RESEARCH ARTICLE

Enhancement of sensitivity and quantification quality in the LC–MS/MS measurement of large biomolecules with sum of MRM (SMRM)

Liang Tang[1] · Robert R. Swezey[1] · Carol E. Green[1] · Jon C. Mirsalis[1]

Abstract

Liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) provides a simple and efficient means for the measurement of analytes in biological matrices with high selectivity and specificity. LC–MS/MS plays an important role in the pharmaceutical industry and biomedical research, but it requires analytes to be in an ionized form in order to be detected. This can pose a challenge for large molecules such as proteins and peptides, because they can exist in multiple charged forms, and this will reduce the total analyte signal by distributing it into multiple ion peaks with a different number of charges in a mass spectrum. In conventional LC–MS/MS analysis of such macromolecules, one charged form is selected as the precursor ion which is then fragmented by collision-induced dissociation (CID) in MS/MS to generate product ions, a process referred to as multiple-reaction monitoring (MRM). The MRM method minimizes interference from endogenous molecules within biological matrices that share the same molecular weight of the precursor ion, but at the expense of signal intensity as compared to precursor ion intensity. We describe here an approach to boost detection sensitivity and expand dynamic range in the quantitation of large molecules while maintaining analytical specificity using summation of MRM (SMRM) transitions and LC separation technique.

Keywords LC–MS/MS · Sum of MRM (SMRM) · Bioanalysis · Large molecules · Charge state distribution

Introduction

The introduction of electrospray ionization (ESI) into mass spectrometry was a milestone in analytical chemistry [1, 2]. The soft ionization principle of ESI [3] made it possible to analyze biomolecules in gas phase and provided an ideal interface to couple liquid chromatography (LC) to mass spectrometry (MS). Tandem mass spectrometry (MS/MS) techniques such as multiple-reaction monitoring (MRM) [4–6] further enhanced selectivity of the assay and thus simplified the sample preparation of biological matrices.

For quantifying protein/peptide drugs in biological matrices, both LC–MS/MS assays and ligand binding assays (LBAs) are powerful techniques [7–11] widely utilized in the biomedical research to analyze drugs, their metabolites, and biomarkers in biological matrices. LBA methods have historically been the most popular technique for quantifying peptides and proteins in biological samples. LBA methods have superior detection sensitivity and are sometimes the only technique to provide the required limit of quantification for large molecules in study. The advantages of LC–MS/MS include superior selectivity, specificity, assay precision, and accuracy. Furthermore, an LC–MS/MS method can be developed much faster than an LBA method, which typically requires months to raise antibodies. Thus, it would be desirable to increase LC–MS/MS sensitivity in the measurement of large molecules for high-throughput research and screening work on a large number of biomolecules.

When the ESI debut demonstrated the capability of studying large biomolecules by mass spectrometry [7], multiply charged peaks are a feature of electrospray mass spectrometry. Forming multiply charged ions from a large molecule provides a means to obtain structural information. However, the distribution of large-biomolecule analytes to multiple ion peaks decreases the detection sensitivity because individual ion peaks are measured. For a neat sample of the analyte, the total ion current (TIC) of all its multiply charged peaks can sum up all analyte ions to increase detection sensitivity.

✉ Liang Tang
Liang.Tang@sri.com

1   SRI Biosciences, SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025, USA

A TIC in LC–MS can help measure the analyte at lower concentration [12, 13]. Nevertheless, the TIC is unsuitable for the quantitative analysis of large molecules in complex samples such as biological samples because endogenous biomolecules can overwhelm the analyte at trace levels. Tandem mass spectrometry is needed to distinguish the analyte from impurities in the sample measurement [14, 15]. The MRM technique in LC–MS/MS can differentiate ions from molecules with the same molecular weight but reduces the intensity of precursor ions by producing various product ions. Using MRM in LC–MS/MS to enhance specificity usually relies on selecting one product ion for analysis, which further decreases the detection intensity of analyte ions. Compared to small molecules, most of which form singly charged molecular ions, large biomolecules suffer more in detection sensitivity when only one charge state among multiple-charged states is selected by MRM.

To improve MRM analyte measurements, researchers started to use more available MRM transitions for the analyte [16–22], that is sum of MRM (SMRM). SMRM intensifies analyte sensitivity by superimposing more ion signals from multiple MRM transitions. However, SMRM in the meantime increases background noise from the multiple MRM transitions [18]. For the measurement of small-molecule analytes, SMRM usually does not have significant advantage than MRM when an analyte only forms a singly charged molecular ion. SMRM could find applications to enhance sensitivity in the measurement of large biomolecules due to their feature of forming multiply charged ions [23–25]. In preclinical research and clinical development [26], large-molecule drugs and biomarkers are typically found at very low levels in biological samples, and thus, sensitivity of measurement is crucial to the successful analysis of the biological samples. The SMRM method is a logical approach to sensitivity enhancement even though the complex nature of the biological sample composition makes the SMRM method more sophisticated. We evaluated the feasibility of SMRM for the quantitative analysis of large molecules in biological samples.

From the perspective of analytical mass spectrometry, large-molecule SMRM is different from small-molecule SMRM. Since small molecules usually form ions of one charge state, small-molecule SMRM is often the sum of MRM from isotopologue ions. Large molecules tend to form multiply charged ions with ESI, so large-molecule SMRM is the sum of MRM of ions from the same molecules but different charges. Here, we demonstrate our approach to LC–MS/MS measurement of large biomolecules with SMRM. In our experiments, we increased the detection sensitivity of large molecules by SMRM with chromatographic separation of the background noise from the analyte signal. After we compared MRM with SMRM, we discovered that SMRM can counterbalance the change of the charge-state distribution with concentration to extend the dynamic range for the quantitative measurement of large molecules. Our approach allows the LC–MS/MS technology to utilize more precursors and product ions from a large-molecule analyte to boost the analyte sensitivity of the large molecules in biomatrices and obtain a wider linear dynamic range for faster quantitative measurement of samples with a wide concentration range of analyte.

Methods

Materials

Acetonitrile and methanol were purchased from Macron Fine Chemicals (Center Valley, PA). Formic acid, trifluoroacetic acid (TFA), teriparatide, ubiquitin, polymyxin B, and colistin sulfate were purchased from Sigma-Aldrich (St. Louis, MO). The molecular weight of the ubiquitin is 8565 Da, which was measured by the high-resolution MS method in 2.3.1 below and is consistent with the reported ubiquitin molecular weight [27]. Sprague Dawley rat serum and CD-1 mouse serum were purchased from BioChemed Services (Winchester, VA). Polymyxin B1, polymyxin B1-Il, and polymyxin B2 were purchased from TOKU-E Company (Bellingham, WA). Columns Acquity UPLC BEH C18 (1.7 μm, 50×2.1 mm), XBridge BEH Phenyl XP (2.5 μm, 50×4.6 mm), and Oasis PRiME HLB 1-cc solid-phase extraction (SPE) columns were purchased from Waters Corp. (Milford, MA).

Sample preparation for serum analysis

2.2.1. Sprague Dawley rat serum was spiked with polymyxin B1, polymyxin B1-Il, or polymyxin B2 and extracted by precipitating proteins in the serum with trichloroacetic acid (TCA). A 100-μL serum sample entailed the addition of 20 μL of internal standard solution (500 ng/mL colistin aqueous solution) and 120 μL of 5% (w/v) TCA aqueous solution [28]. The samples were vortexed for 5 min and centrifuged for 15 min at 18,000 g. The supernatants were transferred to new containers, and the solvent was removed under vacuum in a centrifugal evaporator. The dried residues were reconstituted with 100 μL of 60:40 (v:v) methanol:water, vortexed 3 min, and then clarified by centrifugation (18,000 g) for 15 min. Ten microliters of supernatant was injected into LC–MS/MS for quantitative analysis.

2.2.2. Teriparatide or ubiquitin were extracted from CD-1 mouse serum by SPE. A 100-μL serum sample entailed the addition of 20 μL of internal standard solution (2 μg/mL polymyxin B in water) and 0.5 mL of 0.1% TFA (v:v) aqueous solution. The Oasis PRiME HLB SPE columns were conditioned with 0.25 mL of 0.1% TFA in acetonitrile (ACN)

followed by 0.25 mL 0.1% TFA in water. Samples were then applied to the conditioned SPE columns. The SPE columns were washed twice with 0.5 mL of 0.1% TFA in water, and the analyte was eluted from the SPE column into clean containers by two applications of 0.5 mL of 0.1% TFA/50% ACN in water. The combined eluates were evaporated under vacuum in a centrifugal evaporator. The dried residues were reconstituted with 100 µL of 50:50 methanol:water (v:v) containing 0.1% formic acid, then vortexed for 5 min, and the samples were clarified by centrifugation (18,000 g) for 5 min. The clarified extracts were analyzed by LC–MS/MS.

Instruments

2.3.1. The API Sciex Triple TOF 5600 Plus™ (AB Sciex) was utilized for the accurate measurement of protein/peptide molecular weights and the distributions of multiply charged ions across a wide mass range. The ESI voltage was 5500 V and temperature was 550 °C; the GS1 and GS2 were 40, and curtain gas (CUR) was 30.

2.3.2. The Waters Acquity Ultra Performance LC System equipped with a Waters 2777 Sample Manager and a Waters Xevo TQ-S MS system with an ESI source were utilized for the quantitative LC–MS/MS analyses of peptides and protein extracted from biological matrices.

