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Multiple Time-of-Flight/Time-of-Flight Events in a Single Laser Shot for Improved Matrix-Assisted Laser Desorption/Ionization Tandem Mass Spectrometry Quantification

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Abstract

Quantitative matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) approaches have historically suffered from poor accuracy and precision mainly due to the nonuniform distribution of matrix and analyte across the target surface, matrix interferences, and ionization suppression. Tandem mass spectrometry (MS/MS) can be used to ensure chemical specificity as well as improve signal-to-noise ratios by eliminating interferences from chemical noise, alleviating some concerns about dynamic range. However, conventional MALDI TOF/TOF modalities typically only scan for a single MS/MS event per laser shot, and multiplex assays require sequential analyses. We describe here new methodology that allows for multiple TOF/TOF fragmentation events to be performed in a single laser shot. This technology allows the reference of analyte intensity to that of the internal standard in each laser shot, even when the analyte and internal standard are quite disparate in m/z , thereby improving quantification while maintaining chemical specificity and duty cycle. In the quantitative analysis of the drug enalapril in pooled human plasma with ramipril as an internal standard, a greater than 4-fold improvement in relative standard deviation ($<10\%$) was observed as well as improved coefficients of determination (R^2) and accuracy ($>85\%$ quality controls). Using this approach we have also performed simultaneous quantitative analysis of three drugs (promethazine, enalapril, and verapamil) using deuterated analogues of these drugs as internal standards.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

Supporting Information

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) is a common tool for qualitative analysis due to its extensive mass range, rapid analysis time, high sensitivity, and minimal sample preparation.^{1–4} However, the use of MALDI TOF for quantitative analysis has been less widespread.⁵ Historically, quantitative analyses using this platform have suffered from poor accuracy and precision as well as poor dynamic range, mainly due to sample heterogeneity. Issues of sample heterogeneity persist due to variability in MALDI matrix crystallization resulting in a nonuniform distribution of matrix and analyte across the target surface.⁶ This can cause variations in ion current from laser shots irradiating the same sample position (shot-to-shot reproducibility), at different locations on the target surface (region-to-region reproducibility), and between identical samples on different targets (sample-to-sample reproducibility).

Despite these challenges, MALDI MS quantification has been successfully performed in the analysis of many different types of analytes, including polymers,⁷ drugs,^{8–10} metabolites,¹¹ oligo-nucleotides,¹² oligosaccharides,¹³ peptides,¹⁴ and proteins.¹⁵ Carefully designed sample preparation is extremely important to ensure accurate and precise quantification. For example, the uses of a thin matrix layer,¹⁶ matrix/comatrix approach,¹⁷ ionic liquid matrix,¹⁸ electrospray deposition,¹⁹ acoustic spotting,²⁰ and matrix seeding^{21–23} approaches have all been employed to improve sample homogeneity. In most cases, the use of an internal standard is vital to allow for corrections in sample variability and ionization suppression.^{6,24} In order to combat region-to-region heterogeneity, many spectra are typically averaged across the target surface to generate a more representative mass spectrum. Spectral preprocessing techniques such as background subtraction and normalization can also help improve quantitative MALDI MS experiments.⁶ Despite these advances, accurate quantification by MALDI MS remains difficult.

The accurate quantification of small molecules and metabolites presents an additional challenge because of the vast excess of MALDI matrix signal and other endogenous sample species that can dominate ion signal in the low m/z region of the mass spectrum.^{25–27} One method to combat this issue is the use of high-resolution/accurate mass measurements to

