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Short communication

Expanding the linear dynamic range for multiple reaction monitoring in quantitative liquid chromatography–tandem mass spectrometry utilizing natural isotopologue transitions

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ABSTRACT

We describe a method for expanding the linear dynamic range for multiple reaction monitoring (MRM) in quantitative liquid chromatography/tandem mass spectrometry (LC–MS/MS) using additional transitions for isotopologues. In addition to the regular transition for the highest possible sensitivity, a transition corresponding to the less abundant isotopologue ions was utilized. This decreases saturation at the ion detector; the sensitivity reduction increases the upper dynamic limit. We demonstrated this for a rat plasma assay for a candidate flavor compound; the linear dynamic range increased by an order of magnitude from 3 to 6000 ng/mL with the regular MRM alone to 3–60,000 ng/mL using additionally the isotopologue transition.

1. Introduction

A linear calibration curve is often desired in quantitative analysis and may even be required by official compliance guidelines [1]; liquid chromatography–tandem mass spectrometry (LC–MS/MS), the analytical technique most often used in biomedical analysis, is no exception. Present mass spectrometers offer at least 3 orders of magnitude linear dynamic range, extending up to 5 orders of magnitude, depending on the analyte and the instrument, adequate for the vast majority of applications. However, in some cases, notably pharmacokinetic studies, following an analyte over an even larger concentration span is often desirable. Some samples then require dilution and reanalysis to stay within the linear range. Not only is this time consuming and expensive, insufficient sample availability in small animal studies sometimes makes reanalysis impossible [2].

Multiple reaction monitoring (MRM) in MS/MS uses two stages of mass filtering/selection with fragmentation occurring between the stages. While some other types of mass spectrometers also permit MS/MS analysis, a triple–quadrupole MS is the most commonly used instrument for MRM. A precursor ion is selected in the first quadrupole, fragmented in the second quadrupole, typically by collisional excitation with a neutral gas and a specific fragment ion is then selected by the third quadrupole for detection. The analyte identification and quantitation is thus determined by following the precursor-to-product ion transition rather than the precursor ion itself. The high specificity, sensitivity, precision, and multiplexing capability of the MRM method has made it the analytical tool of choice for the quantification of molecules ranging up to several hundred Thompsons (Th, m/z unit) in many application fields. The linear dynamic range (LDR) can be limited by saturation either at the ion source or at the detector. With an electrospray ionization (ESI) source, perhaps the most commonly used, saturation can be due to space and/or charge limitations [3]. Tang et al. [4] studied the expansion of the LDR for ESI sources both theoretically and experimentally. They pointed out the possibilities for extending the dynamic range using very low flow rates and/or multiple electrospray sources.

A stable isotope labeled internal standard can correct for nonlinear response whether due to the saturation of the ion source or detector to a significant degree, since both the tracer and the analyte behave similarly. Such a tracer can also correct for matrix effect or dilution errors, and is therefore highly desired in general in MS analysis. Unfortunately, an isotopically labeled tracer is rarely available for a new experimental compound until late development stage. Time and cost for in-house custom synthesis is high. Farming out synthesis of an undisclosed promising compound to outside vendors who specialize in such labeled product synthesis is considered risky. Both contribute to the unavailability of a

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labeled tracer. Co-eluting or closely eluting structural analogs (perhaps even a related candidate compound) can sometimes be used as internal standards for expanded LDR [5] but this is not likely to be universally applicable.