(I) The LC–MS/MS method for the separation and quantification of polymyxin B isomers (polymyxin B1, polymyxin B1-Il, polymyxin B2) utilized an Acquity UPLC BEH C18 column (1.7 µm, 50×2.1 mm). Mobile phases were 1% formic acid in water (A) and 1% formic acid in acetonitrile (B). Gradient elution of the column was performed by holding the mobile phase composition at 5% B for 1 min, followed by a gradient to 15% B in 3.2 min to separate the analytes. Elution at 15% B continued for 1.8 min and then increased to 90% B for 2 min to wash the column. The column was re-equilibrated for 2 min at 5% B. The flow rate was 400 µL/min, and the column was operated at 40 °C.

Electrospray ionization (positive ion mode) tandem mass spectrometry was used to detect polymyxin B isomers by MRM analysis. The ESI voltage and source temperature were 2.8 kV and 150 °C, respectively. The desolvation temperature was 640 °C. The SMRM measurements of multiply charged ions of analytes and their MRM profiles are detailed in "Sum of MRM (SMRM) for a large molecule." One MRM transition was used for internal standard, i.e., colistin (386 → 227). The internal standard does not need SMRM in a method [15] while the analyte takes advantage of SMRM to obtain higher sensitivity in the method. Cone voltages and collision energies were optimized to measure the analytes and internal standard.

(II) The LC–MS/MS method for teriparatide or ubiquitin utilized an XBridge BEH Phenyl XP (2.5 µm, 50×4.6 mm) column. Mobile phases were 15 mM TFA in water (A) and

15 mM TFA in acetonitrile (B). Gradient elution of the column was performed by holding the mobile phase composition at 5% B for 1 min, followed by a gradient to 95% B in 5 min to separate the analytes. Elution at 95% B continued for 2 min, and the columns were re-equilibrated for 3 min at 5% B. The flow rate was 400 µL/min, and the column temperature was 40 °C.

Detection of teriparatide or ubiquitin by MRM occurred in the positive ion mode. The ESI voltage and source temperature were 2.8 kV and 150 °C, respectively. The desolvation temperature was 640 °C. The multiply charged ions of analytes and their MRM transitions are detailed in "Sum of MRM (SMRM) for a large molecule" to be the SMRM signal. One MRM transition was used for the internal standard, i.e., polymyxin B (602 → 101). Cone voltages and collision energies were optimized to measure the analytes and internal standard.

Sum of MRM (SMRM) for a large molecule

Developing a SMRM method starts with setting up MRM methods for the various multiply charged precursor ions just as one would for a regular quantitative LC–MS/MS method. The essence of SMRM can be depicted by equations below:

$$SMRM = \sum MRM \tag{1}$$

$$= \sum_{i}^{m} \sum_{j}^{n} \left[MS(P_i)/MS(p_j) \right] \tag{2}$$

For a large molecule (M) analyzed in positive ESI, Eq. (2) becomes

$$\sum_{i}^{m} \sum_{j}^{n} \left[(M + iH)^{i+}/MS(p_j) \right] \tag{3}$$

where.

SMRM: sum of MRM
MRM: multiple-reaction monitoring transition of precursor ion to product ion (MS/MS)
P: precursor
p: product
$MS(P_i)$: precursor ion P_i
$MS(p_j)$: product ion p_j of P_i
M: analyte molecule
H: proton
i to m: number range of charge states of M
j to n: number of product ions (j = 1, 2,…n) from P_i

In a large-molecule SMRM method, all precursor ions are from the same analyte (M). The total number

of precursor ions P_i to P_m is determined by the chemical properties of analyte and experimental conditions, which will be discussed in "SMRM provides a wider dynamic range for the LC–MS/MS method." In practice, precursors with low ion intensities would be empirically excluded because of their low contributions to the SMRM signal. The availability of product ions p_j of P_i ($j = 1, 2, \dots n$) depends on P_i characteristics and CID conditions. The number n varies among P_i and P_m. The product ions interfered by external ions generated from endogenous biomolecules in the biological sample should be excluded. Then, all the MRM methods of major product ions that do not have interference from the biological matrix are combined in a SMRM method by integrating the TIC at the retention time for the analyte of interest. We generated the TIC with the quantitation software equipped on the LC–MS/MS instrument (MassLynx 4.2 SCN986) by summation of the individual MRM chromatograms. The principle of this process is illustrated in Fig. 1. The SMRM compositions

and major mass spectrometric parameters for the peptides and proteins described in the next section are summarized in Tables 1, 2, 3, and 4.

Table 1 The MRM transitions of protonated polymyxin B1 $(PMB1 + nH)^{n+}$ or isomer polymyxin B1-Il $(PMB1\text{-}Il + nH)^{n+}$ and their major MRM parameters utilized for SMRM (Dwell time = 0.02 s for each channel)

Proton number on precursor ion	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (V)
2	602.2	241.1	11	21
2	602.2	202.2	11	22
3	402.0	552.3	20	10
3	402.0	482.2	20	10
3	402.0	241.0	20	11
4	301.7	349.4	31	6

Fig. 1 Illustration of building an SMRM method from MRM methods for polymyxin B1. Six MRM transitions (i to vi) from polymyxin B1 multiply charged ions form the total ion current (TIC) of SMRM
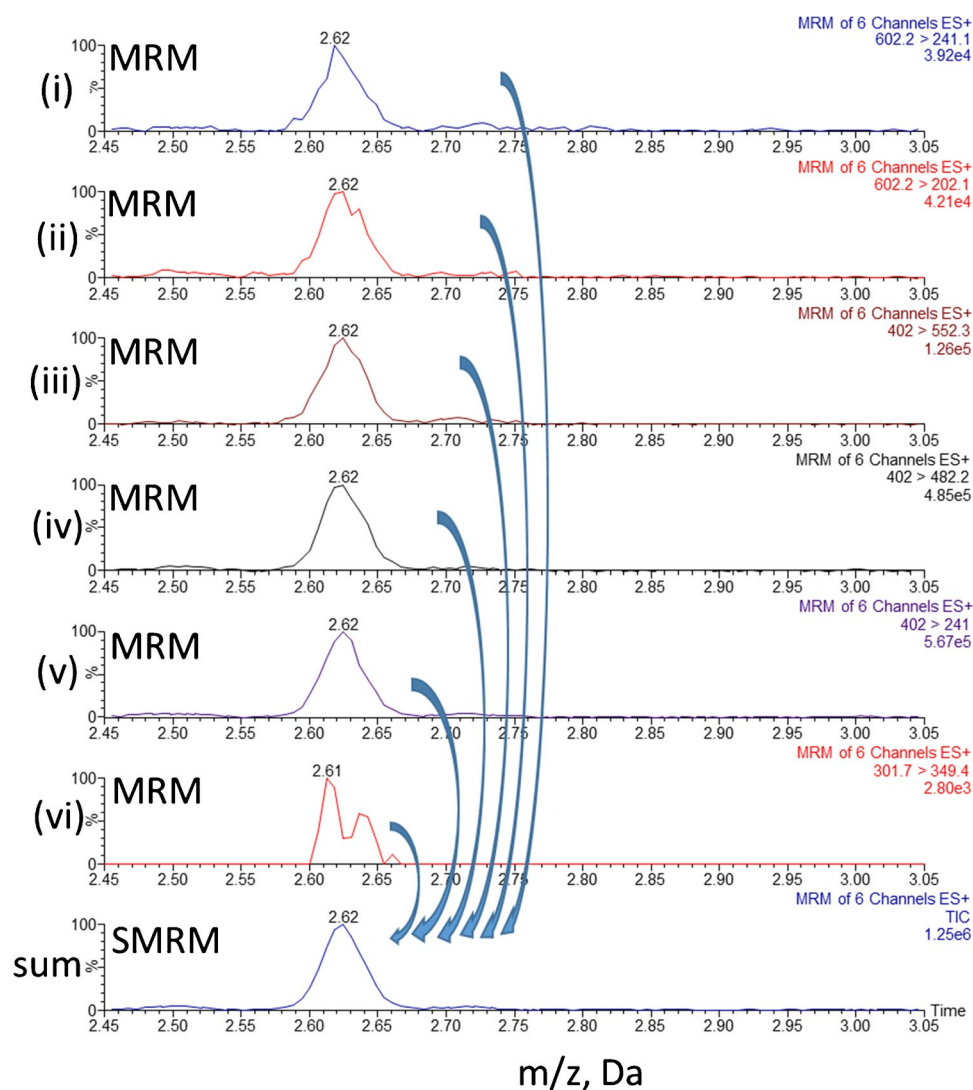
Table 2 The MRM transitions of protonated polymyxin B2 (PMB2 + nH)$^{n+}$ and their major MRM parameters utilized for SMRM (dwell time = 0.02 s for each channel)

Proton number on precursor ion	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (V)
2	595.2	545.2	12	16
2	595.2	482.2	12	16
2	595.2	226.9	12	21
2	595.2	202.0	12	22
3	397.3	240.7	19	26
4	298.1	473.7	29	9
4	298.1	344.5	29	9

Table 3 The MRM transitions of protonated teriparatide (teriparatide + nH)$^{n+}$ and their major MRM parameters utilized for SMRM (dwell time = 0.02 s for each channel)

