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Comparison of MALDI to ESI on a Triple Quadrupole Platform for Pharmacokinetic Analyses

Dietrich A. Volmer,*,† Lekha Sleno,‡ Kevin Bateman,§ Claudio Sturino,§ Renata Oballa,§ Timo Mauriala,§ and Jay Corr||

Medical Research Council, Human Nutrition Research, Elsie Widdowson Laboratory, Cambridge, United Kingdom, Donnelly Center for Cellular and Biomolecular Research, University of Toronto, Ontario, Canada, Merck Frosst Centre for Therapeutic Research, Kirkland, Quebec, Canada, and MDS Analytical Technologies, Concord, Ontario, Canada

This present work describes the systematic experimental comparison of electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) for pharmacokinetic (PK) analysis of two drug candidates from rat plasma using single reaction monitoring (SRM) on a triple quadrupole mass spectrometer. The electrospray assay is an established method using a fast liquid chromatography (LC) separation of the sample extracts prior to mass spectrometry analysis. The novel MALDI assays measured the concentration levels of the drug candidates directly from the spotted sample extracts. Importantly, for both LC-ESI and MALDI the same solid-phase sample extraction protocol, internal standards, triple quadrupole mass analyzer platform, and SRM conditions were used, thus effectively standardizing all experimental parameters of the two assays. Initially, analytical figures of merit such as linearity, limit of quantitation, precision, and accuracy were determined from the calibration curves, indicating very similar performance for both LC-ESI and MALDI. Moreover, the LC-ESI rat plasma concentration time profiles of the drug candidates after orally dosing the animals were accurately reproduced by the MALDI assay, giving virtually identical PK results. The direct MALDI assay, however, was able to generate the data at least 50× faster than the LC-ESI assay. It is shown in this study that analyzing the entire PK curve for one animal took less than 2 min using MALDI (with five replicate analyses per sample), whereas the corresponding LC-ESI assay required 80 min, however, allowing only two replicate measurements in that time frame.

Current drug discovery technologies such as parallel syntheses, combinatorial library analyses, or high-throughput screening in absorption, distribution, metabolism, and excretion (ADME) and pharmacokinetic (PK) studies easily generate many more samples than can be analyzed with any conventional liquid chromatography−mass spectrometry (LC−MS) system. The LC−MS instrument often is the bottleneck in these applications. In PK analyses, cleaned-up plasma samples are most often analyzed by liquid chromatography with MS detection.[1] The extraction of the drugs from plasma by protein precipitation, liquid/liquid extraction (LLE), or solid-phase extraction (SPE) can be readily automated in the 96 or 384-well plate formats to increase throughput levels.[2] The major contributor to total PK analysis time remains the chromatography step, due to the actual separation time as well as LC overhead contributions.[3] Even with new LC developments such as monolithic columns[4] or ultra-performance liquid chromatography (UPLC),[5] total LC analysis times are still on the order of several minutes. Parallel separation and multiplexed mass spectrometry systems have been developed and applied to PK analyses.[3] In practice, however, these systems have not found widespread use in the pharmaceutical industry due to limited ruggedness and carry-over problems. Specialized mass spectrometry platforms such as automated chip-based nanospray[6] or RapidFire mass spectrometry,[7] however, allow ultrahigh-throughput analysis (<5 s sample) for screening of certain biochemical assays without the need for prior chromatography.

In order to significantly improve throughput for regular drug assays, this study investigates matrix-assisted laser desorption ionization (MALDI) as an alternative to LC−MS. MALDI mass spectrometry has been successfully used for quantitative analysis of small molecules, including pharmaceutical drugs.[8−10] A PK assay using MALDI has two inherent advantages. First, the sample "injection" step is much faster. The analogous step to the LC−

* Corresponding author. Dr. Dietrich A. Volmer, Medical Research Council, Human Nutrition Research, Elsie Widdowson Laboratory, Fulbourn Road, Cambridge CB1 9NL, United Kingdom. Phone +44 (0)1223 43 7550, fax +44 (0)1223 43 7515, e-mail Dietrich.Volmer@mrc-hnr.cam.ac.uk.

† Medical Research Council.

‡ University of Toronto.

§ Merck Frosst Centre for Therapeutic Research.

|| MDS Analytical Technologies.

