Response Normalized Liquid Chromatography Nanospray Ionization Mass Spectrometry

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The widely different LC-MS response observed for many structurally different compounds limits the use of LC-MS in full scan detection mode for quantitative determination of drugs and metabolites without using reference standard. The recently introduced nanospray ionization (NSI) technique shows comparable MS response for some compounds under non-LC-MS conditions. However, in the presence of numerous endogenous compounds commonly associated with biological samples such as urine, plasma, and bile, LC-MS is required to separate, detect, identify, and measure individual analytes. An LC-NSI-MS system was devised and the MS response obtained in this system for a variety of pharmaceutical drugs and their metabolites. The set-up involves two high-performance liquid chromatography (HPLC) systems, a chip-based NSI source and a quadrupole-time-of-flight (Q-TOF) mass spectrometer. Herein this is referred to as the response normalized-liquid chromatography NSI-MS (RNLC-NSI-MS) system. One HPLC unit performs the analytical separation, while the other unit adds solvent post-column with an exact reverse of the mobile phase composition such that the final composition entering the NSI source is isocratic throughout the entire HPLC run. The data obtained from four different structural classes of compounds [vicriviroc (VCV), desloratadine (DL), tolbutamide, and cocaine] and their metabolites indicate that by maintaining the solvent composition unchanged across the HPLC run, the influence of the solvent environment on the ionization efficiency is minimized. In comparison to responses obtained from radiochromatograms, responses from conventional LC-ESI-MS overestimated the VCV and DL responses, respectively, by 6- and 20-fold. Although VCV and DL responses obtained using LC-NSI-MS are within 2- to 6-fold from the respective radiochromatographic responses, the response normalization modification results in nearly uniform LC-NSI-MS response for all compounds evaluated. (J Am Soc Mass Spectrom 2007, 18, 1891–1899) © 2007 American Society for Mass Spectrometry

Liquid chromatography-mass spectrometry (LC-MS) with atmospheric pressure ionization (API) has become the preferred tool in the pharmaceutical industry for qualitative and quantitative determination of drugs and their metabolites in biological matrices. The widely different LC-MS responses observed for compounds with structural changes, such as those resulting from metabolism, however, limits the use of LC-MS in full scan detection mode for quantitative determination of drugs and/or metabolites. LC-MS/MS based assays involving selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) techniques are routinely used for quantitative determination of drugs and/or metabolites in biological matrices [1–7]. LC-MS/MS techniques for quantitation require reference and internal standard(s), so that LC-MS/MS responses from unknown quantities of relevant drug-related peaks can be related back to responses

obtained using known quantities of each analyte. Usually a standard curve is generated for each and every analyte to be quantified [6]. Although reference standards may be available for the parent drug, it is very unlikely to have standards for metabolites available in early stage of drug discovery and development thereby making conventional LC-MS/MS based quantitation of metabolites impractical.

Nanospray ionization (NSI), introduced by Wilm and Mann in the 1990s [8], has been used to identify proteins [9–13] and drug metabolites [14], quantify pharmaceutical drugs [15, 16], study absorption, distribution, metabolism and excretion (ADME) properties of drugs [17], and employed in various other applications [12, 13, 15, 16, 18–34]. NSI-MS has the following advantages over conventional flow ESI-MS: (1) higher sensitivity, (2) lower rate of sample consumption, and (3) improved signal-to-noise ratio (S/N). Although both on- and off-line NSI-MS have been developed, off-line NSI-MS methods have been more frequently used because of the technical difficulties associated with interfacing conventional flow LC systems with NSI and

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interrogating an ion of interest with multiple MS and MS/MS experiments on a given LC time scale. Furthermore, the flow rate at which a compound is introduced into the mass spectrometer influences ionization efficiency and therefore variability in LC-MS response [35–37].

Recently, using a silicon microchip, Hop et al. [36] and a custom-built pulled capillary, Valaskovic et al. [29], studied the NSI-MS response of different classes of drugs and their metabolites by infusing directly into the NSI-MS system. Hop et al. [36] compared ionization efficiencies of drugs and metabolites in conventional flow (500- μ L/min) LC-MS using electrospray ionization with that in a microchip based infusion NSI-MS and showed that in the latter technique the ionization efficiencies vary to a much lesser extent. Valaskovic et al. [29] demonstrated that as the flow rate in nanospray is reduced, the differences in ionization efficiencies among various compounds also reduce and at a flow rate of about 10 nL/min most compounds show similar ionization efficiency.