Improvements in MS detectors have extended the ion detector saturation limit and have thus increased the LDR [6]. Curtis et al. [2] reported on a different strategy of expanding LDR: multiple product ions that are generated with different abundance are monitored. We report herein a different generally applicable strategy that relies on the fact that almost all synthetic compounds of interest contain one or more (typically all) of the elements C, H, N, and O. Further, no compound is made in an isotopically pure form. Made from starting reagents with isotopic compositions of natural abundance, the product molecule will also have an isotopic composition close to that dictated by natural abundance, subject to kinetic isotope effects playing a role in certain syntheses. Consider the popular β-blocker pharmaceutical propranolol, $C_{16}H_{21}NO_2$. The primary precursor ion $(M+H)^+$ is composed of the ^{12}C, ^{1}H, ^{14}N and ^{16}O isotopes and has an exact mass of 260.1645 that shows up at 260.17 or 260.2 Th (depending on instrument resolution) in a typical well-calibrated tandem mass spectrometer. The transition most commonly monitored is that to $C_6H_{14}NO^+$, exact mass 116.1070 that shows up at 116.11 (or 116.1) Th. The 1 Th higher isotopologue of the primary precursor ion at 261.17 Th has an abundance of 17.6% [7] because of a presence of a ^{13}C, ^{2}H, ^{17}O or ^{15}N atom (most commonly due to ^{13}C because of its much larger relative abundance). A similar value for the 261.17 Th response, given a molecular formula of $C_{16}H_{22}NO_2$, are predicted by other sources, e.g., a web-based calculator [8] albeit such calculators can generally only assume an uncharged molecule. The same fragmentation path for the 261.17 Th isotopologue then produces a fragment ion at 117.11 Th, with a 6.7% abundance relative to the 116.11 Th ion from the 260.17 Th species. (Note that the daughter isotopologue ion abundance is much less than that of the parent because of the smaller number of atoms in the fragment and a proportionally reduced probability of a heavier isotope substitution.) Obviously, were we to monitor the 261.17 → 117.11 Th transition rather than the 260.17 → 116.11 Th transition, it would be proportionally less sensitive. If we pick an isotopologue transition that is 2 Th higher, the ion abundance will be even lower. We thus propose and elucidate in this paper a method to substantially increase the upper limit of the LDR in MRM-based tandem mass spectrometry by choosing a less abundant isotopologue transition and refer to this as isotopologue-MRM (abbreviated *i*MRM hereinafter) method; it is understood that *i*MRM includes the regular MRM transition. Implementing *i*MRM does not require any additional tracer or special ion sources or adjustment of flow rates.

We demonstrate *i*MRM for a proprietary flavor compound X, for which we normally monitor the 344.2 → 136.1 Th transition, here we additionally use the 345.2 → 137.1 Th transition in a rat plasma sample matrix. From an LDR of 3.0–6000 ng/mL for the regular transition, *i*MRM easily extends the upper limit of the LDR by an order of magnitude.

2. Experimental

2.1. Materials

Compound X (monoisotopic mass 343.2 Da) and the internal standard (IS), an analog of X, were synthesized in-house. All quantitation was performed with reference to the internal standard. Formic acid (www.sial.com), acetonitrile and ethanol (HPLC grade, www.fishersci.com) were obtained as indicated. Deionized water was produced in-house (PureLab Ultra, www.water.siemens.com). The analyte stock standard was prepared at the 100 μg/mL level in 0.02% $HCOOH_{aq}$. The IS stock was prepared at 100 μg/mL in 0.02% HCOOH in ethanol.

Using 0.02% $HCOOH_{aq}$ as diluent, calibration standards were prepared at 0, 3.0, 6.0, 15.0, 30.0, 60.0, 150.0, 300 and 600 ng/mL and1.5, 3.0, 6.0 15,30, 60 and 150 μg/mL. The working IS solution (100 ng/mL) was prepared using 0.02% HCOOH in ethanol as diluent. Rat plasma samples were from an in-house preliminary pharmacokinetics (PK) study of X with a single oral dose level of 100 mg/kg body weight.