distinguish matrix ions from analyte ions on the basis of mass defect. However, these types of measurements typically require more specialized instrument platforms, such as Fourier transform or quadrupole-TOF (Q-TOF) mass spectrometers. Off-line chromatographic separation and standard addition approaches have been used to simplify the analysis of highly complex samples, but these approaches are still sensitive to matrix effects.²⁸ However, tandem mass spectrometry (MS/MS) can be used to ensure chemical specificity as well as to improve signal-to-noise ratios by eliminating interferences from chemical noise, alleviating some concerns about dynamic range. MS/MS experiments typically rely on the use of isotopically labeled internal standards to perform quantitative analysis.^{26,27,29} In these experiments, the quantification is either performed at the MS level²⁸ or the MS/MS isolation window can be widened sufficiently to pass both the analyte and internal standard ions^{30–33} or multiple isolation windows can be used to isolate ions of disparate m/z .^{34,35} These experiments have largely been performed on ion trap-based platforms. For example, Reich et al. used wide isolation windows and deuterated internal standards to quantify cocaine levels in the brain via MALDI MS/MS on a linear ion trap mass spectrometer.³⁰ The use of multiple isolation windows has been achieved on ion trap systems through the use of isolation waveforms with multiple frequency notches (e.g., synchronous precursor selection) and has also been used recently in quantitative proteomics analyses.³⁶ However, the method by which ions are selected for MS/MS analysis is much different in an ion trap compared to a TOF/TOF instrument. Whereas an ion trap discriminates ions on the basis of ion secular frequency, a TOF/TOF instrument achieves precursor ion separation based on ion flight time through the first time-of-flight (TOF-1) region of the instrument. An ion trap instrument can then perform isolation on the basis of secular frequency by, for example, using tailored waveforms to selectively eject ions from the trap. Conversely, a TOF/TOF instrument uses a timed ion selector (TIS) as a voltage gate for ion transmission based on flight time. The TOF/TOF experiment is a “tandem in space” measurement, where ion isolation, activation, and mass analysis take place in different regions of the instrument, while the ion trap experiment is a “tandem in time” measurement, where ion isolation, activation, and mass analysis can take place in the same region of the instrument but at different times during the scan function (though hybrid instruments can also provide elements of “tandem in space” experiments). Though an ideal choice because of their speed, MALDI TOF/TOF measurements have not been shown to perform multiple isolation events in a single laser shot and have seen far less use in quantitative MALDI MS analyses.

Herein, we describe the use of a novel instrument and data acquisition paradigm designed to perform multiple TOF/TOF events in the same laser shot. For the purposes of this discussion, a TOF/TOF “event” is defined as the isolation, fragmentation, and reacceleration of one m/z window. Analyte intensity can be referenced to that of an internal standard in each laser shot even when the analyte and internal standard are significantly different in m/z , thereby improving quantification while maintaining chemical specificity and duty cycle. This is useful in instances where simply broadening the isolation window would pass an excess amount of chemical noise. A unique benefit to this TOF/TOF approach is the ability to resolve fragment ions of the exact same mass that are derived from different precursor ions. This is due to the precursor ion separation achieved in the TOF-1 region of the instrument and is demonstrated here using enalapril and ramipril, two angiotensin-

converting enzyme (ACE) inhibitors used in the treatment of hypertension. Additionally, this approach is shown to allow the simultaneous quantification of three separate drugs (promethazine, enalapril, and verapamil) using their deuterated analogues as internal standards. While assays of multiple analytes have been previously reported, unique here is the acquisition of a complete fragment ion spectrum and the separate fragmentation of each analyte in every laser shot.

EXPERIMENTAL SECTION

Materials

See the Supporting Information for details.

Sample Preparation

See the Supporting Information for details.

Mass Spectrometry

All experiments were performed on a MALDI TOF/TOF mass spectrometer (300 Tandem, SimulTOF Systems, Sudbury, MA) operated in positive ion MS/MS mode at 4 kV³⁷ and capable of performing multiple TOF/TOF events in a single laser shot as described by Marvin Vestal^{38,39} and used in quantitative imaging mass spectrometry.⁴⁰ This instrument is equipped with a 349 nm, diode-pumped, frequency-tripled Nd:YLF laser capable of laser repetition rates up to 5 kHz (Spectra-Physics, Santa Clara, CA). The laser energy for all experiments was ~66 μ J/pulse (as measured prior to attenuation). This instrument utilizes continuous laser raster sampling.^{37,41–43} Data were acquired in typewriter imaging mode using a 1-kHz laser repetition rate, 1 mm/s stage velocity, 100 hardware averages, and 100- μ m vertical step size (resulting in 100- μ m by 100- μ m pixel sizes). During acquisition, an intensity filter was used to only record spectra above a specified intensity threshold. Following acquisition, spectral averages were manually exported using a region of interest selection tool from each MALDI spot. Instrument operation and data acquisition were controlled using the SimulTOF Controller, and data analysis was performed using the SimulTOF Viewer (SimulTOF Systems, Sudbury, MA) as well as Microsoft Excel (Redmond, WA). External calibration was performed in both MS and MS/MS mode using spotted drug standards.