Proton number on precursor ion	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (V)
3	1373.3	1801.7	60	48
3	1373.3	1737.6	60	52
3	1373.3	1680.6	60	52
3	1373.3	1615.0	60	52
4	1030.3	1311.3	60	33
4	1030.3	1201.3	60	32
4	1030.3	1077.3	60	34
5	824.1	963.2	78	25
5	824.1	900.8	78	25
5	824.1	786.7	78	25
6	686.9	786.7	78	18

Table 4 The MRM transitions of protonated ubiquitin (ubiquitin + nH)$^{n+}$ and their major MRM parameters utilized for SMRM (dwell time = 0.02 s for each channel)

Proton number on precursor ion	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (V)
7	1224.5	1307.1	30	24
8	1071.2	1307.1	30	32
8	1071.2	1141.8	30	34
8	1071.2	1089.2	30	29
8	1071.2	260.1	30	40
9	952.6	1141.9	30	31
9	952.6	1089.6	30	27
9	952.6	933.9	30	31
9	952.6	913.8	30	28
10	857.4	933.9	30	20
10	857.4	817.3	30	19
11	779.5	817.3	30	17
11	779.5	726.5	30	16
12	714.7	817.3	30	14
12	714.7	726.5	30	14

one product ion in an MRM method makes use of just a portion of total ions produced by CID. Utilizing more ions from a large-molecule analyte is an approach to improve sensitivity for measurement of the analyte. In this report, we demonstrate our method to implement SMRM in the analysis of large biomolecules in complex matrices.

SMRM enhances analyte detection sensitivity

The more sensitive a bioanalytical method is, the more meaningful the data; this is particularly true when measuring drugs administered at very low levels, as in preclinical research and clinical trials [26]. Typically, for small-molecule analytes, a single and optimal MRM transition of its singly charged molecular ion provides sufficient sensitivity for the analyses. However, for a large-molecule analyte, a conventional LC–MS/MS method with a single MRM value from a specific charged ion species has limited sensitivity. A SMRM method is superior to each of its composing MRM methods because it provides higher sensitivity for the LC–MS/MS measurement of a large molecule; however, SMRM can accumulate impurity ions with the same MRMs as well as the product ions from the analyte. Therefore, for the SMRM method to achieve a lower detection limit, it is necessary to separate noise ion peaks of impurities, i.e., endogenous biomolecules, from analyte peaks. In our experiments, the chromatography in the LC–MS/MS method played an important role in separating trace impurities that remained in analytical
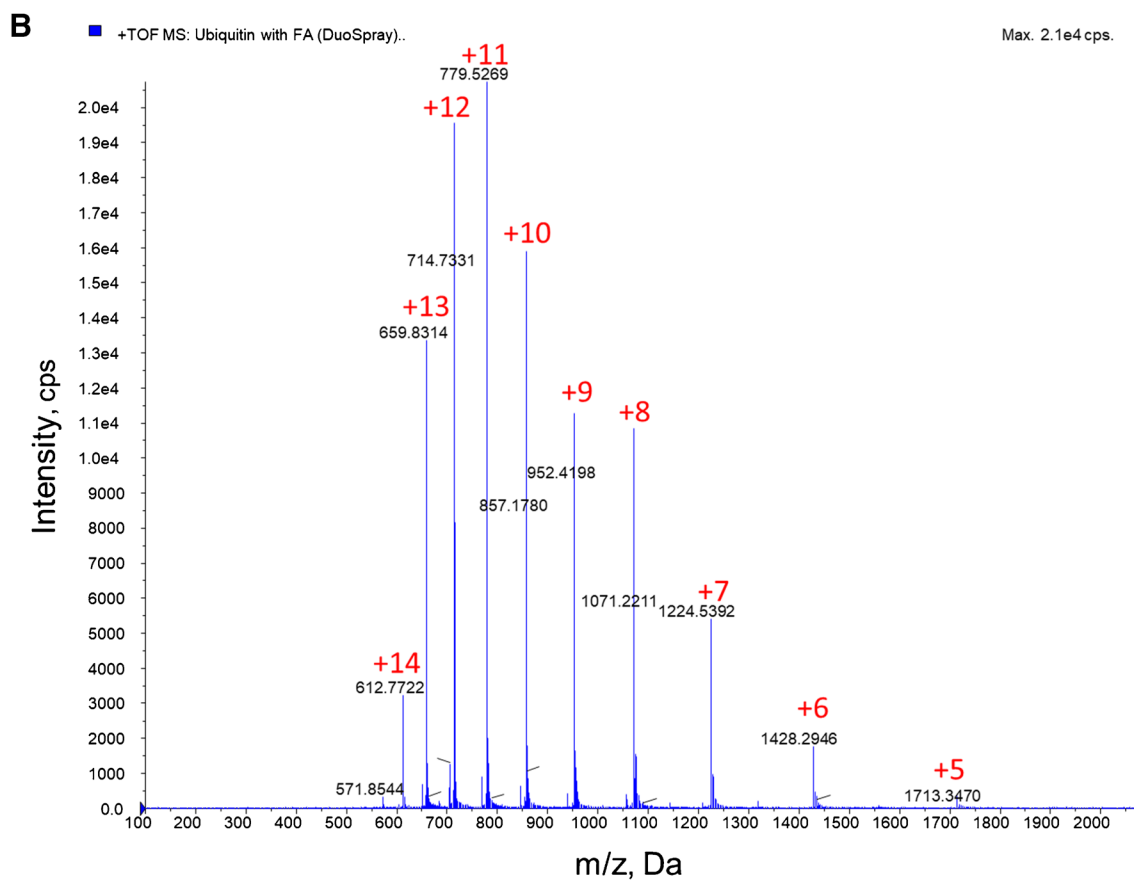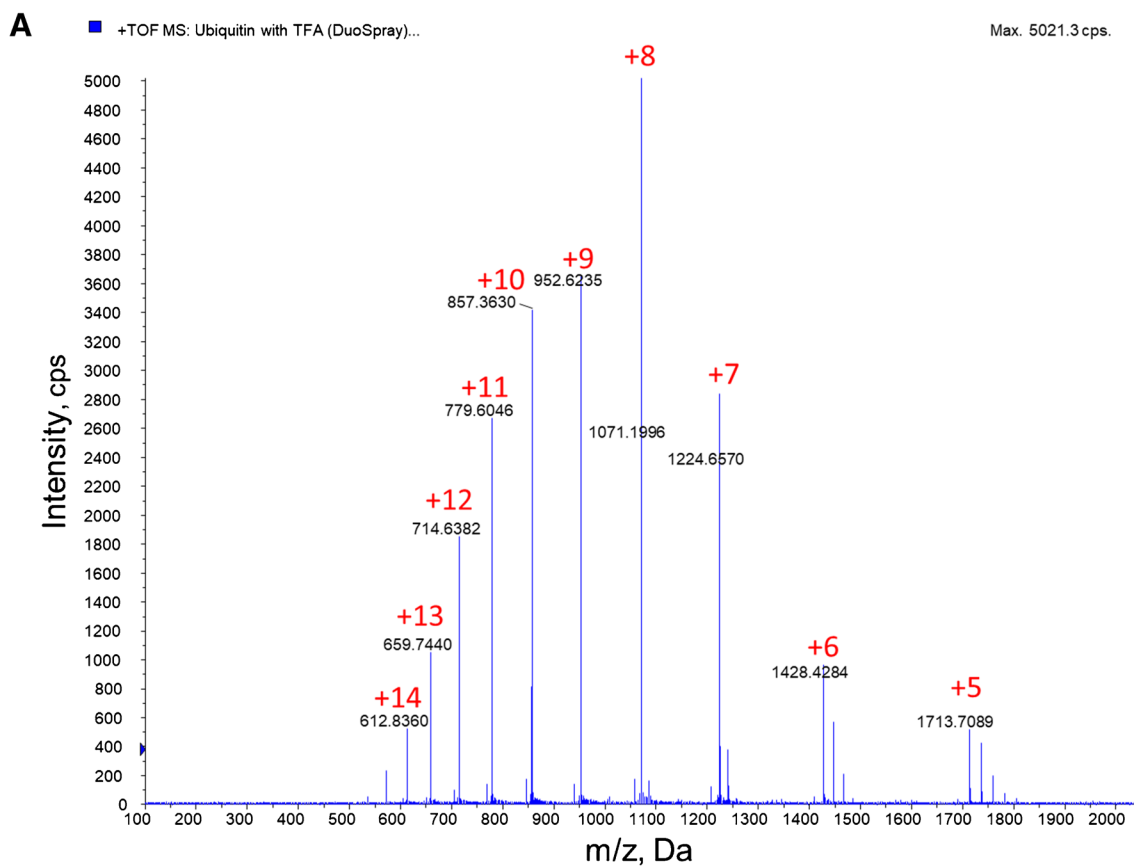
Results and discussion

When using ESI–MS to study proteins and peptides, analytes often produce multiple ion peaks with different numbers of charges [27–30]. An example is given in Fig. 2, which shows the mass spectrum of ubiquitin contains multiple peaks. The distribution of peaks can change with solution conditions like pH and the additives in solutions.

Multiple ion peaks from a large molecule can provide information about its structure [24, 27, 29], but the distribution of analyte molecules into differently charged ion species reduces ion peak signal intensities as compared to those of the analyte in just one charged state. Therefore, selecting only the most prominent charged state as the precursor ion in an MRM method will exclude potential contributions from the other charged forms in quantitative analysis. Likewise, selecting only

A

■ +TOF MS: Ubiquitin with TFA (DuoSpray)...   Max. 5021.3 cps.