2.2. Sample preparation

For calibration standards, 20 μL of the standard solution was mixed with 20 μL of blank plasma. For rat plasma samples, to 20 μL of the plasma 20 μL of 0.02% aqueous HCOOH was added. The standards and samples thus prepared above were then mixed with 160 μL of the working IS, vortexed for 15 s, refrigerated at ∼4 °C for ≥2 h, and centrifuged (15,000 × *g*, 15 min). Aliquots (100 μL) of the supernatant were then transferred to a 96-well plate and mixed with 300 μL of 0.02% $HCOOH_{aq}$. To validate performance at the low end and demonstrate the feasibility of avoiding sample dilution through *i*MRM, an additional set were prepared by diluting the above samples 100× with 0.02% $HCOOH_{aq}$.

2.3. Instrument and operating conditions

The LC–MS/MS analyses were performed on an LC system (model 1100, www.agilent.com), equipped with a binary pump, an in-line mobile phase vacuum degasser and an autosampler maintained at 10 °C. The separation column (HALO RP-amide, www.mac-mod.com, 2.1 mm × 50 mm, 2.7 μm particles) effluent was monitored by a triple–quadrupole mass spectrometer with an ESI source (Q-Trap 3200, www.appliedbiosystems.com). The chromatographic conditions were as follows (solvent percentages are volume fractions, A = 0.1% $HCOOH_{aq}$; B = 0.1% HCOOH in CH_3CN, times are given in minutes); *t* = 0, 2% B; *t* = 0.5, 2% B; *t* = 4, 40% B; *t* = 5, 100% B; *t* = 10, 100% B; *t* = 10.1, 2% B. The flow rate was 200 μL/min. Compound X with a retention time of ∼3.4 min was measured using the transitions 344.2 → 136.1 (regular MRM transition) and in addition 345.2 → 137.1 Th, and both were ratioed to the IS with a retention time of ∼4.7 min, which was measured using the transition 342.2 → 135.1 Th. The injection volume was 20 μL. The other instrumental parameters used for MS/MS detection were: TEM = 600 °C; CAD = Medium; IS = 4500 V; DP = 41 V; EP = 4.5 V; CE = 37 V; Dwell = 150 ms.

3. Results and discussion

A prior study with a close analog of X was already suggestive that the initial plasma concentration of X would exceed the upper limit of the linear calibration range of the regular MRM transition. Additional dilution would have solved this but uniform additional dilution will compromise the lower limit of quantitation. Doing different dilutions for different samples in the same study is acceptable in a research laboratory but tedious in a high throughput operation. This was the impetus for the present work. As the data for the regular MRM transition and the less sensitive isotopologue MRM transitions are acquired simultaneously in the *i*MRM method, the overall linear calibration range was expanded, as shown in the response factor plot in Fig. 1 [9]. For a satisfactory linear fit, we followed the recommended practice that $1/x^2$ weighted [10] linear correlation coefficient (*r*) must be ≥0.99 (see Supporting information, Figs. S1–S2) and the predicted concentration value for any standard must not deviate by >20% from the known value. The 344.2 → 136.1 Th transition met these criteria from 3.0 to 6000 ng/mL, and the 345.2 → 137.1 Th transition