A schematic is shown in Figure 1 to explain the process of individually selecting and reaccelerating two distinct precursor ions (the blue and red ions in Figure 1) in a single laser shot. Initially, ions are extracted from the first source region of the instrument via pulsed extraction ($t = 0 \mu$ s) and then enter the first field-free drift (TOF-1) region of the instrument. In MS/MS mode, a precision timed ion selector (TIS, 500 fwhm resolution, 6 ns transition speed) located at the velocity focal distance is used to isolate a precursor ion of interest ($t = 30 \mu$ s and $t = 38 \mu$ s). The TIS can be “opened” and “closed” multiple times within the same laser shot, allowing for the individual isolation of multiple ions of increasing m/z ($t = 25 \mu$ s to $t = 45 \mu$ s). Following TIS isolation, the ions enter an 8.6 cm long collision cell region ($t = 30 \mu$ s and $t = 38 \mu$ s) and are then reaccelerated to 2 kV in the second source region ($t = 37 \mu$ s and $t = 45 \mu$ s). Similar to TIS isolation, the second source region can be ramped multiple

times within the same laser shot to reaccelerate ions from precursor ions of increasing m/z . The ability to individually isolate and reaccelerate ions of similar m/z is limited by ion transit time through the devices as well as the rise and fall times of the relevant power supplies and the length of the voltage pulses, which can be partially controlled by user. Consequentially, precursor ions must differ by at least ~6–7% in m/z to be successfully isolated and reaccelerated in this fashion. Fragmentation can be achieved using either laser-induced dissociation (LID) (i.e., postsource decay [PSD] using no collision gas) or high energy collision induced dissociation (CID) (i.e., using collision gas).⁴⁴ For experiments herein, PSD was used for fragmentation. Following reacceleration, the ion beam then enters the TOF-2 region of the instrument including passage through a two-stage ion mirror (not shown in Figure 1) before reaching the detector (High Mass Bi-Polar TOF detector, Photonis, Sturbridge, MA). While spectra shown here represent averages of 1 000 laser shots to show good spectral quality, multiple TOF/TOF events are indeed performed in each laser shot.

RESULTS

Multiple MS/MS Events in a Single Laser Shot

The TOF/TOF instrument described here uniquely provides for the individual selection and reacceleration of multiple precursor ions in a single laser shot. An example of such an experiment is shown in Supplemental Figure 1 using two peptides, angiotensin II and [glu1]-fibrinopeptide B (glu-fib). The timing of the isolation and reacceleration of these ions approximately correlates with the timing illustrated in Figure 1; the blue ion in Figure 1 corresponds to protonated angiotensin II and the red ion corresponds to protonated glu-fib. Initially, the mass spectrum is reported as a function of the ion arrival time in microseconds at the detector (Supplemental Figure 1a). The mass spectrum can then be calibrated using either angiotensin II as the precursor ion (Supplemental Figure 1b) or glu-fib as the precursor ion (Supplemental Figure 1c). In this simple case, the fragment and precursor ions of the lower mass ion, angiotensin II, all arrive at the detector prior to the arrival of the lowest mass fragment ion of glu-fib (i.e., the two fragment ion spectra do not overlap with one another). Here, assigning fragment ion peaks to their proper precursor ion is relatively easy. Once the proper precursor for a given fragment ion has been identified, the correct mass can be assigned based on the proper mass calibration. As seen in Supplemental Figure 1b,c, the fragment ions are mainly b- and y-type ions common to CID analyses of peptides.⁴⁵

However, one can imagine a more complicated instance where the fragment ion spectra of two precursor ions overlap. In such an instance, the user must have *a priori* knowledge of the fragmentation behaviors of the precursor ions of interest in order to ensure each fragment ion is accurately assigned to the appropriate precursor ion. An example of this type of experiment is shown using the drugs ramipril and enalapril (Figure 2), where the lowest molecular weight fragment ion (purple m/z 235) of the higher mass precursor ion (ramipril, m/z 417) arrives at the detector before the lower mass precursor ion (enalapril, m/z 377) (Figure 2c). In this experiment, the enalapril precursor ion appears in the spectrum between the fragment and precursor ions of ramipril. Even with the relatively simple fragmentation