B

■ +TOF MS: Ubiquitin with FA (DuoSpray)..   Max. 2.1e4 cps.

◀**Fig. 2** Mass spectra of ubiquitin from a high-resolution mass spectrometer (QTOF). Ubiquitin forms multiply charged ions by electrospray ionization. Charge distribution is related to the compositions in solution. The most abundant ion peak of multiply charged ions varies with the additives in solutions. **A** 500 µg/mL ubiquitin in 0.1:50:50 (v/v/v) TFA:water:acetonitrile. The ion peak of ubiquitin with 8 protons (m/z = 1071) has the highest abundance. **B** 500 µg/mL ubiquitin in 0.1:50:50 (v/v/v) FA:water:acetonitrile. The ion peak of ubiquitin with 11 protons (m/z = 779.5) has the highest abundance. Flow rate = 0.4 mL/min

samples following the extraction of the analyte from biological samples so that more MRM transitions could be utilized in SMRM. Figure 3 shows chromatograms of a double-blank extract (lacking the analyte and internal standard), a blank sample extract (lacking the analyte but containing the internal standard), and a lower limit of quantitation (LLOQ) extract for teriparatide. There is no significant peak in the blank extract at the retention time for teriparatide that arises from the summation of the individual MRM chromatograms. The impurities contributing to the major background noise were separated from the teriparatide, so chromatographic separation made it possible to achieve a lower detection limit in LC–MS/MS analysis using SMRM. Another example to show SMRM capability in sensitivity enhancement is illustrated with the SMRM of teriparatide at a higher concentration in Fig. 4; the SMRM chromatography (TIC, bottom chromatogram) has approximately fourfold higher signal intensity than the most sensitive single MRM chromatography (top chromatogram) of the teriparatide in the experiment. Figure 4 also shows that the impurity mainly affects the detection limit rather than the high concentration measurement of the analyte.

The SMRM method has another advantage over MRM for measuring an analyte at high concentration. Conventional MS detectors are prone to saturation effects when measuring analytes at high concentration. A more sensitive method has to measure analytes at lower concentration before saturation effects occur. In the past, researchers either diluted samples or employed a range of detuning strategies for the MS to measure high concentrations [32], but these affect sensitivity at the detection limit. Since an SMRM ion signal is composed of multiple MRM ion signals, the SMRM method obtains a stronger total ion signal at high concentration without sacrificing MS sensitivity. The SMRM method boosts the detection signal at low analyte concentration and is less prone to saturation by a merged strong signal at high analyte concentration. Therefore, the SMRM method is conducive to a wider dynamic range in quantitative analysis of large molecules when used with LC–MS/MS. The importance of a wider dynamic range will be discussed further in "SMRM provides a wider dynamic range for the LC–MS/MS method."

The SMRM method does not change the properties of analytes. For example, polymyxin B isomers have different sensitivities in LC–MS/MS. Polymyxin B1 was found to have higher sensitivity among polymyxin B isomers in LC–MS/MS analyses [33, 34]. In our study, the trend of sensitivity difference among polymyxin B isomers still existed with SMRM methods although SMRM increased their sensitivities as compared to each of their MRM methods. All polymyxin B isomers (PMB1, PMB1-Il, PMB2) had an LLOQ of 1 ng/mL by SMRM. The PMB1 has better intrinsic sensitivity than the other two isomers and can be measured at a lower concentration (≤ 0.1 ng/mL) with SMRM. In Fig. 5, the signal-to-noise ratio (*S/N*) of PMB1 at a plasma concentration of 0.1 ng/mL is > 25, which exceeds the Food and Drug Administration (FDA) acceptance criteria for LLOQ [35]; the analyte response at the LLOQ should have a *S/N* of ≥ 10.

SMRM provides a wider dynamic range for the LC–MS/MS method

A wide dynamic range is a desirable quality of an analytical mass spectrometric method. Achieving such a wide dynamic range for a bioanalytical assay is crucial to rapid development of a new drug [36, 37]. For instance, safety assessments involve measuring drug exposures at both low and high levels [38]. Therefore, in addition to sensitivity and precision, a wide dynamic range is beneficial for a quantitative LC–MS/MS method. Our results suggest a wider linear dynamic range can be achieved by using SMRM as compared to MRM for large-molecule compounds.

Among the peptides and protein analyzed in this study, a trend was discovered; that is, the relative abundance of multiply charged ions of an analyte was related to the concentration of the analytes. As the solution concentration increased, the relative abundance of low-charge-state ions decreased, and the relative abundance of high-charge-state ions increased. Figure 6 shows this charge conveyance phenomenon in large-molecule LC–MS/MS. In Fig. 6A, $(PMB1+4H)^{4+}$ ions of polymyxin B1 are almost undetectable at low concentration but provide an obvious contribution to the total ion current at high concentration; correspondingly, there are fewer ions of lower-charge state $(PMB1+2H)^{2+}$. This trend is also seen for polymyxin B isomers B1-Il and B2 in Figs. 6B and C, respectively. In some cases, the most abundant ion peak observed can change with an increase of concentration, as seen for teriparatide (Fig. 6D) and ubiquitin (Fig. 6E). This charge conveyance phenomenon can result in a concave or convex calibration curve that can limit the dynamic range of an LC–MS/MS method if an unsuitable MRM transition is selected for the quantification of a large-molecule
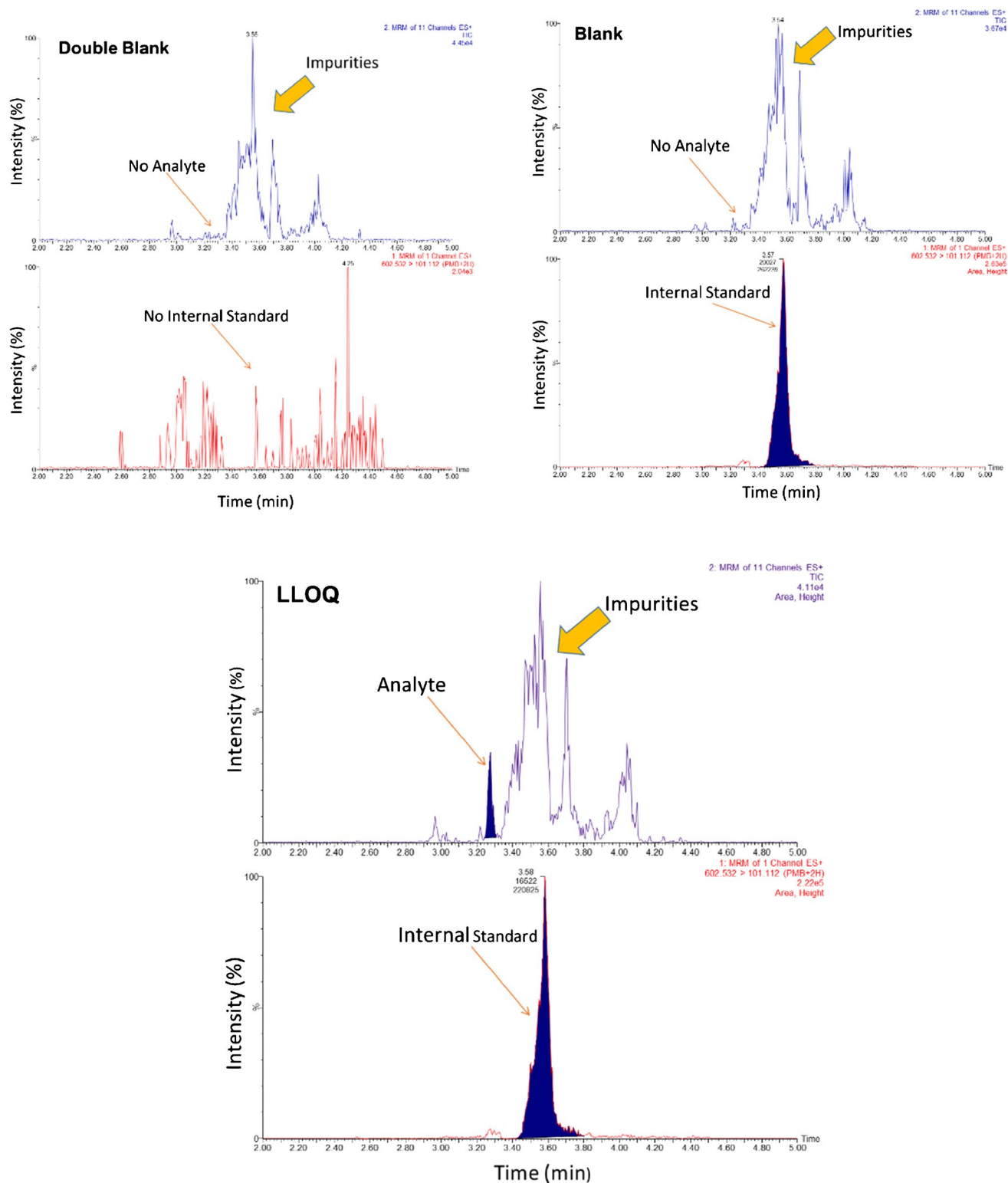
Fig. 3 The SMRM method achieves higher detection sensitivity for analysis of teriparatide by separating the analyte from endogenous impurities. Chromatograms of a double-blank extract (top left), a blank sample extract (top right), and a LLOQ [5 ng/mL] extract (bottom) for analyte teriparatide
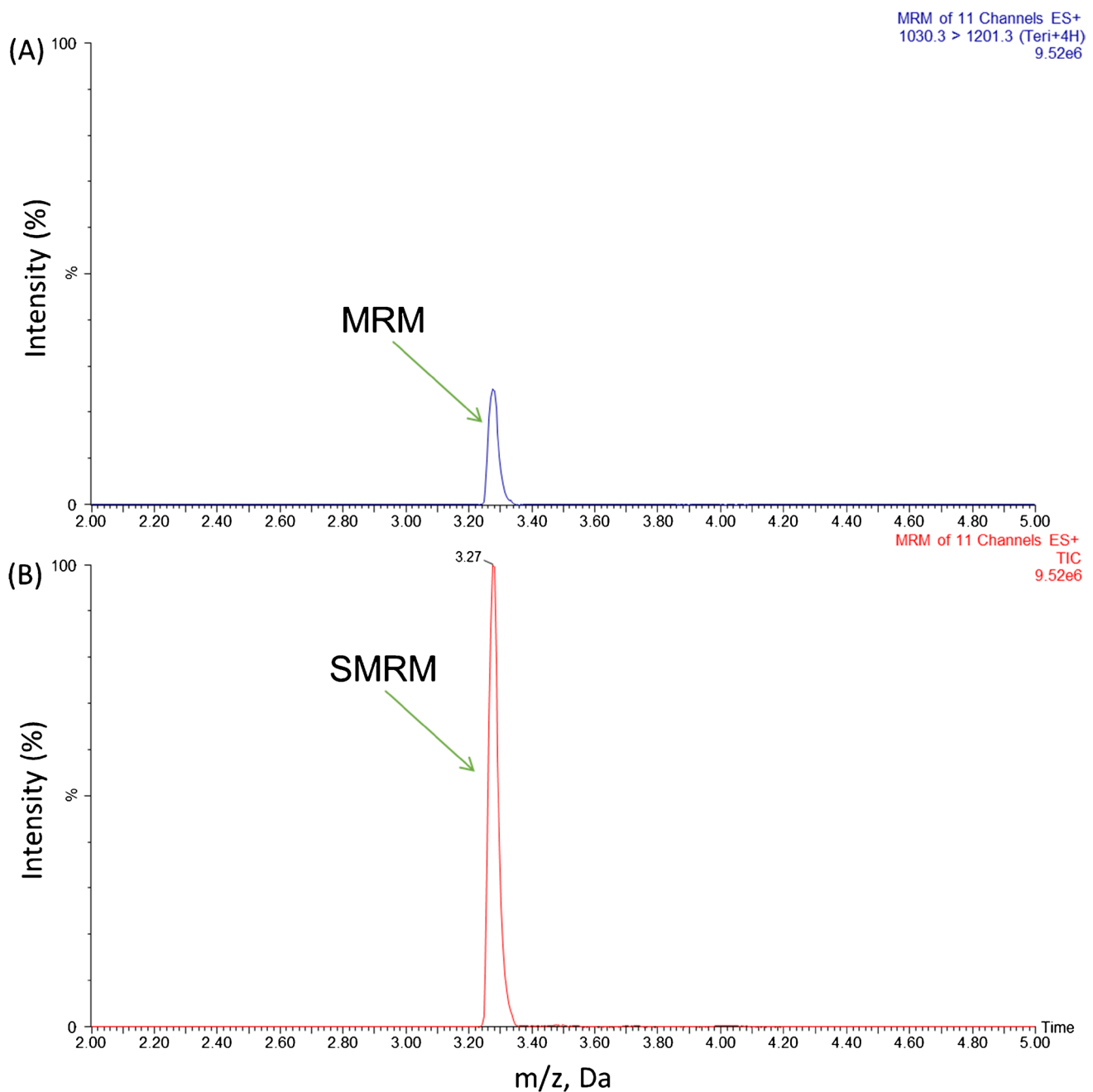
Fig. 4 The SMRM method provides higher detection sensitivity than MRM in the measurement of large-molecule analyte by LC–MS/MS. A comparison of the teriparatide ion signal between MRM and SMRM at a concentration of 5000 ng/mL: **A** the highest MRM ion signal is from (teriparatide + 4H)$^{4+}$ (m/z 1030.3 ->1201.3) and **B** the SMRM ion signal is more than fourfold higher than the highest single MRM signal