These encouraging results were obtained by direct infusion of analytes into NSI-MS systems. However, analysis of drugs and metabolites are usually performed from biological matrices or extracts. Metabolites, which are likely to be isobaric (same modification at different sites) and accompanied by endogenous salts, fatty acids, peptides, and proteins require chromatographic separation before analysis. Therefore, an NSI-MS system coupled to a HPLC is the most desirable approach for metabolite characterization.

In this paper, results from an investigation of an on-line LC-NSI-MS system and the role of mobile phase composition on the variation of ionization efficiencies of different classes of drugs [vicriviroc (VCV), desloratadine (DL, Clarinex), tolbutamide (TOL) and cocaine], and their metabolites are described. The analysis of a series of DL- and VCV-related compounds were performed directly from urine. These data were further compared with those from conventional LC-MS with electrospray ionization using the same gradient for separation.

Experimental

Materials

HPLC grade acetonitrile and methanol were from Burdick and Jackson (Muskegon, MI). Water was purified using the Millipore Milli-Q_{plus} water purification system (Bedford, MA). Acetic acid and ammonium acetate were from Sigma-Aldrich (St. Louis, MO). Cocaine and benzoylecgonine were obtained from Cerilliant Corporation (Round Rock, TX) as 1 mg/mL free base in acetonitrile and methanol, respectively. Solutions were further diluted in 1:1 mixture of methanol:acetonitrile to a final solution containing 20 μ M each cocaine and benzoylecgonine. Tolbutamide (TOL) and hydroxytolbutamide (OH-TOL) were purchased from Sigma-Al-

drich as powders, dissolved and diluted in methanol to a final concentration of 20 μ M. All other reagents and solvents were obtained from commercial sources. A 25 μ L aliquot of the standard mixture was injected for all LC-MS experiments.

Capsules containing 14 C-VCV {1-[[[(4,6-dimethyl-5-pyrimidinyl)carbonyl]-4-[4-[2-methoxy-1(R)-(4-trifluoromethyl)phenyl]ethyl]-3(S)-methyl-1-piperazinyl]-4-methylpiperidine, 14 C-SCH 417690} were formulated, manufactured and released at the Schering-Plough Research Institute (Kenilworth, NJ). As detailed elsewhere [38–41], urine was collected in block intervals for 10 d following a single 50 mg administration of 14 C-VCV (~100 μ Ci) to six healthy volunteers. This was a Phase I open-label study designed to characterize the absorption, metabolism, and excretion of 14 C-VCV. Following centrifugation, a 100 to 150 μ L aliquot of urine (0 to 6 h) from a single subject was injected for all LC-MS experiments. The radioactivity content in the urine was about 60 DPM/ μ L.

14 C-DL [8-chloro-6, 11-dihydro-11-(4-piperidylidene)-5H-benzo [5,6] cyclohepta [1,2-b]pyridine, 14 C-SCH 34117] was synthesized at Schering-Plough Research Institute (Kenilworth, NJ) and had >99% radiochemical purity. As described elsewhere [42, 43], monkeys ($n = 3$) were administered a single oral dose in 0.4% aqueous methylcellulose by gavage at a targeted dose level of 6.5 mg [14 C]DL/kg (80 μ Ci/kg). Urine was collected at selected intervals through 240 h post-dose. Urine samples were pooled by taking 10% to 25% of the volume collected over each time interval. Equal volumes from each individual pool were mixed together to obtain a pooled 0 to 24 h composite urine. Following centrifugation, a 100- μ L aliquot of the pooled composite urine sample was injected for LC-MS analysis with in-line radioactivity detection (FSA). The radioactivity content of the urine was about 500 DPM/ μ L.

LC-ESI-MS

All LC-ESI-MS experiments were performed using a TSQ Quantum mass spectrometer (Thermo Electron Corp., San Jose, CA) equipped with a vendor supplied source operated in the positive ionization mode. ESI source and MS conditions were optimized using reserpine (10 ng/ μ L) to avoid preferential MS conditions for any one of the metabolites or drugs. The mass spectrometer was nominally operated with the spray needle voltage, capillary offset and capillary temperature set at 4.0 kV, 35 V, and 300 $^{\circ}$ C, respectively. For all LC-MS experiments, the Q1 resolution (FWHM, full width at half-maximum) and the first/last mass were set at 0.7 Th and 100/1100 Da, respectively.