patterns of these two drugs, one can imagine that *de novo* interpretation of the spectrum in Figure 2c would be difficult. Without knowing which mass calibration to apply for each ion (the calibration using ramipril as the precursor ion or the calibration using enalapril as the precursor ion), it would be challenging to properly assign the mass of fragment ions based on the appropriate precursor ion given only detector arrival time information. Therefore, experiments should be conducted which only fragment one ion at a time in order to obtain a product ion spectrum for each precursor ion alone (Figure 2a and 2b). This fragmentation fingerprint can then be used to accurately assign fragment ions to precursor ions in experiments where multiple ions have been selected for fragmentation. This assignment is performed by comparing the arrival times between the different experiments. As the TIS and reacceleration timings and voltages can be kept constant in each experiment, the arrival times of the ions observed in a single precursor experiment will exactly correlate with those observed in a multiple precursor experiment, allowing for proper peak assignment.

Figure 2 also highlights a unique aspect of this TOF/TOF methodology: the ability to resolve fragment ions of the exact same m/z that are derived from different precursor ions. Ramipril and enalapril are structural analogues of one another and differ only by a cyclopentapyrrole group versus a pyrrolidine group, respectively. Not surprisingly, the two drugs fragment extremely similarly under CID conditions and the resulting structures of the dominant fragments do not contain the disparate portion of the molecules (see structure insets in Figure 2a,b). As such, the main fragment ions of the two drugs are exact isomers of one another and would be indistinguishable even on any high-resolution/accurate mass, ion trap MS/MS, or ion mobility instruments in a single measurement. For example, a trap-based approach such as using a wide isolation window^{30,34} or the use of synchronous precursor selection³⁵ would be unable to resolve these two fragments based solely on the m/z of the fragment ion. With the TOF/TOF platform, however, the separation in time of the precursor ions in the TOF-1 region of the instrument allows for the facile separation of the isomeric fragment ions. This is similar in principle to a triple quadrupole (QqQ) precursor ion scan, where the separation of precursor ions is performed via a scan of the first resolving quadrupole (Q1) while the third quadrupole (Q3) remains fixed on the fragment ion of interest. However, in contrast to a triple quadrupole, an entire fragment ion spectrum is obtained, and for each precursor ion, in every laser shot.

Enalapril TOF/TOF Assay

In characterizing this methodology, we sought to explore the use of multiple TOF/TOF events in a single laser shot for improved quantification. By using one TOF/TOF event for an analyte and a second TOF/TOF event for an internal standard, one could relate the intensity of the analyte to that of an internal standard in each laser shot even in instances where the two ions are different in m/z , such as is the case with enalapril (m/z 377) and ramipril (m/z 417). In such instances, simply broadening the isolation window to allow the transmittance of both precursor ions is not practical; the required ~40 Da isolation window would transmit an excess of chemical noise which would diminish sensitivity and mitigate the benefits of MS/MS specificity.

As enalapril and ramipril possess similar physical and chemical properties, this drug pair represents an ideal test for MALDI MS/MS quantification. For these experiments, seven sets of enalapril/ramipril spots were prepared and analyzed on the TOF/TOF platform, five of which were included in quantitative calculations based on accuracy (passing QC), spot homogeneity and quality, and successful instrument data acquisition. For each replicate analysis, data were acquired in imaging mode (data not shown) using an intensity filter to only record a spectrum when at least 2 peaks reached 0.06 V in intensity. The TIS was set for a difference of 40 (TISB delay equation = 1880, TISB pulse end equation = 1920). This results in pulse lengths of ~500 and ~525 ns for the TIS isolation events for enalapril and ramipril, respectively. In practice, this corresponds to ~4–5 Da isolation windows.³⁷ The source 2 pulse end slope was set to 1.03, resulting in ~690 and ~725 ns source 2 pulse lengths for enalapril and ramipril, respectively. Following data acquisition, peak detection settings were optimized to detect both the ¹²C and ¹³C isotopes of both the enalapril and ramipril fragment ions (doublet preset; minimum SNR, 1; max peak width, 3×10^{-8} s; deisotope width, 0.0001). Regions of interest were manually selected in the SimulTOF Viewer for each MALDI spot to yield an average spectrum. A list of masses and corresponding peak areas from each average spectrum was exported to Excel, where the ¹²C and ¹³C isotopes of each drug fragment were summed and quantitative calculations performed.