compound. The effect of charge conveyance on the distortion of the calibration curve is demonstrated in Fig. 7 with three MRM transitions of polymyxin B1. Using SMRM can correct for this limitation of a single MRM by summing ions from various charge states. As shown in Fig. 8, SMRM provided wide linear calibration curves for analytes ranging from peptides of different sizes to protein.

Therefore, SMRM data is more representative of the total analyte concentration for macromolecules than data from MRM methods that rely on measures of individual multiple-charged ion species.

The mechanism of the charge conveyance in the abundance of multiply charged ions of the large-molecule analyte shown in Fig. 6 can be rationalized by the

Fig. 5 The SMRM method enhances the analyte signal in LC–MS/MS measurement. Polymyxin B1 can be measured at 100 pg/mL with a *S/N* > 25
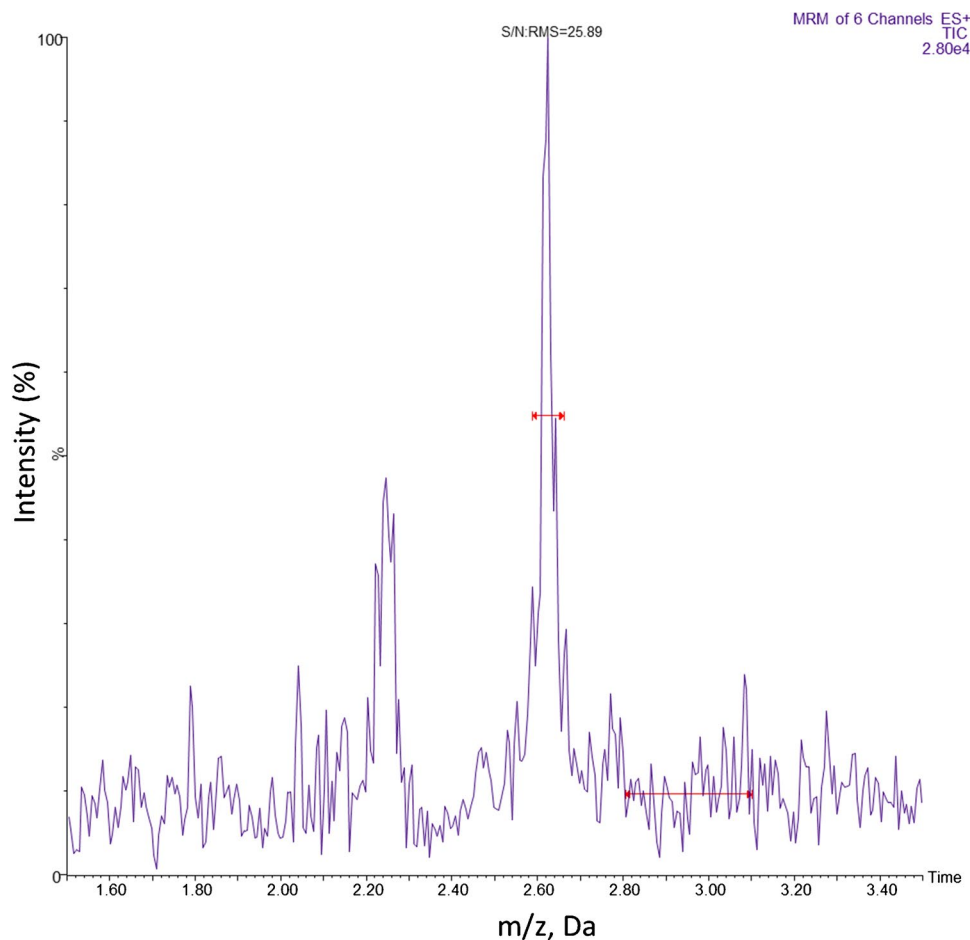
charge-state distribution (CSD) of the large molecule in ESI. The CSD phenomenon has been extensively studied [29, 30, 39–48]. For instance, the chemical properties of large biomolecules, their solution chemistry, and experimental conditions can affect CSD [29, 39–43], and the CSD can even be modified using special techniques [44–47]. In our experiments, concentration was a variable and other experimental conditions were kept constant in a batch analysis. The effect of concentration on CSD has been studied by other researchers; for example, Cole and Wang predicted that increasing concentrations of analyte increase the degree of protonation in solution phase [39]. Our experimental results are in line with their prediction. Figure 6 illustrates higher average CSD at higher analyte concentration. Contrary observations have been reported [30, 31, 44]; these experiments involved change in electrospray conditions such as the change of the ratio of the number of excess charges to the analyte molecules in droplets. In our LC–MS/MS experiments, mobile phase solutions were acidified to provide sufficient protons and a high ESI current provided sufficient charges for the

analyte. Our experimental conditions were kept constant throughout a study, and our observations are similar to what could be expected using theoretical prediction in CSD.