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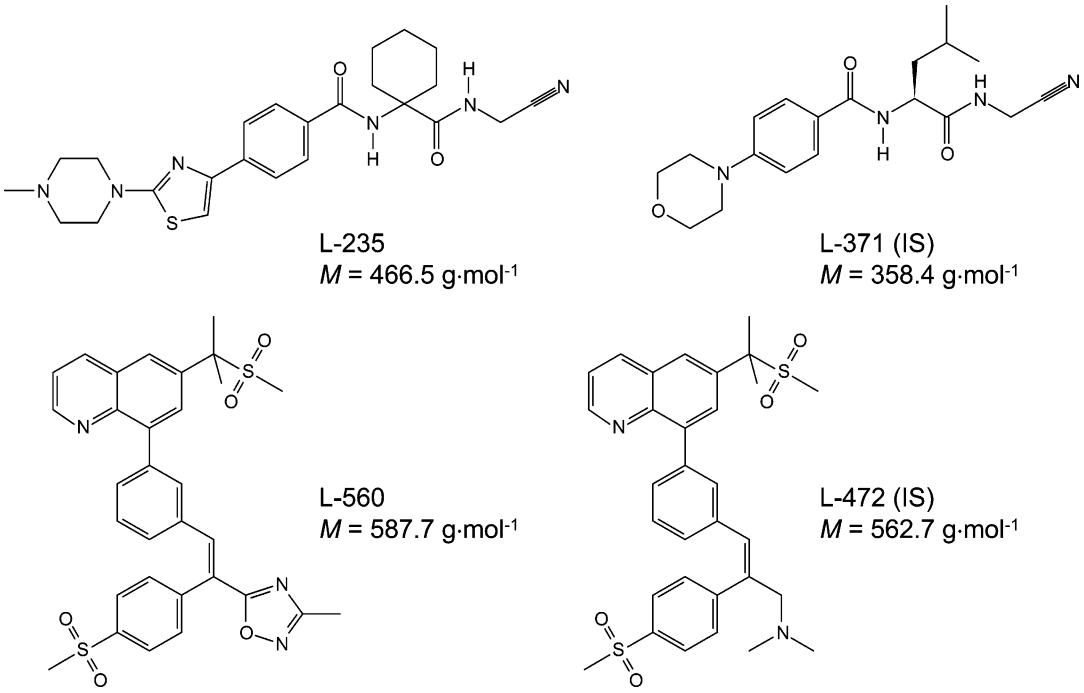

Figure 1. Chemical structures of the two investigated drug candidates and their internal standards.

MS autosampler injections is the sample spotting process onto the MALDI plates. High-density spotting of extracts from SPE plates can be readily done in parallel using multichannel liquid dispensing systems in a highly automated fashion with negligible overhead contributions. More importantly, the employed MALDI technique has no LC separation stage; extracts are directly spotted onto the plates. Sampling of MALDI spots using high-frequency lasers usually takes no more than a few seconds. We have previously demonstrated[11−14] the suitability of MALDI for quantitative analysis of small drug molecules from complex sample matrixes. In those studies, we utilized a high repetition rate laser MALDI source installed on a triple quadrupole (QqQ) instrument, allowing us to circumvent the interfering isobaric interferences from the MALDI matrix in the low m/z range by implementing the single reaction monitoring (SRM) mode. The figures of merit of the MALDI QqQ assays were quite comparable to similar published electrospray ionization (ESI) LC−MS assays,[13,15] because the large number of spectra generated by the high-frequency laser improved the precision of analysis over conventional MALDI applications. The use of SRM on the QqQ instrument enabled[12] specific and sensitive detection in complex biological materials. Furthermore, it was recently demonstrated[16,17] that established sample preparation techniques for plasma (i.e., SPE and LLE) provide sufficiently clean extracts, from which cocrystallization of analyte and MALDI matrix was readily possible for quantitative MALDI analysis.

This study provides a systematic comparison of discovery PK analyses for two drug candidates in rat plasma samples, using electrospray ionization and MALDI on a triple quadrupole mass spectrometer. Discovery PK is aimed at analyzing many compounds in the shortest possible time to enable fast decision-making. The approach is error-tolerant since at this stage the compounds do not show favorable PK characteristics, and the goal is to eliminate these compounds quickly so that the resources to develop more definitive and rigorous methods is aimed at the "better" compounds. The structures of the two investigated candidate drugs are shown in Figure 1 along with the internal standards chosen for analysis. L-006235 ("L-235") was previously a candidate drug for treatment of osteoporosis[18] and L-454560 ("L-560") was developed for asthma.[19] Initially, it was of interest to investigate whether the new MALDI approach was giving accurate results, by comparing the measured concentrations to those from the established LC-ESI assay. Other important figures of merit such as precision, dynamic range, limits of quantitation, speed of the assay, as well as the PK curves were subsequently determined and compared. Importantly, the rat plasma samples were processed using a well-established sample preparation protocol and

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simply split for LC-ESI and MALDI, and the analyses were conducted on virtually identical triple quadrupole mass analyzer platforms, with the same internal standards and tandem mass spectrometry conditions

EXPERIMENTAL SECTION

Chemicals. Two drugs, L-006235 ("L-235") and L-454560 ("L-560"), as well as their internal standards, L-406371 ("L-371") and L-457472 ("L-472") (Figure 1), were from Merck Frosst (Montreal, QC, Canada). Formic acid was purchased from Sigma-Aldrich (Mississauga, ON, Canada). Acetonitrile, methanol (Caledon, Georgetown, ON, Canada), and Milli-Q organic-free water (Millipore, Bedford, MA) were used as solvents. The MALDI matrix stock solution was α-cyano-4-hydroxycinnamic acid (CHCA) solution (6.2 mg mL^{-1} in 90/10 methanol/acetonitrile) from Agilent (Wilmington, DE).