Quantification of Enalapril

The relative standard deviations (RSDs) of enalapril without normalization to the internal standard were well above 20% at every concentration (“raw standard deviation” in Table 1). However, upon normalization to the ramipril internal standard, the RSDs of enalapril at every concentration dropped below 9% (“ratio standard deviation” in Table 1). This resulted in a >4-fold improvement in RSD at most concentrations and provided for a dynamic range of over 2.5 orders of magnitude and a limit of quantification (LOQ) of 0.03 μ M. This LOQ of ~11 ng/mL is well below the expected serum level following a single 10 mg dose, which is reported to be 50–250 ng/mL.^{46,47} This LOQ compares favorably with values obtained via HPLC-UV (12 ng/mL)⁴⁸ but is roughly an order of magnitude higher than results reported using HPLC-MS.⁴⁹ In addition to an improvement in precision, improvements in enalapril linearity (Supplemental Figure 2) and accuracy (Table 2) were also observed upon normalization to ramipril. As determined in the range of 0.03 to 10 μ M, a least-squares linear regression analysis showed an improvement in the correlation coefficient from $R^2 \approx 0.9966$ to $R^2 \approx 0.9998$ upon normalization (Supplemental Figure 2). Without normalization, the average of the absolute values of the accuracies for the 0.5 and 5 μ M QCs were 66.8% and 28.2%, respectively, and as low as 10.2% in one instance (“raw accuracy” in Table 2). However, upon normalization these accuracies improved dramatically to 92.9% and 93.4%, with accuracies for every trial above 85% and most above 93% (“ratio accuracy” in Table 2). The SimulTOF Viewer determines peak areas for a selected region of interest by computing a sum of all the individual spectra. While the number of spectra averaged per spot is not controlled for in this experiment, normalizing these data to the number of spectra per MALDI spot yields no improvement in linearity and only minor improvements in accuracy and precision (Supplemental Figures 3–5). While not surprising that normalization to an internal standard improves accuracy, precision, and linearity in quantification, the use of

multiple TOF/TOF events in a single MALDI laser shot represents novel methodology. This example highlights the quantitative capabilities of this technique within clinically relevant therapeutic ranges.

Multidrug TOF/TOF Assay

As the ion transit times through the devices and the rise and fall times of the relevant power supplies necessitate that precursor ions differ by ~6–7% in m/z to be successfully isolated and reaccelerated, the use of multiple TOF/TOF events for the discrete analysis of a small molecule and an isotopically labeled internal standard is impractical. However, when using stable isotope analogues as internal standards, the TOF/TOF isolation window can be broadened to accommodate both the analyte and internal standard. In fact, multiple TOF/TOF events in a single laser shot can then be leveraged to increase the throughput of a quantitative assay. By using each event to examine a different drug and internal standard pair, the analysis can be multiplexed. This is demonstrated here using three drugs: promethazine (PMZ), enalapril (ENP), and verapamil (VPM). PMZ is a phenothiazine used to treat a variety of conditions, including allergy symptoms and nausea. VPM is phenylalkylamine class L-type calcium channel blocker used to treat hypertension. For these analyses, isotopically labeled drug analogues, promethazine-d3 (PMZ-d3), enalapril-d5 (ENP-d5), and verapamil-d3 (VPM-d3), were used as internal standards.

Seven sets of drug panel spots were prepared and analyzed on the TOF/TOF platform, five of which were included in quantitative calculations based on accuracy (passing QC), spot homogeneity and quality, and successful instrument data acquisition. For each replicate analysis, data were acquired in imaging mode (data not shown) using an intensity filter to only record a spectrum when at least 3 peaks reached 0.06 V in intensity. In order to accommodate the isotopically labeled internal standards, the TIS isolation windows were broadened. The TIS was set for a difference of 75 (TISB delay equation = 1870, TISB pulse end equation = 1945). This results in pulse lengths of ~740, ~845, and ~920 ns for the TIS isolation events for PMZ and PMZ-d3, ENP and ENP-d5, and VPM and VPM-d3, respectively. In practice, this corresponds to ~8-Da isolation windows. The source 2 pulse end slope was decreased slightly to 1.022 to ensure the multiple pulses did not overlap. This resulted in source 2 pulse lengths of ~450, ~510, and ~560 ns for PMZ and PMZ-d3, ENP and ENP-d5, and VPM and VPM-d3, respectively, which were all long enough to fully cover the ~8-Da range isolated by the TIS. Following data acquisition, peak detection settings were optimized to detect both the ^{12}C and ^{13}C isotopes of both the enalapril and ramipril fragment ions (wavelet preset; minimum SNR, 1; max peak width, 0 s; deisotope width, 0.0001). Regions of interest were manually selected in the SimulTOF Viewer for each MADLI spot to yield an average spectrum. A list of masses and corresponding peak areas from each average spectrum was exported to Excel, where the ^{12}C and ^{13}C isotopes of each drug fragment were summed and quantitative calculations performed.