The charge conveyance phenomenon seen in our experiments may involve other mechanisms. There was evidence that gas phase ions are produced directly from charged droplets [49]. Kaltosov and Mohimen's work demonstrated that protein ion charge is a measure of the surface area of a large molecule [45]. Ion competition is a well-known phenomenon [3, 50, 51], and electrospray ionization efficiency (IE) can be different for different ions [52]. Ion competition among multiply charged ions in ESI droplets could explain the peak shift phenomenon in our experiments. The competition of multiply charged ions of the analyte at higher concentration could favor the ions of higher-charged states to be gas phase ions from ESI droplets. The shift of CSD is subtle even with the 100-fold change in the analyte solution as shown in Fig. 6; it was not obvious when its precursors were monitored. This is due to signal interference
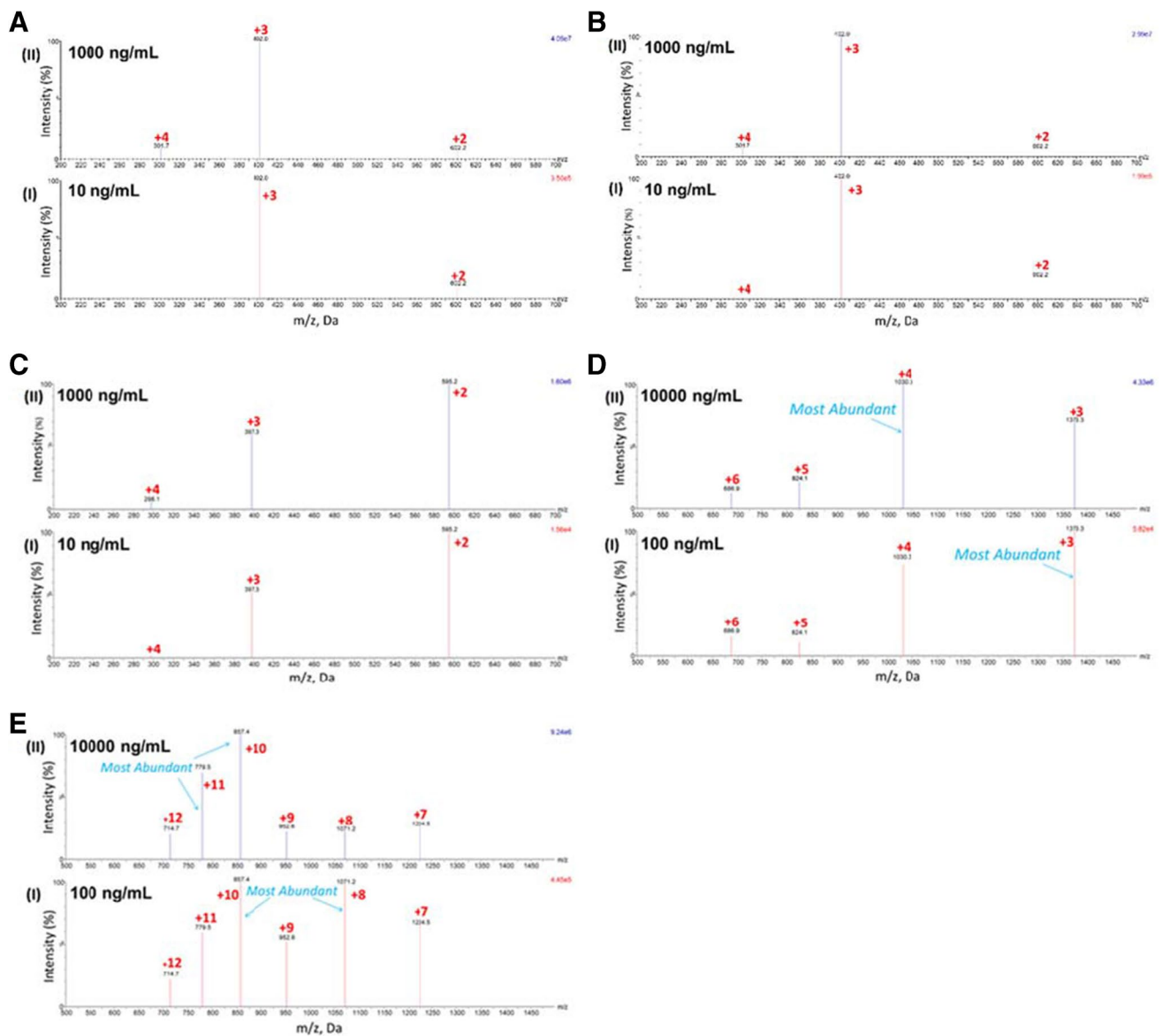
Fig. 6 Comparison of product ion abundance at low concentration (I) and high concentrations (II). Relative abundance of product ion peaks shifted from lower-charge-state precursor ions to higher-charge-state precursor ions with increase in analyte concentration (100-fold). **A** Polymyxin B1, **B** polymyxin B1-Il, **C** polymyxin B2, **D** teriparatide, **E** ubiquitin
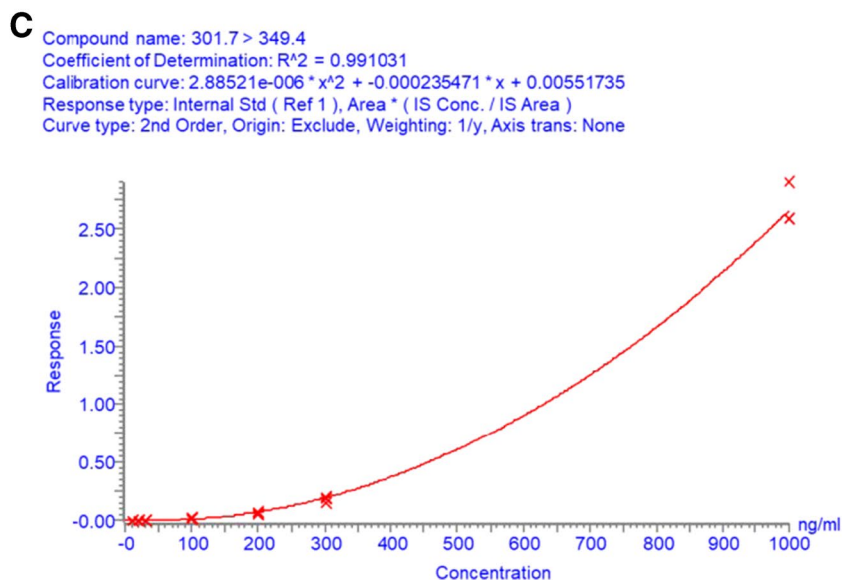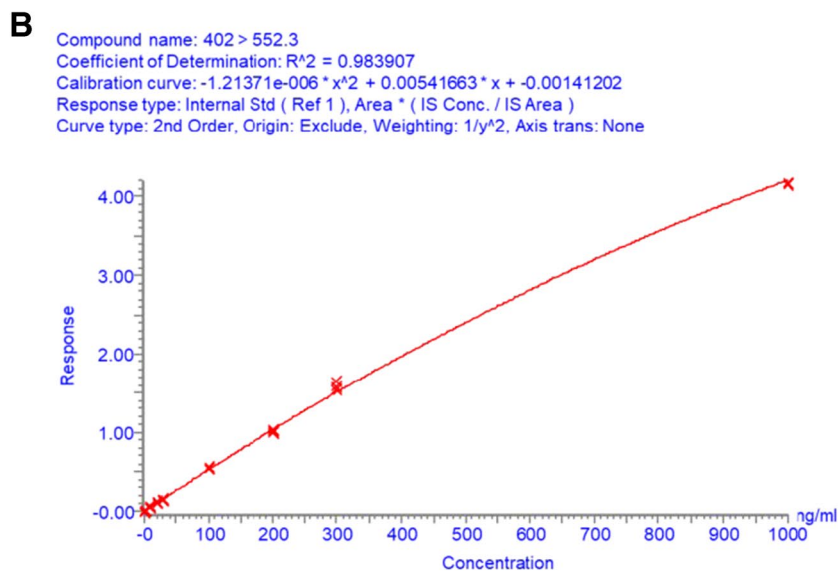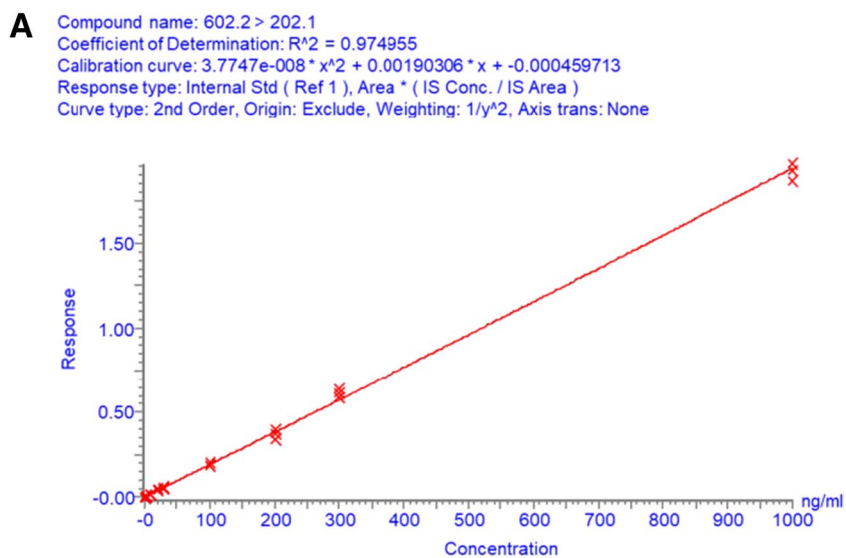
of ions such as solvent clusters and trace-level impurities to the analyte at low concentration, and there is a peak saturation and distortion effect on the analyte at high concentration [32]. Due to the high selectivity and specificity of MRM, the phenomenon of a charge conveyance to higher-charge states becomes noticeable in SMRM. In analytical mass spectrometry of large biomolecules, SMRM can counterbalance the variation of CSD with concentration to achieve a wider linear dynamic range to measure large-molecule analytes, although the mechanism of charge conveyance may be worthy of further investigation. As compared to the feature of narrow dynamic range in LBA methods, the wider dynamic range provided by SMRM can make LC–MS/MS more powerful for the analysis of large biomolecules.
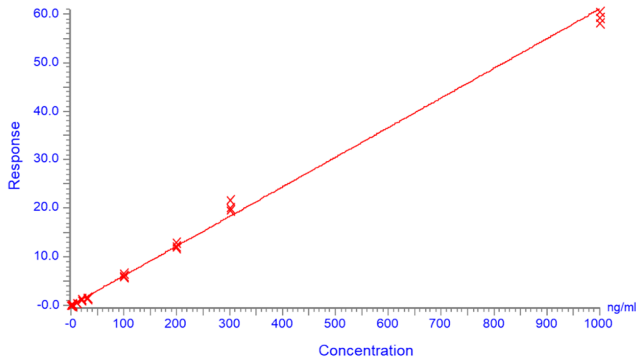
Conclusions