Animals. The animal experiments were conducted at the Merck Frosst Centre for Therapeutic Research in Montreal, QC, Canada. The Animal Care Committee at Merck Frosst approved all procedures according to the guidelines established by the Canadian Council on Animal Care. Sprague Dawley rats ($n = 3$ per compound) with an average mass of 300 g were used in these studies.

Plasma Collection and Preparation. L-235 and L-560 were dosed orally at 25 mg kg^{-1} in 1% methocel as vehicle and 20 mg kg^{-1} in 0.5% methocel, respectively ($n = 3$ rats each). Blood samples were collected at 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h postdosing in addition to a predose sample. Whole blood (400 μL) was collected into heparinized tubes and centrifuged (10 min, 1000*g*) to separate plasma and red blood cells. All samples were prepared in one batch, at the same time, and simply split for ESI and MALDI analysis. Plasma (125 μL) was loaded into preconditioned (200 μL of methanol, 200 μL of H_2O) solid-phase extraction plates (Oasis HLB μelution; Waters, Milford, MA) followed by internal standard solution (150 μL) and a wash step (H_2O, 200 μL). The compounds were eluted with acetonitrile (100 μL) containing 0.1% formic acid. Standard curves for calibration were obtained from blank plasma using the above sample preparation procedure after spiking 270 μL of plasma with 30 μL of stock solution of the drugs (0, 0.1, 0.25, 0.5, 1.0, 2.0, 5.0, 10.0, 25.0, 50.0 μg mL^{-1}). Quality control (QC) samples were prepared at 0.035, 0.075, 0.35, and 0.75 μg mL^{-1} in plasma using the same procedure as for the standard curves. For LC-ESI-MS, the samples were taken to dryness under vacuum (Genevac Evaporator, Valley Cottage, NY) and reconstituted in a 1:1 mixture of H_2O and acetonitrile (100 μL). For MALDI analysis, the samples were evaporated to dryness and reconstituted in a 1:1:1 mixture of methanol, H_2O, and stock MALDI matrix solution (50 μL). In-house software was used for PK calculations.

LC-Electrospray-MS. Quantitation of L-235 and L-560 in plasma was performed using an MDS Sciex 4000 QTRAP (Concord, ON, Canada) tandem MS instrument equipped with a Turbo V Ionspray source operated in SRM mode under positive ionization. A collision energy (CE) of 35 V was used for L-235 and its IS and 40 V for L-560 and IS. The following transitions were monitored: L-235, m/z 467 → 286; L-371 (IS), m/z 359 → 190; L-560, m/z 588 → 509; L-472 (IS), m/z 563 → 484. The LC system consisted of Shimadzu (Columbia, MD) LC-10AD pumps, DGU-14A degasser, and Sil-HTC autosampler. The separations were performed on a Phenomenex (Torrance, CA) Luna C18 (50 mm × 2.1 mm) column at 500 μL min^{-1} flow rate. An acetonitrile/H_2O gradient (10−90% acetonitrile in 1.5 min) was used to elute the target compounds after injection of 5 μL of sample solution.

MALDI-MS. The sample solutions (1 μL) were manually spotted on polished stainless steel plates (123 mm × 81 mm, 384 OptiTOF plates; Applied Biosystems, Framingham, MA) with wells arranged in a 24 × 18 array. An MDS Sciex API 4000 triple quadrupole instrument with a prototype MALDI source containing a high repetition rate (1 kHz) frequency-tripled (355 nm) Nd:YAG laser (PowerChip NanoLaser, JDS Uniphase, San José, CA) was employed in the positive ion mode. Sample spots for each analyte/IS pair were analyzed with linear rastering of the MALDI plate (~750 ms/spot) and, subsequently, plotting peak area ratios of analyte/IS vs concentration of analyte. With the use of this procedure, ~750 laser shots were acquired and averaged for the analyte and the IS pair. More details on the experimental setup can be found elsewhere.[12−14] Each standard curve and QC series were spotted three times with each individual concentration level repeatedly dispensed for five times. Similarly, three PK sample sets from three rats for each compound were spotted fives times for each time point. The collision energies and SRM transitions were identical to those described in the LC-ESI-MS section above.