A Waters Alliance HPLC system (Alliance Model 2690; Waters Corp., Milford, MA) and a 4.6 mm $\times$ 250 mm Luna Phenyl-Hexyl column (Phenomenex, Torrance, CA) were used for all LC separations. The column was maintained at room temperature. The mobile phase, 95% 10 mM ammonium acetate with 5%

Table 1. Linear LC-MS gradients used in this study

VCV and DL Experiments			Tolbutamide Experiments			Cocaine Experiments		
Time (min)	%A	%B	Time (min)	%A	%B	Time (min)	%A	%B
0	90	10	0	98	2	0	98	2
10	70	30	15	10	90	20	10	90
40	30	70	15.1	98	2	20.1	98	2
40.1	10	90	20	98	2	25	98	2
50	10	90						
50.1	90	10						
60	90	10						

acetonitrile, adjusted to pH 6.0 with acetic acid (A) and 95% acetonitrile with 5% water (B), was delivered at a constant flow rate (1 mL/min). The linear gradients used in this investigation are shown in Table 1.

For all experiments involving radioactive samples, 15% of the column effluent was utilized for LC-ESI-MS experiment while 85% of the column effluent was diverted to a Model 500TR (Packard Instrument Co., Meriden, CT) flow scintillation analyzer (FSA) equipped with a 250- μ L liquid flow cell (Packard Instrument Co., Meriden, CT). Flo-Scint III cocktail (Packard Instrument Co., Meriden, CT) was used as scintillation fluid at a flow rate of 2.4 mL/min. FSA was controlled by FLO-ONE for Windows software (version 3.65, Packard Instrument Co., Meriden, CT). All radioactivity data were processed and integrated using the same software. This simultaneous detection of all drug-derived material by an MS and an FSA provided confirmation of the molecular weight of all radioactive peaks in a simple LC-MS experiment. For all non-radioactive samples, 15% of the flow was diverted to the mass spectrometer and the remainder to waste.

Following LC-MS/FSA analysis, the area of each detected radioactive peak in the FSA was expressed as a percent of peak area of the most abundant peak (set to 100%) in the radiochromatogram. Similarly, from LC-MS spectra, peak areas of the metabolites and the corresponding parent drug were integrated using Xcalibur software (ThermoFinnigan, San Jose, CA) and then normalized to the peak area of the most abundant peak. For comparison the integrated peak areas of metabolites and the parent drug from radiochromatograms (only for VCV and DL) and LC-MS spectra were plotted using Excel (Microsoft Corporation, Red Bank, WA) to generate bar graphs.

Nanoelectrospray Ionization (NSI) Source

All LC-NSI experiments were performed using an automated chip-based Triversa NanoMate (Advion BioSciences, Ithaca, NY) source operated by ChipSoft software operating under Windows XP. The Triversa NanoMate source provided options to perform: (1) infusion from a 96-well sample plate using a rack of

96-disposable conductive pipette tips connected to a robotic mandrill, (2) on-line fraction collection into 96-well plate during LC-MS experiments, and (3) LC-MS experiments using a conductive re-usable pipette tip based LC coupler. All experiments described in this paper used a silicon wafer based microchip consisting of a 20×20 array of non-reusable nozzles fabricated by proprietary etching techniques. Each nozzle had an i.d. of 5.5 μ m and the nozzle design allowed flow rates between 200 and 500 nL/min. For optimal performance, the microchip was positioned about 4.5 mm from the sampling cone of a quadrupole time-of-flight (Q-TOF Global) mass spectrometer (Waters Corporation, Beverly, MA). To improve the nozzle-to-nozzle reproducibility and to maintain continuous spray during an LC run, the ChipSoft software monitored the ion current. When the ion current fell below 20 nA or went over 400 nA, the spray was automatically switched over to the next nozzle.