The SMRM approach has advantages over MRM in the LC–MS/MS technology for quantitative measurement of large molecules such as protein and peptides. Compared to

Fig. 7 The MRM method provides linear or concave or convex calibration curves susceptible to the selection of both a multiply charged precursor and a suitable product ion. **A** MRM (602.2 →202.1) of $(PMB1 + 2H)^{2+}$ shows a linear calibration curve, **B** MRM (402→552.3) of $(PMB1 + 3H)^{3+}$ shows a concave calibration curve, **C** MRM (301.7→349.4) of $(PMB1 + 4H)^{4+}$ shows a convex calibration curve in the same concentration range
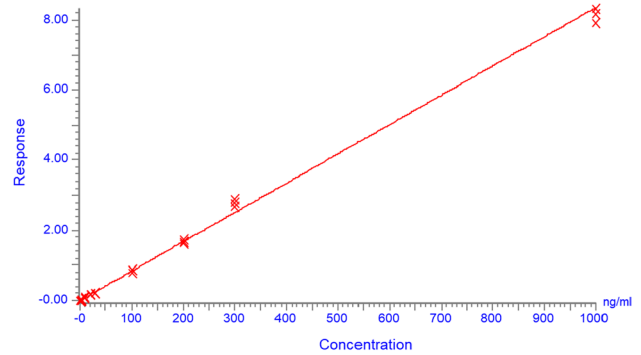
A
Compound name: 602.2 > 202.1
Coefficient of Determination: R^2 = 0.974955
Calibration curve: 3.7747e-008 * x^2 + 0.00190306 * x + -0.000459713
Response type: Internal Std (Ref 1), Area * (IS Conc. / IS Area)
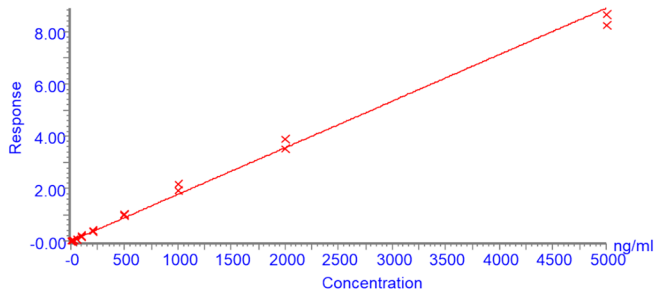Curve type: 2nd Order, Origin: Exclude, Weighting: 1/y^2, Axis trans: None

B
Compound name: 402 > 552.3
Coefficient of Determination: R^2 = 0.983907
Calibration curve: -1.21371e-006 * x^2 + 0.00541663 * x + -0.00141202
Response type: Internal Std (Ref 1), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: 1/y^2, Axis trans: None

C
Compound name: 301.7 > 349.4
Coefficient of Determination: R^2 = 0.991031
Calibration curve: 2.88521e-006 * x^2 + -0.000235471 * x + 0.00551735
Response type: Internal Std (Ref 1), Area * (IS Conc. / IS Area)
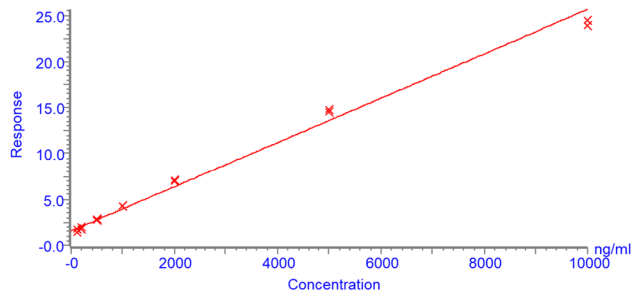Curve type: 2nd Order, Origin: Exclude, Weighting: 1/y, Axis trans: None

Fig. 8 The SMRM method provides a wide linear dynamic range for the quantitative analysis of peptide and protein. Three-decade calibration curves are achieved for small peptides [concentration = 1–1000 ng/mL for all polymyxin B1, polymyxin B1-Il, and polymyxin B2], large peptide [concentration = 5–5000 ng/mL for teriparatide], and protein [concentration = 100–100,000 ng/mL for ubiquitin]

MRM, SMRM provides better sensitivity for the quantitative analysis of large-molecule compounds in biological matrices. Among the large molecules analyzed, a trend was discovered, which indicated that the relative abundance of multiply charged ions of an analyte was directly related to the concentration of the analytes. This means that as the solution concentration increased, the relative abundance of low-charge state ions decreased, and the relative abundance of high-charge state ions increased. The SMRM approach can counterbalance the variation of charge-state distribution (CSD) with concentration to achieve a wider linear dynamic range to measure large-molecule analytes.

The SMRM method development is not dependent on generating reagents or antibodies, and this enables rapid method development and accurate measurements of isomers and metabolites of large molecules.

Declarations

Ethics approval The manuscript is approved by SRI International.

Conflict of interest The authors declare no competing interests.

Source of biological material As included in section "Methods" ("Materials") of the manuscript.

Statement on animal welfare. Not applicable.