RESULTS AND DISCUSSION

This study methodically compares results from discovery PK analyses of two Merck drug candidates from rat plasma samples using a novel MALDI approach and an established LC-electrospray-MS assay and assesses the suitability of quantitative MALDI for PK analysis. While the electrospray assay utilized a short LC separation followed by triple quadrupole (QqQ) MS/MS detection, the system used for quantitative MALDI was a combination of a high-frequency laser MALDI source with a QqQ mass spectrometer, without prior LC separation. The use of specific precursor ion/product ion combinations for the target analytes circumvents the isobaric chemical noise from the MALDI matrix. In the MALDI assay, the samples were spotted on the MALDI plates and then analyzed by linear rastering of the sample spots (Figure 2a). The analyte ion signals were monitored in the single reaction-monitoring (SRM) mode of the MS and the peak areas were subsequently integrated. The high repetition rate laser of the MALDI source employed here generates a virtually continuous ion beam during the sample ablation and ionization from the sample spots, which is then ideally combined with the almost 100% duty cycle SRM mode of the QqQ instrument. We have previously shown[13] that by operating above a certain laser pulse energy level, a complete ablation of the cocrystallized matrix/sample mixture in the laser's path can be achieved without significant losses from fragmentation reactions in the ion source. Complete ablation ensures that laser energy-dependent fluctuations in the ablation and ionization rate do not affect precision and accuracy of the assay. In addition, the high laser sampling rate (1 kHz) leads to a significant number of spectra for massive data averaging and significantly improved analytical precision. Naturally, an internal standard with similar physicochemical properties gives optimum results in quantitative analysis. In a previous study, we demonstrated[14] that closely matching the crystallization behavior of analyte and internal standard yields significantly improved precision and linearity, even though a homogeneous distribution

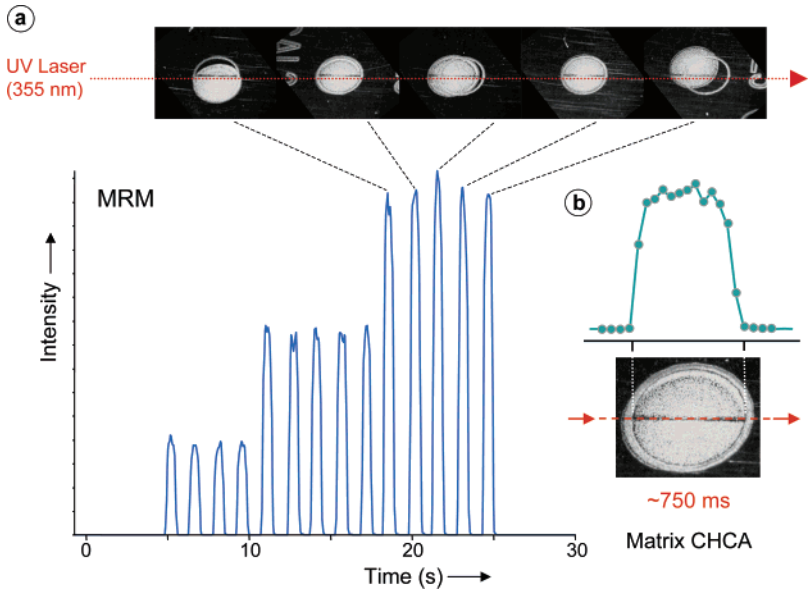

Figure 2. Schematic representation of direct MALDI QqQ analysis. Sampling was achieved by linear rastering down each column of the 384-well format sample plate, which translates into intensity traces for the SRM transitions with subsequent integration for quantitative results (a). The rastering speed and dwell times for the SRM transitions (analyte and IS) were chosen to yield approximately 15 data points across each peak (~750 ms width at the base, depending on sample spot quality) (b).

throughout the sample spot was not necessarily achieved. In the present study, however, we deliberately chose the same internal standard for the investigated drug candidates as was used in the established LC−MS assay, to mimic current practice in PK laboratories.

For this comparison, it was important that all experimental parameters were standardized for both assays, except for the ionization source. In addition to using the same sample preparation procedure and internal standards, the triple quadrupole platform was identical for both ESI and MALDI, allowing identical SRM transitions and collision cell conditions to be implemented.

MALDI and LC-ESI Assay Description. As described above, the MALDI analyses were performed directly from the SPE extracts, without prior chromatographic separation. The SRM dwell times were chosen to give a sufficient number of data points across each peak. With spot sizes on the order of 1.5 mm, monitoring the analyte and its internal standard for 20 ms each (plus 10 ms electronic pause and settle times) resulted in ~15 data points during ablation of one spot, thus allowing an accurate reconstruction of the time profile of the peak (Figure 2b). The total analysis time for each spot was approximately 750 ms.