LC-NSI-MS

All LC-NSI-MS analyses were performed using a Q-TOF Global (Waters Corporation) mass spectrometer equipped with an automated chip based Triversa NanoMate NSI source described above. All MS conditions and NSI source orientations were optimized by infusing reserpine. Typical NanoMate infusion conditions are described elsewhere [15]. For all LC-NSI-MS experiments, the Q-TOF mass spectrometer was operated as follows: ionization mode, positive; spray voltage, 1.7 to 1.9 kV; cone voltage, 45 V; source temperature, 45 °C; collision gas, 1.5 mTorr; scan range, 100 to 1100; inter-scan time, 0.1 s. Data for each sample was acquired over the entire LC run time in the profile mode with mass resolution for reserpine set at 8000 (full width at half maximum).

Chromatographic separations in LC-NSI-MS experiments were achieved using the same linear gradients, mobile phases and analytical column (4.6 mm $\times$ 250 mm Luna Phenyl-Hexyl; Phenomenex, Torrance, CA) used for LC-ESI-MS. The HPLC unit consists of Shimadzu LC-10AD *vp* pumps and a SIL-10AD *vp* autosampler (Shimadzu Corporation, Kyoto, Japan). This system is hereafter termed the "analytical HPLC" system. For all LC-NSI-MS experiments (Figure 1), the column effluent was split such that most (~85%) of the effluent was directed into the FSA (for VCV and DL experiments) or to waste and the remainder (~15%) diverted to the TriVersa NanoMate NSI source. Within the TriVersa NanoMate, the HPLC flow (~15% or 150 μ L/min) was further split to deliver about 200 to 500 nL/min to the NanoMate chip via the LC coupling device and the rest to NanoMate fraction collector or waste. All split ratios were adjusted by varying ID and lengths of PEEK tubing (all connections exterior to NanoMate) and fused silica capillary (all connections within the NanoMate). Split ratios were verified by injecting radioactive samples, collecting effluent, and

Figure 1. A schematic of the experimental set-up for response normalized liquid chromatography nanospray ionization mass spectrometry (RNLC-NSI-MS).

counting aliquots using liquid scintillation spectrometry (LSS). All LC-MS data were processed and integrated using the MassLynx software.

RNLC-NSI-MS

The response normalized liquid chromatography (RNLC)-NSI-MS (Figure 1) experiments utilized two HPLC systems: an “analytical HPLC” and a “response normalizing (RN) HPLC”. The RN-HPLC is used to deliver mobile phase post column with a gradient program exactly reversed from that used for analytical separation [37]. A Waters Alliance HPLC system (Alliance Model 2690; Waters Corp., Milford, MA) was used as the RN-HPLC. The flow rate of the RN-HPLC system was varied between 50 $\mu\text{L}/\text{min}$ to 250 $\mu\text{L}/\text{min}$ and optimized using an equimolar mixture of TOL and OH-TOL. RN-HPLC flow was optimized at 0.15 mL/min. The presplit flow rate of the “analytical LC” system was 1.0 mL/min.

Results and Discussion

The response of different analytes under LC-MS conditions was investigated for both higher flow rate (~ 150 $\mu\text{L}/\text{min}$) ESI-MS and lower flow rate (200 to 500 nL/min) NSI-MS using DL, VCV, and their respective metabolites. The amounts of material injected in the

four experiments reported here are provided in Table 2. The ionization efficiency in LC-NSI-MS was further investigated by reducing the variability in solvent composition that occurs during a gradient analysis by adding a solvent mixture post-column with an exact reverse gradient to that used for analytical separation. The latter procedure is referred hereafter as RNLC-NSI-MS.

The first set of experiments involved LC-MS of urine collected following administration of 50 mg ^{14}C -VCV to healthy male volunteers. A comparison of LC-MS responses of VCV and its metabolites (M2/M3, M15, M35, and M41) (Figure 2) obtained using ESI and NSI as ionization modes with and without RN flow along with the corresponding radioactivity (LC-FSA) data is shown in Figure 3. All LC-MS and LC-FSA data (peak areas) are an average of three separate analyses and the

Table 2. The amounts of material injected

Sample	Volume injected	Amount on column
Tolbutamide and hydroxytolbutamide	25 μL	125 μg each
Cocaine and benzoylecgonine	25 μL	125 μg each
^{14}C -VCV derived radioactivity	150 μL	1.9 μg total
^{14}C -DL derived radioactivity	50 μL	1.6 μg total

Figure 2. Structures of Vicriviroc and its metabolites that were evaluated using NRLC-NSI-MS.