References

1. Yamashita M, Fenn JB. Electrospray ion source. Another variation on the free-jet theme. J Phys Chem. 1984;88:4451–4459.
2. Yamashita M, Fenn JB. Negative ion production with the electrospray ion source. J Phys Chem. 1984;88:4671–5.
3. Kebarle P, Tang L. From ions in solution to ions in the gas phase. Anal Chem. 1993;65(22):972A-986A.
4. Kondrat RW, McClusky GA, Cooks RG. Multiple reaction monitoring in mass spectrometry/mass spectrometry for direct analysis of complex mixtures. Anal Chem. 1978;50(14):2017–21.
5. Kortza L, Dorowa J, Ceglareka U. Liquid chromatography–tandem mass spectrometry for the analysis of eicosanoids and related lipids in human biological matrices: a review. J Chromatography B. 2014;964:1–11.
6. Guo B, Chen B, Liu A, Zhua W, Yao S. Liquid chromatography-mass spectrometric multiple reaction monitoring-based strategies for expanding targeted profiling towards quantitative metabolomics. Curr Drug Metab. 2012;13:1226–43.
7. van den Broeka I, Sparidansa RW, Schellensa JHM, Beijnena JH. Quantitative bioanalysis of peptides by liquid chromatography coupled to (tandem) mass spectrometry. J Chromatography B. 2008;872:1–22.
8. van de Merbel NC. Protein quantification by LC–MS: a decade of progress through the pages of bioanalysis. Bioanalysis. 2019;11(07):629–44.
9. Ewles M, Goodwin L. Bioanalytical approaches to analyzing peptides and proteins by LC–MS/MS. Bioanalysis. 2011;3(12):1379–97.
10. Thway TM. Fundamentals of large-molecule protein therapeutic bioanalysis using ligand-binding assays. Bioanalysis. 2016;8(1):11–7.
11. Zhang S, Jian W. Recent advances in absolute quantification of peptides and proteins using LC-MS. Rev Anal Chem. 2014;33(1):31–47.
12. Burns SA, Hong YJ, Mitchell AE. Direct liquid chromatography–mass spectrometry method for the detection of glutathione S-transferase isozymes and investigation of their expression in response to dietary flavone. J Chromatography B. 2004;809:331–7.
13. Mayr BM, Kohlbacher O, Reinert K, Sturm M, Gropl C, Lange E, Klein C, Huber CG. Absolute myoglobin quantitation in serum by combining two-dimensional liquid chromatography-electrospray ionization mass spectrometry and novel data analysis algorithms. J Proteome Research. 2006;5:414–21.
14. Liebler DC, Zimmerman LJ. Targeted quantitation of proteins by mass spectrometry. Biochemistry. 2013;52:3797–806.
15. Jenkins R, Duggan JX, Aubry AF, Zeng J, Lee JW, Cojocaru L, Dufield D, Garofolo F, Kaur S, Schultz GA, Xu K, Yang Z, Yu J, Zhang YJ, Vazvaei F. Recommendations for validation of LC-MS/MS bioanalytical methods for protein biotherapeutics. AAPS J. 2015;17(1):1–16.
16. Nitin M, Rajanikanth M, Lal J, Madhusudanan KP, Gupta RC. Liquid chromatography–tandem mass spectrometric assay with a novel method of quantitation for the simultaneous determination of bulaquine and its metabolite, primaquine, in monkey plasma. J Chromatography B. 2003;793:253–63.
17. Swamy JM, Kamath N, Shekar AKR, Srinivas NR, Kristjansson F. Sensitivity enhancement and matrix effect evaluation during summation of multiple transition pairs—case studies of clopidogrel and ramiprilat. Biomed Chromatogr. 2010;24:528–34.
18. Movassaghi CS, McCarthy DP, Bhandari D, Blount BC, De Jesús VR. Multiple ion transition summation of isotopologues for improved mass spectrometric detection of N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine. J Am Soc Mass Spectrom. 2019;30:1213–9.
19. Asara JM, Christofk HR, Freimark LM, Cantley LC. A label-free quantification method by MS/MS TIC compared to SILAC and spectral counting in a proteomics screen. Proteomics. 2008;8:994–9.
20. Lamont L, Eijkel GB, Jones EA, Flinders B, Ellis SR, Siegel TP, Heeren RMA, Vreeken RJ. Targeted drug and metabolite imaging: desorption electrospray ionization combined with triple quadrupole mass spectrometry. Anal Chem. 2018;90:13229–35.
21. Li X, Li S, Kellermann G: Method for the ultra-sensitive determination of catecholamines and their metabolites. PCT Int Appl. 2017;WO 2017077401 A1 20170511.
22. Maráková K, Rai AJ, Schug KA. Effect of difluoroacetic acid and biological matrices on the development of a liquid chromatography–triple quadrupole mass spectrometry method for determination of intact growth factor proteins. J Sep Sci. 2020;43:1663–77.
23. Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. Electrospray ionization for mass spectrometry of large biomolecules. Science. 1989;246(4926):64–71.
24. Mann M, Meng CK, Fenn JB. Interpreting mass spectra of multiply charged ions. Anal Chem. 1989;61:1702–8.
25. Pan P, McLuckey SA. Electrospray ionization of protein mixtures at low pH. Anal Chem. 2003;75:1491–9.
26. Derendorf H, Schmidt S: Chapter 21, Protein drugs. In: Rowland and Tozer's clinical pharmacokinetics and pharmacodynamics: concepts and applications. 5th ed., 2020. pp 687–730.
27. Loo RRO, Smith RD. Investigation of the gas-phase structure of electrosprayed proteins using ion- molecule reactions. J Am Soc Mass Spectrom. 1994;5:207–20.
28. Hee KH, Leaw YKJ, Ong JL, Lee LS. Development and validation of liquid chromatography tandem mass spectrometry method quantitative determination of polymyxin B1, polymyxin B2, polymyxin B3 and isoleucine-polymyxin B1 in human plasma and its application in clinical studies. J Pharm & Biomedical Anal. 2017;140:91–7.
29. Banerjee S, Mazumdar S. Electrospray ionization mass spectrometry: a technique to access the information beyond the molecular weight of the analyte. Int J Anal Chem. 2012;1–40.
30. Smith RD, Loo JA, Edmonds CG, Barinaga CJ, Udseth HR. New developments in biochemical mass spectrometry: electrospray ionization. Anal Chem. 1990;62:882–99.
31. Chowdhury SK, Katta V, Chait BT. An electrospray-ionization mass spectrometer with new features. Rapid Commun Mass Spectrom. 1990;4(3):81–7.
32. Wei AAJ, Joshi A, Chen Y, McIndoe JS. Strategies for avoiding saturation effects in ESI-MS. Int J Mass Spec. 2020;450:116306.
33. Thomas TA, Broun EC, Abildskov KM, Kubin CJ, Horan J, Yin MT, Cremers S. High performance liquid chromatography–mass spectrometry assay for polymyxin B1 and B2 in human plasma. Ther Drug Monit. 2012;34:398–405.
34. Meng M, Wang L, Liu S, Jaber OM, Gao L, Chevrette L, Reuschel S. Simultaneous quantitation of polymyxin B1, polymyxin B2 and polymyxin B1–1 in human plasma and treated human urine using solid phase extraction and liquid chromatography–tandem mass spectrometry. J Chromatogr B. 2016;1012–1013:23–36.
35. U.S. Department of Health and Human Services FDA. Bioanalytical method validation: guidance for industry. May 2018.

36. Welink J, Xu Y, Yang E, Wilson A, Henderson N, Luo L, Fraser S, Kavita U, Musuku A, James C, Fraier D, Zhang Y, Goykhman D, Summerfield S, Woolf E, Verhaeghe T, Hughes N, Behling A, Brown K, Bulychev A, Buonarati M, Cherry E, Cho SJ, Cludts I, Dillen L, Dodge R, Edmison A, Garofolo F, Green R, Haidar S, Hottenstein C, Ishii-Watabe A, Jang HG, Ji A, Jones B, Kassim S, Ma M, Lima Santos GM, Norris DA, Owen T, Piccoli S, Ramanathan R, Röhl I, Rosenbaum AI, Saito Y, Sangster T, Savoie N, Stebbins C, Sydor J, de Merbel NV, Verthelyi D, Vinter S, Whale E. 2018 white paper on recent issues in bioanalysis: 'A global bioanalytical community perspective on last decade of incurred samples reanalysis (ISR)' (Part 1 –small molecule regulated bioanalysis, small molecule biomarkers, peptides & oligonucleotide bioanalysis). Bioanalysis. 2018;10(22):1781–801.
37. Spriggs FP, Zhong ZD, Safavi A, Jani D, Dontha N, Kant A, Ly J, Brilando L, Österlund K, Rouleau N, Fischer SK, Boissonneault M, Ray C. Ligand binding assays in the 21st century laboratory: platforms. AAPS J. 2012;14(10):113–8.
38. Buckley LA, Bebenek I, Cornwell PD, Hodowanec A, Jensen EC, Murphy C, Ghantous HN. Drug development 101: A Primer. Int J Toxicology. 2020;39(5):379–96.
39. Wang G, Cole RB. Mechanistic interpretation of the dependence of charge state distributions on analyte concentrations in electrospray ionization mass spectrometry. Anal Chem. 1995;67:2892–900.
40. Iavarone AT, Jurchen JC, Williams ER. Effects of solvent on the maximum charge state and charge state distribution of protein ions produced by electrospray ionization. J Am Soc Mass Spectrom. 2000;11:976–85.
41. Krusemark CJ, Frey BJ, Belshaw PJ, Smith LM. Modifying the charge state distribution of proteins in electrospray ionization mass spectrometry by chemical derivatization. J Am Soc Mass Spectrom. 2009;20:1617–25.
42. Kuprowski MC, Konermann L. Signal response of coexisting protein conformers in electrospray mass spectrometry. Anal Chem. 2007;79:2499–506.
43. Lomeli SH, Peng IX, Yin S, Loo RRO, Loo JA. New reagents for increasing ESI multiple charging of proteins and protein complexes. J Am Soc Mass Spectrom. 2010;21:127–31.
44. Li Y, Cole RB. Shifts in peptide and protein charge state distributions with varying spray tip orifice diameter in nanoelectrospray Fourier transform ion cyclotron resonance mass spectrometry. Anal Chem. 2003;75:5739–46.
45. Kaltashov IA, Mohimen A. Estimates of protein surface areas in solution by electrospray ionization mass spectrometry. Anal Chem. 2005;77:5370–9.
46. Alves S, Fournier F, Afonso C, Wind F, Tabeta JC. Gas-phase ionization/desolvation processes and their effect on protein charge state distribution under matrix-assisted laser desorption/ionization conditions. Eur J Mass Spectrom. 2006;12:369–83.
47. Petroff JT II, Tong A, Chen LJ, Dekoster GT, Khan F, Abramson J, Frieden C, Cheng WWL. Charge reduction of membrane proteins in native mass spectrometry using alkali metal acetate salts. Anal Chem. 2020;92:6622–30.
48. Thinius M, Polaczek C, Langner M, Bräkling S, Haack A, Kersten H, Benter T. Charge retention/charge depletion in ESI-MS: experimental evidence. J Am Soc Mass Spectrom. 2020;31:773–84.
49. Hager DB, Dovlchi NJ, Klassen J, Kebarle P. Droplet electrospray mass spectrometry. Anal Chem. 1994;66:3944–9.
50. Tang L, Kebarle P. Dependence of ion intensity in electrospray mass spectrometry on the concentration of the analytes in the electrosprayed solution. Anal Chem. 1993;65:3654–68.
51. Tang K, Page JS, Smith RD. Charge competition and the linear dynamic range of detection in electrospray ionization mass spectrometry. J Am Soc Mass Spectrom. 2004;15:1416–23.
52. Oss M, Kruve A, Herodes K, Leito I. Electrospray ionization efficiency scale of organic compounds. Anal Chem. 2010;82:2865–72.