The quantitative assay of these three drugs involves three separate TOF/TOF events in a single laser shot (Figure 3a), effectively tripling the throughput for this type of assay. Zoomed-in regions of the x -axis show the relevant time (mass) range for each drug transition (Figure 3b–d), with the figure insets showing the chemical structures of the three drugs. For

each of PMZ (Figure 3b), ENP (Figure 3c), and VPM (Figure 3d), the isotopically labeled internal standard (denoted with an asterisk) and analyte precursor and fragment ions are all clearly visible in the mass spectrum. When performing multiple TOF/TOF events in a single laser shot, the complexity of the resulting mass spectrum is greatly increased. Especially in a quantitative assay, it is important that peaks from one TOF/TOF event do not exactly overlap with peaks from another TOF/TOF event, as this would give inaccurate measures of ion intensity. That is, if two peaks from two different TOF/TOF events were not resolved from one another, the recorded intensity of the peak would not solely represent the contribution of a single m/z but rather the sum of two ions of varying m/z . For example, the fragment ions of VPM/VPM-d3 (m/z 304 and 307* in Figure 3d) nearly overlap with the precursor ions of ENP/ENP-d5 (m/z 377 and 382* in Figure 3c). While in this instance these ions are all still clearly resolved from one another, this highlights the care that should be taken with this type of multiplexed assay. With this assay, standards of each drug were individually spotted and analyzed in order to confirm the accurate assignment of each fragment ion to the proper precursor ion (data not shown).

Multidrug Quantification

Normalization of the three drug analytes to their appropriate isotopically labeled internal standard resulted in improved metrics of quantification. The RSDs of PMZ, ENP, and VPM prior to normalization were almost all above 20% at every concentration, with most RSDs above 30% and as high as 52% ("raw standard deviation" in Table 3). However, upon normalization to the isotopically labeled internal standards, the RSDs for all three drugs improved dramatically ("ratio standard deviation" in Table 3). For PMZ, the standard deviations for the quantified concentration range improved from an average of 27.77% to an average of 7.20% with no RSD higher than 13%. For ENP, the standard deviations for the quantified concentration range improved from an average of 45.58% to an average of 15.35% with only one RSD higher than 17%. For VPM, the standard deviations for the quantified concentration range improved from an average of 34.86% to an average of 8.33% with no RSD higher than 13%. It should be noted that, in contrast to the multiple discrete TOF/TOF events used for analyte and internal standard analysis in Tables 1 and 2, the results here are derived from using a wide isolation technique to analyze both the analyte and deuterated standard in the same TOF/TOF event (i.e., one TOF/TOF event is used for each analyte and internal standard pair). The LOQ for ENP reported here (0.7 μM) is higher than that reported in Table 1 and is most likely due to the increase in chemical noise transmitted by the wider TOF/TOF window and/or the two additional MS/MS isolation windows. Although the LOQ is somewhat compromised here, a 3-fold improvement in throughput is achieved while maintaining the selectivity of a MS/MS approach.