The established LC-ESI assay used a fast generic LC gradient allowing the separation of the drug candidates an their internal standards within 2 min (Figure 3a,d). The total analysis time for each sample, including chromatographic run time plus injection and column equilibration overhead contributions, was approximately 5 min.

For both assays, identical SRM transitions were selected. The CID spectra of both drug candidates and their internal standards are shown in Figure 3. Except for L-235, the spectra were very simple, with one major fragment ion, making them ideal for sensitive SRM analyses. The main fragmentation routes for L-235 involved cleavage of the central amide bond to yield the product ion at m/z 286, which was used for SRM (Figure 3b). Other abundant ions can be rationalized by neutral loss reactions of C_3H_7N (from the piperazinyl group) and HCN from both precursor ion and m/z 286. L-560 and its internal standard L-472 almost exclusively exhibited a single radical loss of $SO_2CH_3^{\bullet}$ (Figure 3e,f).

Importantly, analogous fragmentation channels were chosen for the analyte and its internal standard, for both drug candidates under investigation using LC-ESI and MALDI.

Analytical Figures of Merit. The investigated concentration range was 0.01−50 μg mL^{-1} for L-235 and L-560. Three calibration curves in blank plasma were prepared for each drug candidate over this concentration range, with each calibration point measured five times (3× for LC-ESI). Four QC samples were assayed throughout the concentration range.

The calibration curves showed a good linear range up to 2.5 μg mL^{-1} for L-235 and L-560, for both ionization techniques, as seen in Table 1. The slopes of both assays were very similar, with the MALDI assay exhibiting a slightly more negative intercept. The correlation coefficients (r^2) were ≥0.996 for LC-ESI and MALDI, thus meeting generally accepted criteria. The limit of quantitation (LOQ) was set at a level of 0.025 μg mL^{-1} for the MALDI assay as the precision values seen for $c \geq 0.025$ μg mL^{-1} were always <10% relative standard deviation (RSD) in plasma. If this criterion was lowered to 15% RSD, the LOQ would improve to 0.01 μg mL^{-1}. Furthermore, if the LOQ was defined using a signal-to-noise-ratio, e.g., S/N = 10, as is often done in published ADME or PK studies, the LOQ values would be drastically lower, viz., less than 1 ng mL^{-1}. The calibration and LOQ results for the established LC-ESI assay were very similar (Table 1).

Typical precision values for the ratios of analyte-to-IS for LC-ESI were approximately 10% RSD between injections within the quantitation range (0.025−2.5 μg mL^{-1}), while MALDI consistently delivered significantly better results of <5% RSD as illustrated in Figure 4 (the precision decreased for $c \ll$ LOQ, as shown for the 24 h time point, where the level of the parent drug was approaching the detection limit). This is an important finding for the MALDI assay, and we attribute it to the minor instrumental