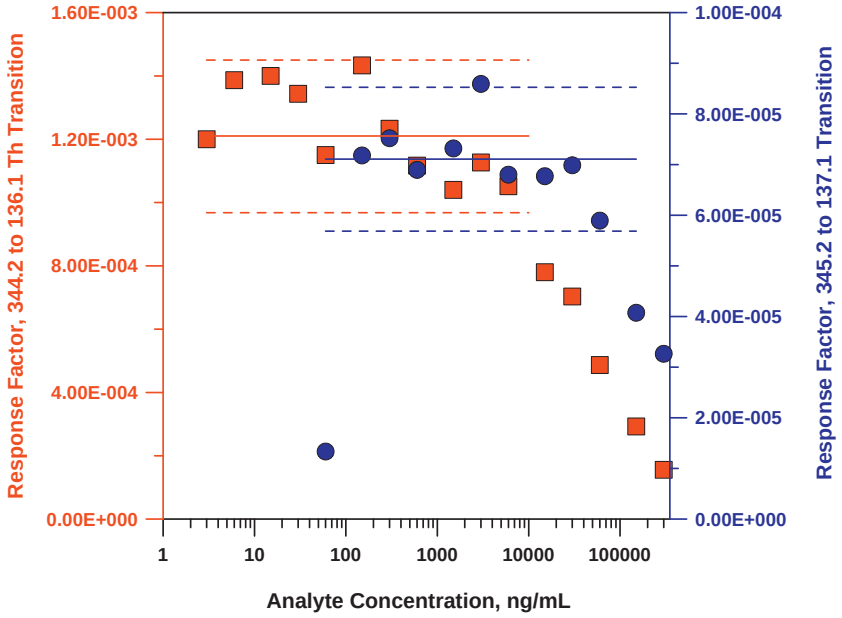

Fig. 1. Sensitivity plots for the transition 344.2 → 136.1 Th (left ordinate, squares) and the 345.2 → 137.1 transition (right ordinate, circles). The ±20% ranges are marked by the respective dashed lines.

from 0.15 to 60 μg/mL. The accuracy data for the calculated concentrations for the standards based on the different transitions are listed in Table 1. The upper limit of quantitation (ULOQ) was extended to 60 μg/mL for the less sensitive transition. Since compound X isotopologues are not chromatographically separated, they are ionized under essentially identical conditions; the reason for the reduced ULOQs for the more sensitive transition is detector and not source saturation. This demonstrates the capability of the *i*MRM approach to identify the limiting factor for signal saturation. If source saturation is the problem, other options, e.g., operating the source with a lower flow, will be warranted to expand the LDR. The benefits of the *i*MRM approach to expand the LDR are expected to be most pronounced for systems prone to ion detector saturation.

Table 2 shows the results for the plasma samples. The results in columns 2–4 were from 100× diluted samples (relative to the data in columns 5–7) and utilize the regular transition; the data are corrected for the dilution. For the undiluted samples, all but one (rat 3 at 4 h) exceeded the upper LDR limit for the 344.2 → 136.1 Th transition and dilution must be made. The agreement between the results from the undiluted samples using the 345.2 → 137.1 Th transition and the diluted samples using the 344.2 → 136.1 Th transition, best holistically seen in Fig. 2, demonstrates the feasibility of obviating any need for dilution by simply acquiring additional data for the 345.2 → 137.1 Th transition. While it may seem that collecting the regular transition data was superfluous in this case, the results were not of course known *a priori*. Further, in a real typical single dose PK study, the plasma sample collection will continue until the compound is nearly eliminated from the system, requiring a lower limit of quantitation (LLOQ) as low as possible, and thus needing the most sensitive transition. In practice, the *i*MRM approach does not involve additional analysis time, making it attractive in any situation where different degrees of dilution are potentially needed to measure a full sample set. However, following *n* transitions reduces the dwell time on each transition proportionally. In principle, this will deteriorate the LLOQ by $\sqrt{n}$. The gain in the ULOQ must be

Table 1
Accuracy for the calculated concentrations for the standards based on different transitions.

Concentration, ng/mL	Accuracy for *m/z* 344.2/136.1 based calibration, %	Accuracy for *m/z* 345.2/137.1 based calibration, %
3	99.8	
6	91.3	
15	118	
30	108	
60	94.4	
150	120	98.6
300	104	103
600	93.9	94.6
1500	87.3	101
3000	94.9	118
6000	88.7	116
15,000		92.6
30,000		95.8
60,000		80.7