In addition to improvements in precision, improvements in PMZ, ENP, and VPM linearity (Supplemental Figure 6) and accuracy (Supplemental Figure 7) were also observed. As determined in the concentration range of 0.7–30 μM , a least-squares linear regression analysis showed dramatic improvements in the correlation coefficients of each drug upon normalization; PMZ improved from $R^2 \approx 0.8262$ to $R^2 \approx 0.9974$ (Supplemental Figure 6a,b), ENP improved from $R^2 \approx 0.6793$ to $R^2 \approx 0.9622$ (Supplemental Figure 6c,d), and VPM improved from $R^2 \approx 0.8772$ to $R^2 \approx 0.9991$ (Supplemental Figure 6e,f). Without

normalization, the average accuracies for the QCs were 50.2%, 23%, and 55.0% for PMZ, ENP, and VPM, respectively (“raw accuracy” in Supplemental Figure 7). However, upon normalization these accuracies once again improved dramatically to 84.7%, 85.0%, and 89.5%, respectively, with only one measurement less than 80% (“ratio accuracy” in Supplemental Figure 7). While the LOQ in these examples (~199 ng/mL for PMZ, ~264 ng/mL for ENP, ~318 ng/mL for VPM) is slightly higher than most clinically relevant therapeutic levels, the LOQ for each of these drugs is still well within toxic dose levels.^{50–53} A multidrug assay such as the one presented here could then be used to screen samples for toxic levels of the sampled drugs and serves as a proof of principle experiment for the quantitative MALDI TOF/TOF analysis of multiple drugs in a single laser shot.

CONCLUSIONS

We have demonstrated the individual isolation and reacceleration of multiple precursor ions in a single laser shot on a MALDI TOF/TOF instrument. The use of a MS/MS platform ensures chemical specificity and improves sensitivity through the elimination of chemical noise. This novel methodology is shown to provide accurate quantification of drug samples in complex environments of pooled human serum. Using one TOF/TOF event to perform MS/MS on an analyte (enalapril) and a second event to perform MS/MS on an internal standard (ramipril), the intensity of the analyte ion can be referenced to that of the internal standard in every single laser shot, providing for dramatic increases in accuracy, precision, and linearity and allows for the accurate quantification of enalapril within clinically relevant therapeutic ranges. Because of precursor ion separation in the TOF-1 region of the instrument, the isomeric fragment ions of enalapril and ramipril are easily resolved using this approach. Using isotopically labeled internal standards, multiple TOF/TOF events in a single laser shot can also be used to perform simultaneous quantification on multiple drug analytes. In these instances, a wider MS/MS window is used to transmit both the analyte and internal standard. The quantification of PMZ, ENP, and VPM is shown as a proof of principle experiment which demonstrates the potential use of this methodology as a multiplexed toxicity assay. In general, the type of analytes and internal standards under investigation will determine the applicability of this TOF/TOF approach. When a structural analogue that differs sufficiently (i.e., at least 6–7% in m/z) in molecular weight is available, multiple TOF/TOF events can be used to discretely analyze the analyte and internal standard separately in each laser shot (Figure 2). When the desired internal standard is a stable isotope form of the analyte, the TOF/TOF window can be broadened to accommodate the analyte and internal standard. Multiple TOF/TOF windows can then be used to analyze multiple analyte and internal standard pairs (Figure 3), thereby increasing throughput, albeit at the cost of some sensitivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Scheme showing the individual selection and reacceleration of two distinct precursor ions in a single laser shot. (a) Ions are extracted from the source 1 region of the instrument and separated according to m/z in TOF-1. (b) The TIS opens to allow the first (blue) ion through. (c) As the first ion is fragmented and passes through the collision cell to the source 2 region, the TIS closes again to prevent the second (yellow) ion from passing. (d) While the first (blue) ion is reaccelerated into the TOF-2 region, the TIS reopens to allow the third (red) ion to pass into the collision cell. (e) The second (blue) ion is traversing the TOF-2 region while the third (red) ion has reached the source 2 region and the TIS has closed again. (f) The first (blue) ion is still traversing the TOF-2 region and the third (red) ion has been reaccelerated. (g) The fragment and precursor ions of both the blue and red ions have reached the detector and are visible in the resulting spectrum. Note: fragment ions are shown here to arise from CID whereas actual experiments employed PSD. The reflectron in the TOF-2 region has been omitted for simplicity. Ion intensities and fragment ion sizes are not shown to scale.

Figure 2.

(a) Fragmentation of ramipril and (b) enalapril both produce the exact same m/z 235 fragment ion. (c) Using the arrival time information obtained in parts a and b, fragment ions can be accurately assigned to the proper precursor ion. The isomeric m/z 235 fragment ions of the two drugs are separated due to precursor ion separation in the TOF-1 region of the instrument. The colored portions of each structure inset correspond to the fragment ion of each species that was detected. The mass resolution of the precursor ions was sacrificed to maximize fragment ion intensity. Each spectrum represents an average of ~1,000 laser shots.