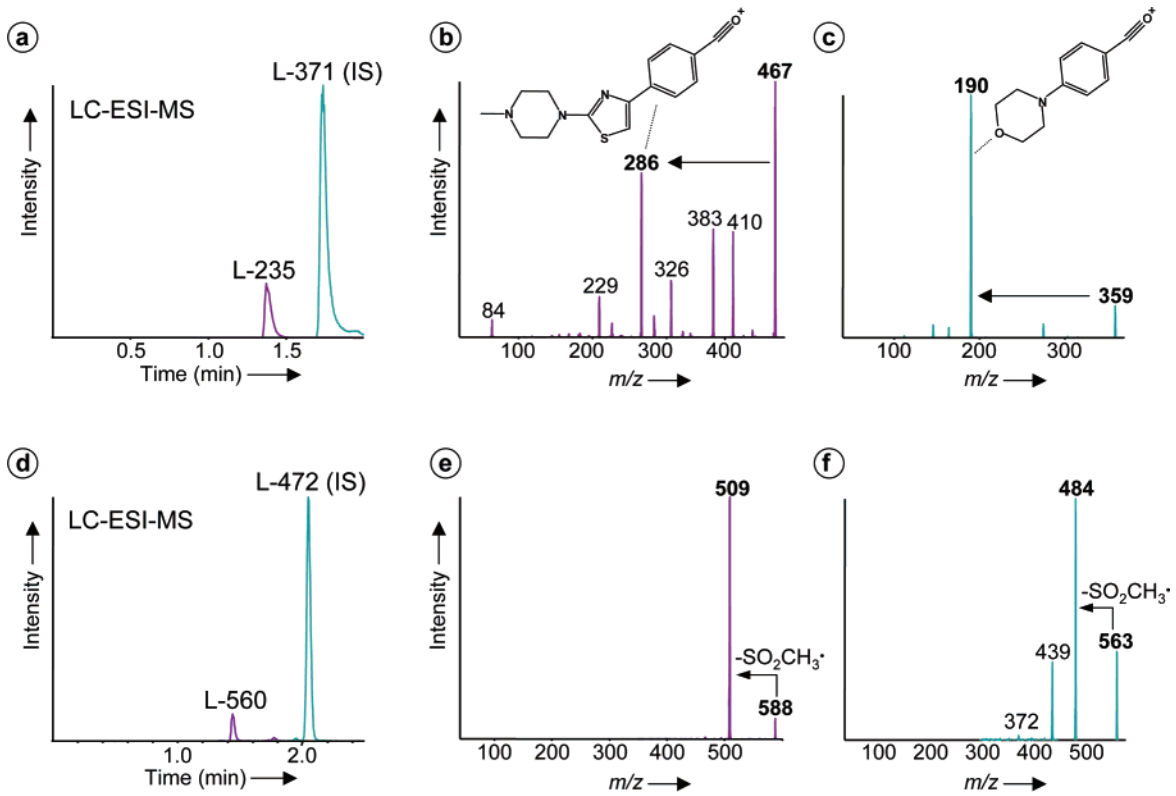

Figure 3. Electrospray SRM chromatograms for the investigated drug candidates and their internal standards. The chromatograms show examples of plasma PK samples for L-235 (a) and L-560 (d). MALDI CID spectra and selected SRM transitions are shown in panels b and c for L-235 and its IS and in panels e and f for L-560 and IS.

Table 1. Assay Calibration Data and PK Results for LC-ESI and MALDI Analyses of Drugs L-235 and L-560 in Rat Plasma Samples[a]

	$y = ax + b$, r^2 (0.025−2.5 μg mL^{-1})	LOQ (μg mL^{-1})	c_{max} (μg mL^{-1})	$t_{1/2}$ (h)	AUC (μg mL^{-1} h)
L-235					
LC-ESI	$0.5058x - 0.0005$, 0.998	0.025	0.57	1.1	1.3
MALDI	$0.4029x - 0.0117$, 0.997	0.025	0.53	1.1	1.6
L-560					
LC-ESI	$0.5133x + 0.0002$, 0.997	0.025	0.53	8.1	5.4
MALDI	$0.3862x - 0.0103$, 0.996	0.025	0.59	10.5	5.9

[a] The calibration curves were obtained from spiked blank plasma samples using the sample preparation procedure outlined in the Experimental Section; the PK data (c_{max}, $t_{1/2}$, and AUC) were determined from averaged ($n = 3$) animal data (Figure 5).

fluctuations on this prototype instrument (for example from laser light delivery and movements of the experimental *x,y* stages), observed in the very short time frame of repeat analyses (five replicates of a sample are analyzed in ~10 s including the spot-to-spot laser positioning time) as compared to LC-ESI, where 5 min go by before the same sample is repeated.

The accuracies of the two assays were determined by analyzing QC samples (see Experimental Section for details on the concentration levels). They were expressed as relative errors (% RE) of the averaged measured concentration from the theoretical values. For LC-ESI, the intraday accuracy was between −9 and +11% RE for L-235 and L-560, with a similar deviation range seen for the MALDI assay (−7 to +12% RE), in the quantitation range of the assay.

Obviously, the most important advantage of the MALDI assay over the LC-ESI assay is the speed of analysis. This will be discussed in the next section.

Application to Rat PK Studies. The new MALDI assay was compared with LC-ESI in two drug discovery studies, using three animals each (see Experimental Section for details on animal dosing and sample preparation). Figure 4 shows a representative MALDI analysis of an entire PK curve of drug candidate L-235 for one animal. Note that the analysis time for the complete time profile (0−24 h), including five replicates for each sample, took less than 1.8 min, including large dead times due to inefficient manual operation of the prototype MALDI source in the rastering mode of operation. Figure 5 summarizes and compares the time profiles for drug candidates L-235 and L-560 obtained from the MALDI and LC-ESI assays. As is obvious from the figure, there is very good agreement between PK data produced from both assays (note that the error bars in Figure 5 represent the variations from the individual animals. The same similarity between LC-ESI and MALDI, as seen in these averaged time profiles, is also reflected in the PK curves for each individual animal.). Table 1