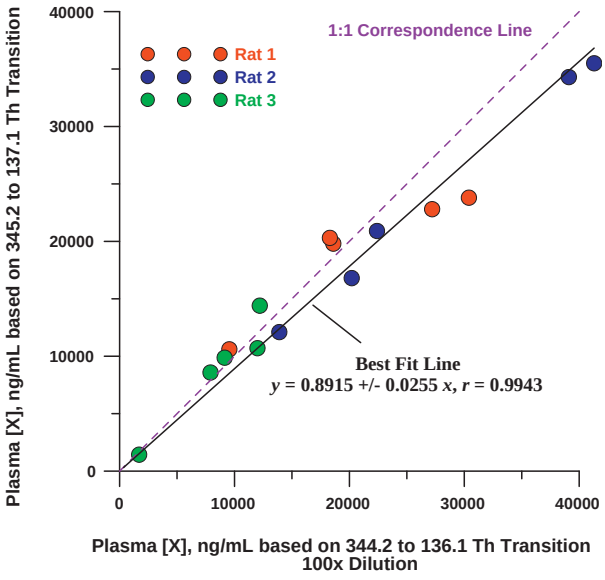

Fig. 2. The correlation between results from 344.2 to 136.1 Th transition (100 × diluted samples) and those from 345.2 to 137.1 Th transition.

Table 2
Comparison of the results based on different transitions.

Time, h	Concentrations from 100× diluted samples based on 344.2/136.1 transition, ng/mL			Concentrations from undiluted samples based on 345.2/137.1 transition, ng/mL			Relative difference between the concentrations from the diluted and undiluted samples, %		
	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3
0.25	18,600	13,900	12,000	19,800	12,100	10,700	6.3	−13.8	−11.5
0.5	27,200	22,400	12,200	22,800	20,900	14,400	−17.6	−6.9	16.5
1	30,400	39,100	9140	23,800	34,300	9870	−24.4	−13.1	7.7
2	18,300	41,300	7910	20,300	35,500	8580	10.4	−15.1	8.1
4	9550	20,200	1700	10,600	16,800	1430	10.4	−18.4	−17.3

larger than this to have a net gain in the LDR. In a typical *i*MRM method for a single analyte, the number of transitions will be three (one for the IS, and two for the analyte, instead of one for the analyte in the corresponding regular MRM method), and thus the LLOQ will deteriorate by a factor of $\sqrt{(3/2)} = 1.22$.

Because the isotope abundances are intrinsic characteristics of a compound, the sensitivity for the MRMs corresponding to the isotopes can be estimated *a priori* and thus appropriate MRM transitions can be selected according to the desired dynamic response range. In an *i*MRM method, all the MRM transitions share the same fragmentation pathway and a prior study of different product ions that have different fractional yields [2] (this cannot be predicted *a priori* and will likely depend on the collision energy as well) is not required. There are a few parameters in an MRM method such as Declustering Potential (DP) and Collision Energy (CE) that affect the ion current or the sensitivity. However, the appropriate settings for the desired sensitivity need to be established beforehand and likely can only be applied on a specific mass spectrometer. In contrast, the sensitivity of the extra transition in *i*MRM is predictable and virtually independent of the mass spectrometer used, making it more straightforward to expand the LDR of an existing MRM method.

Some caveats are necessary, however. The *i*MRM approach is only applicable when the LDR is limited by detector saturation, particularly common in pulse counting ion detection techniques due to pulse pileup effects where multiple ion impact at high incidence rates cannot be distinguished [6]. Second, when an isotopically labeled analog of the analyte is used as an IS, it must be labeled to an extent that it will not contain any parent ions used for the *i*MRM quantitation; this condition generally will not be difficult to meet. Third, the *i*MRM approach must be validated if rather than the parent compound, it is desired to monitor a particular metabolite. Depending on where the isotopic substitution is in the isotopologues, different isotopologues of the parent compound may not be metabolized at the same rate [11].

While this discussion and our experience to date have been limited to the MRM scan mode, it is logical to believe that the concept will be applicable to other MS methodologies. There is much current interest in quantitative analysis in the selected ion monitoring (SIM) mode using high resolution mass spectrometers that offer high selectivity through narrow mass filtration windows [12,13]. Following SIMs for different isotopologues should be simple and straightforward, constituting an effective way to expand the LDR, we hope to report on such extensions in the near future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.09.063.

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