-noise ratio of 1600 (Figure 4d).

Table IV lists quantitative results for the impurities of an aged drug product formulation using LC–MS–MS and compares them with results obtained from a previously validated LC UV method (9).

The accuracy of the LC–MS–MS method was excellent and within regulatory requirements for drug product analysis (10–12). The quinaprilat quantitation data provide better precision than those for the cyclic impurity, most likely due to the inclusion of the deuterated stable label. In addition, the run time for the LC–MS–MS work was only 4 min, compared with 20 min for the LC UV method, thereby enabling higher sample throughput.

Clearly, MS–MS detection is capable of detecting drug product impurities at or below the levels required for quantitation. The additional modes of selectivity offered by MS, or more dramatically MS–MS, analysis allow for more-rapid method development in that baseline chromatographic resolution of impurities and matrix components is nonessential.

Often, analysts perceive that MS detection does not provide the precision required for quantitation of pharmaceutical drug products. This perception might be true in the case of determining active components, which typically require tighter precision (2.0% RSD); however, the determination of drug product impurities involves a more relaxed precision criterion of 10–15% RSD. As demonstrated, tandem MS in the multiple-reaction monitoring mode can achieve these levels of precision with extremely low analyte concentrations.

Conclusion

The data we presented in this article demonstrate that LC–MS–MS allows for the accurate and precise determination of pharmaceutical drug product impurities and meets recognized method validation requirements for selectivity, sensitivity, lin-

earity, range, accuracy, and precision. Furthermore, the quantitative results as determined by the LC–MS–MS method are equivalent to those obtained by the previously validated LC UV method.

The LC–MS–MS method offers the potential for improved sensitivity; detection limits in our study were in the range of 10 pg/mL. This detection approach seems particularly viable for detecting drug entities or related substances that lack chromophores or fluorophores, and it could be preferable to the use of refractive index, electrochemical, chemiluminescence, or evaporative light-scattering detection.

The additional modes of selectivity offered by tandem MS obviate the need for baseline chromatographic resolution, thereby allowing rapid development of fast chromatographic methods. In cases that involve more-complicated formulations or difficult-to-separate impurities, improvements in both the method development and sample run times can be substantial when using tandem MS. In the above case, the LC–MS–MS run was five times shorter than that of the LC UV method. In situations in which resources are time limited, the greater throughput enabled by LC–MS–MS could quickly justify the added costs; for example, it is less expensive to buy one LC–MS system than five LC UV systems.

Admittedly, pharmaceutical quality-control groups and regulatory agencies are not necessarily prepared to implement tandem MS detection for routine use in drug product analysis. However, as the pace of drug development quickens, we can ignore neither the potential for its application nor the time savings it can present. As commercial instrumentation matures and costs decrease, instruments will become more rugged and require less user intervention, which will make the approach more accessible to manufacturing environments.

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