Figure 3.

(a) Quantitative assay of PMZ, ENP, and VPM involves three separate TOF/TOF events in a single laser shot. (b–d) Zoomed-in regions of the *x*-axis show the relevant time (mass) range for each drug transition, with the figure insets showing the chemical structures of the three drugs. For each of (b) PMZ, (c) ENP, and (d) VPM, the isotopically labeled internal standard (denoted with an asterisk) and analyte precursor and fragment ions are all clearly visible in the mass spectrum. The colored portions of each structure inset correspond to the fragment ion of each species that was detected. The asterisks in the structures denote the locations of the heavy isotope labels. Spectrum represents an average of ~1 000 laser shots.

Table 1

Normalization to the Ramipril Internal Standard Improves Precision in Enalapril Quantification (n = 5)

concn (μ M)	avg raw (abundance)	avg ratio (enalapril/ramipril)	raw standard deviation (%)	ratio standard deviation (%)
0	974.94	0.036 106	51.02	13.88
0.0300	8 733.56	0.082 025	37.57	6.64
0.0700	9 949.36	0.090 460	25.18	7.59
0.100	13 258.70	0.166 99	37.89	8.36
0.300	34 067.25	0.375 98	35.42	6.10
0.700	30 910.39	0.978 84	28.00	6.94
1.00	96 651.56	1.2374	35.59	7.70
3.00	229 541.16	3.9656	25.55	3.51
7.00	580 569.41	8.8850	22.63	4.41
10.0	771 085.47	12.882	28.32	1.65
low QC (0.500)	31 374.80	0.649 16	54.80	8.38
high QC (5.00)	120 606.44	6.8504	67.65	6.10

Table 2Normalization to the Ramipril Internal Standard Improves Accuracy in Enalapril Quantification (n = 5)^a

trial	quality control (μ M)	raw accuracy (%)	ratio accuracy
1	0.500	75.4	89.7
	5.00	18.7	99.7
2	0.500	50.2	97.2
	5.00	17.4	88.8
3	0.500	81.9	96.7
	5.00	48.5	85.4
4	0.500	97.6	94.9
	5.00	45.9	99.4
5	0.500	28.7	86.0
	5.00	10.2	93.8
avg	0.500	66.8	92.9
	5.00	28.2	93.4

^a Accuracies are reported as the difference from 100% of the absolute value of the percent error.

Table 3

Normalization to the Isotopically Labeled Internal Standard Improves Precision in the Quantitative Analysis of PMZ, ENP, and VPM (n = 5)

	concn (μ M)	avg raw (abundance)	avg ratio (promethazine/promethazine-d3)	raw standard deviation (%)	ratio standard deviation (%)
PMZ	0.700	61.40	0.1286	16.72	3.54
	1.00	107.79	0.17000	22.23	12.92
	3.00	409.13	0.52653	36.60	9.57
	7.00	747.87	1.1193	32.97	6.43
	10.0	735.92	1.8532	34.83	6.18
	30.0	1 214.63	4.8739	23.28	4.55
QC (5.00)		481.02	0.73438	32.52	8.23
	concn (μ M)	avg raw (abundance)	average ratio (enalapril/enalapril-d5)	raw standard deviation (%)	ratio standard deviation (%)
ENP	0.700	96.47	0.1780	42.78	15.08
	1.00	128.44	0.21830	38.24	16.50
	3.00	436.16	0.74059	52.52	21.22
	7.00	527.00	1.4113	51.99	11.14
	10.0	720.31	2.6003	49.39	16.06
	30.0	832.51	4.9275	38.57	12.09
QC (5.00)		381.43	0.97135	51.95	16.34
	concn (μ M)	avg raw (abundance)	avg ratio (verapamil/verapamil-d3)	raw standard deviation (%)	ratio standard deviation (%)
VPM	0.700	118.14	0.1664	27.00	12.82
	1.00	268.46	0.29214	29.31	12.89
	3.00	1 028.24	0.90239	38.06	7.68
	7.00	1 785.75	1.9939	44.35	2.59
	10.0	1 924.66	3.2225	41.40	5.89
	30.0	3 349.35	9.1365	29.04	8.10
QC (5.00)		1 141.22	1.3501	39.68	5.47