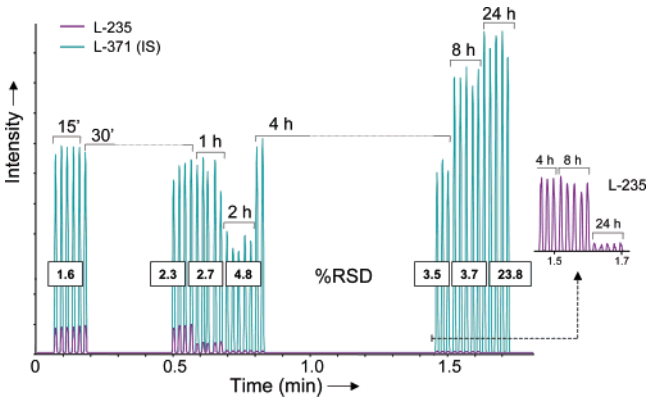

Figure 4. Direct MALDI analysis of entire rat PK curve for L-235. The precision for the ratios of analyte-to-internal standard ($n = 5$) is shown in the boxes for each peak cluster (time point). Note that time gaps between peak clusters are due to the inefficient manual setting and control of the laser raster across sample spots. Differences of absolute peak intensities for IS and analyte between PK time points are caused by small variations of the SPE process and sample pipetting, which are compensated for by the IS.

further illustrates the excellent correlation between the two methods: the c_{max}, $t_{1/2}$ as well as the area-under-the-curve (AUC) values derived from the time profiles are in very good agreement for both L-235 and L-560. Finally, we summarized all experimental data points from QC samples as well as animal experiments in the correlation diagram seen in Figure 6. Again, an excellent agreement was found between LC-ESI and MALDI; r^2 was >0.99 for the shown comparison in the figure.

With the equality of the analytical output of the two assays established, it was important to compare the throughput levels, where MALDI was expected to clearly excel. Not surprisingly, the speed advantage over LC-ESI was remarkable: while analysis of one PK curve (one rat; for reasons of practicality, the LC-ESI samples were only analyzed in duplicate) took approximately 80 min (16 injections), the same sample set could always be analyzed in less than 1.8 min on the MALDI-QqQ instrument (in pentuplicate, 40 sample spots). Analyzing the entire PK study of three animals per drug took approximately 4 h with LC-ESI but only slightly more than 5 min with MALDI. Using higher spotting density would further speed up the MALDI analysis. The manual spotting routine used here was not very efficient in terms of utilizing the available spots on the plate. Thus, more frequent repositioning of the laser to the beginning of a new sample row was required, as can be seen from the gaps in Figure 4, resulting in approximately 50% overhead contribution during most of our analyses. This effect was amplified with the prototype MALDI source due to the requirement to manually select and initiate laser rasters across sample spots.

One major concern during this study was the potential for ion suppression in MALDI, as neither an LC separation nor isotope standards were used for quantitation. If the SPE cleanup procedure does not efficiently remove endogenous interferences, ion suppression can be expected. The excellent agreement of the measured concentrations between LC-ESI and MALDI, for QC and PK samples (Figure 6), however, does indicate that the extracts were sufficiently clean for efficient crystallization and desorption in MALDI and that ion suppression was negligible.

Obviously, there are potential areas of concern for MALDI in PK analyses. Specifically, the described MALDI assay may not be able to resolve certain isomeric interferences, unless an isomer-specific fragment ion can be selected for SRM. If isomers of the

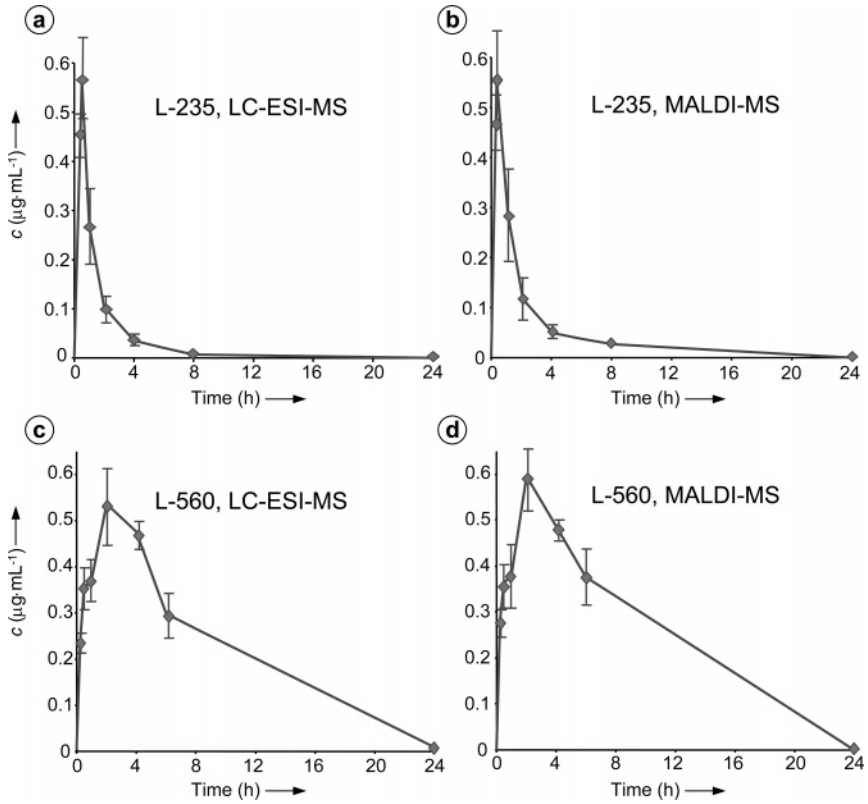

Figure 5. Averaged rat plasma concentration profiles after oral dosing of L-235 and L-560 ($n = 3$ rats for each compound) obtained from the LC-ESI (a,c) and MALDI (b,d) assays. The error bars represent the variation between animals (SD, $n = 3$).