Figure 3. Comparison of LC-MS responses obtained using conventional flow LC-ESI-MS, RNLC-NSI-MS, and radioactivity detection for vicriviroc and its metabolites. [All responses were normalized to the major metabolite (M35)]

responses were normalized to the major radioactive peak. M35 or VCV-O-desmethyl-glucuronide was the major human urinary metabolite and therefore MS responses of all other drug-derived analytes were normalized to this compound to facilitate a comparison. LC-FSA detection is nondiscriminating with respect to molecular weight, chemical structure and is relatively

immune to most matrix effects. As demonstrated in Figure 3, the LC-ESI-MS detector signal differed much more widely for VCV and its metabolites in comparison to those obtained using a radioactivity detector (FSA). Had this study been conducted using non-radiolabeled drug and only the LC-ESI-MS response used (without deriving concentrations from a calibration curve) to esti-

Figure 4. Structures of desloratadine and its metabolites that were evaluated using NR-LC-ESI-MS.

mate relative amounts of metabolites in human urine, unchanged drug (VCV) would have been grossly overestimated (identified as the major urinary drug-related material). Similarly, the relative amounts of all metabolites would have been underestimated. With M35 the major metabolite underestimated by a factor of six.

The MS response obtained in the LC-ESI-MS for the same urine sample shows a marked contrast to those data obtained using LC-ESI-MS (Figure 3). Without any RN flow in LC-ESI-MS, M35 is the most abundant drug-related material as shown by radioactivity traces. The responses for M41, M2/M3, and VCV decreased relative to M35, although, M2/M3 and VCV would still have been overestimated by a factor of 2 to 3. The differences in the NSI-MS response of various compounds in LC-ESI-MS were further reduced when response normalizing (RN) flow was added. The LC-ESI-MS responses of VCV and metabolites with RN flow rates of 0.15 and 0.2 mL/min are comparable to

those obtained on an LC-FSA system. Additional experiments employing 0.16 and 0.18 mL/min RN flow rates did not bring the LC-ESI-MS responses any closer to the radioactivity data. Therefore, the RN flow rate was close to optimal at about 0.15 mL/min. This could be potentially due to the similarity between the RN flow and the flow reaching the NSI source as described in the experimental section, resulting in an isocratic final solvent composition (50:50 = aqueous:organic) reaching the NSI source throughout the LC run in this RNLC-ESI-MS experiment.

While LC-FSA responses showed that M15 was at about 25% of M35, RN normalized LC-ESI-MS responses showed M15 to be about 15% of M35. This discrepancy was carefully evaluated and later attributed to a metabolite at an *m/z* 494 co-eluting with M15. The contribution of this metabolite was inseparable in the radioactivity peak but did not contribute to the NSI response of M15.

The second set of experiments involved pooled (*n* = 3) 0 to 24 h urine collected following oral administration of 8 mg ¹⁴C-DL/kg to male monkeys (Figure 4). In addition to obtaining radiochromatograms using LC-FSA, LC-MS analysis was performed in four different modes: LC-ESI-MS, RNLC-ESI-MS, LC-ESI-MS, and RNLC-ESI-MS. The data from these four experiments and from LC-FSA are provided in Table 3. A comparison of the responses of DL and its metabolites in five different detection/analysis modes showed that under conventional flow LC-ESI-MS conditions, the levels of DL and M31/M33 are overestimated by about twenty and ten times, respectively when compared to the radioactivity responses. The responses in LC-ESI-MS without RN normalization are much closer to the data obtained using LC-FSA (Table 3). The addition of the RN flow brought down the response of DL closer to the radioactivity value, however, the responses for M31/M33 were reduced. In LC-ESI-MS with and without RN flow, the response for M7 was underestimated. This was again attributed to two other co-eluting metabolites contributing to LC-FSA integrated peak area for M7. Because of the *m/z* difference, these metabolites did not contribute to the XIC peak area for M7. It should be noted that the RN flow addition had no effect on the MS response of DL and metabolites in the conventional flow LC-ESI-MS.