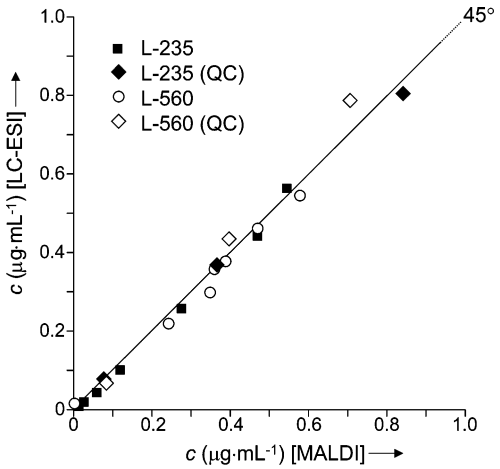

Figure 6. Correlation of the averaged rat plasma concentrations of L-235 and L-560 as well as QC samples using LC-ESI versus direct MALDI (slope = 1.03, $r^2 = 0.991$). Note that the line in the *x,y* diagram corresponds to the ideal behavior, i.e., slope = 1.

drug or metabolite are present in the sample and the isomers' MS/MS spectra are the same, the MALDI assay may overestimate the concentration of the compound of interest, as there is no chromatographic separation. In addition, fragile ions of circulating metabolites such as acyl-glucuronides or *N*-oxides could potentially revert back to the precursor ion of the parent drug after formation in the source, which again would lead to an overestimation of the parent drug's concentration. Finally, if the sample cleanup procedure does not efficiently remove endogenous interferences, ion suppression may become important, especially if no suitable internal standards are available for the drugs of interest. From work conducted in our laboratory and results shown by other groups,[12,17] however, it can be concluded that sample preparation techniques commonly used for LC−MS assays of drugs in plasma, such as protein precipitation and solid-phase and liquid/liquid extraction, provide sufficiently clean extracts, from which most of the analyte's signal is recovered as compared to the pure standards.

Obviously, more studies are required, including extensive method validations, to fully evaluate the performance of direct MALDI triple quadrupole MS in ADME or PK assays. The present authors will leave this comparison to established drug discovery laboratories in the pharmaceutical industry.

CONCLUSIONS

The present work compared two ionization sources for discovery PK analysis of drug candidates from rat plasma. Both assays used the same sample preparation protocol and triple quadrupole platform, thus effectively standardizing the most important parameters of the two techniques. The established method is an LC−MS assay utilizing electrospray ionization, to which the novel MALDI assay was directly compared in terms of quantitation limits, linearity, precision, and accuracy. In our opinion, this study clearly demonstrates that the MALDI QqQ technique, in the SRM mode, is a serious alternative to the established LC-ESI method. All analytical figures of merit were very similar for the two investigated drug candidates, and the PK curves determined for rat plasma were in excellent agreement. The direct MALDI assay, however, was at least 50× faster than the LC-ESI assay. In our study, the MALDI samples were manually spotted, which added approximately 10 min to the previously described total instrumental analysis time. This spotting time, however, is still considerably shorter than the equivalent contribution in the LC−MS assay, viz., the autosampler injection time. Furthermore, these spotting times can be shortened significantly by using readily available commercial multichannel liquid dispensers for MALDI.

Importantly, the analytical methods for MALDI do not necessarily have to be developed on an LC−MS system first and then transferred. It is entirely feasible to develop assays on the MALDI QqQ platform alone. With the use of CHCA as a matrix compound, it has been shown[16] that the applicability range of MALDI is comparable to electrospray in terms of ionizing ability for a wide range of drug compounds. Optimizing SRM conditions from the precursor ions formed from the MALDI spots as well as calibration and validation experiments are virtually identical to the corresponding ESI assay development experiments. In some cases it is advantageous to initially conduct LC−MS experiments, however, viz., when interferences from endogenous or exogenous compounds in the samples can be expected. As described above, isomeric interferences could potentially lead to an overestimation of the concentration as well as the presence of fragile metabolites such as *N*-oxides or acyl-glucuronides could. Finally, endogenous plasma components have to be efficiently removed to avoid ion suppression if no suitable internal standards are available for the drugs of interest.

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