As a further demonstration of the potential for response normalization in LC-ESI-MS, the NSI-MS responses of equimolar mixtures (20 μM each, 25 μL

Table 3. Comparison of peak area normalized data for conventional flow LC-ESI-MS and nano flow LC-ESI-MS for desloratadine and its metabolites

Drug/metabolites (mass-to-charge ratio)	Radioactivity	LC-ESI-MS		LC-ESI-MS	
		Without normalizing HPLC flow	0.15 mL/min normalizing HPLC flow	Without normalizing HPLC flow	0.15 mL/min normalizing HPLC flow
DL (<i>m/z</i> 311)	2.63	61.5	50.8	5.0	2.52
M31&M33 (<i>m/z</i> 327)	37.0	378	371.2	33.1	26.6
M9 (<i>m/z</i> 503)	100	100	100	100.0	100
M7 (<i>m/z</i> 503)	38.2	23.9	23.4	13.4	14.8

Figure 5. LC-MS chromatograms of tolbutamide and hydroxy-tolbutamide obtained with (top panel) and without (bottom panel) response normalizing (RN) HPLC flow.

injected) of tolbutamide (TOL), hydroxyl-tolbutamide (OH-TOL), cocaine, and benzoylecgonine were examined. As shown in Figure 5, without the use of RN flow, the LC-NSI-MS response (peak area) of equimolar mixtures of TOL and OH-TOL were 25 and 8, respectively (Figure 5). With the addition of RN flow (0.15 mL/min), equal LC-MS responses were obtained. This experiment was repeated 15 times within a span of 6 mo and with 0.15 mL/min RN flow rate, the response of OH-TOL was within 95% to 105% of the LC-NSI-MS response of TOL. Under LC-ESI-MS conditions, the XIC response of OH-TOL was in the range of 25% to 28% of TOL from an equimolar mixture of TOL and OH-TOL. The ESI-MS data are comparable to the results reported by Valaskovic et al. [29]. In an attempt to achieve MS response comparable to the concentrations of analytes, Valaskovic et al. [29] reduced the NSI flow rate to 7 nL/min. Even at 7 nL/min, the response ratio for TOL:OH-TOL was 1:0.8. With the use of RN flow on a chip-based nanospray system (Nanospray), a response ratio of 1:0.9 or better was routinely achieved for equimolar mixture of TOL and OH-TOL.

A similar set of experiments was performed using an equimolar mixture of cocaine and benzoylecgonine. Again, RNLC flow helped to achieve near equal LC-NSI-MS responses. Without the RN flow, the LC-NSI-MS response of cocaine was almost three times higher than that for benzoylecgonine; however, nearly equal responses were obtained following the addition of RN flow (Figure 6). The response normalized ratio ranged from 1:0.85 to 1:1.2. By reducing the NSI flow rate to 3.7 nL/min, Valaskovic et al. [29] achieved an MS response ratio of only 1:0.5 for an equimolar mixture of cocaine and benzoylecgonine.

Although RNLC-NSI-MS provides an improvement in normalization of MS response in LC-NSI-MS, there was an

overall reduction of the sensitivity of analyte responses following the addition of RN flow. The reduction in ion intensities can be attributed to two factors. First, the addition of RN flow reduces the analyte concentration by a factor of two. Second, response normalization maintains a constant mobile phase composition (50:50 organic: aqueous) throughout the LC run. Therefore, the responses of those analytes that elute at >50% organic content of the mobile phase are reduced in RNLC-NSI-MS. Nonetheless, the data presented here demonstrates that the sensitivity in RNLC-NSI-MS is suitable for characterization and estimation of drugs and metabolites directly from biological matrices.

The results presented here are encouraging and indicate that the RN flow addition reduces the variability in NSI-MS response due to differences in the mobile phase composition in a gradient LC experiment. A variety of different compounds yielded relative NSI-MS response comparable to their concentration in biological matrices concurrently established using radiometric detection. These data suggest that RNLC-NSI-MS can be used to simultaneously acquire semiquantitative information during structural characterization of drugs and metabolites. Results reported here indicate that metabolite levels can be estimated from studies conducted with non-radiolabeled drugs in early exploratory drug metabolism and clinical programs.

Conclusions

The addition of response normalization flow to the LC effluent in LC-NSI-MS minimizes the effect on the analyte response resulting from changes in the solvent composition in gradient elution experiments. Response normalization results in similar MS responses for all analytes in a related series. Therefore, RNLC-NSI-MS can be used to

Figure 6. LC-MS chromatograms of cocaine and benzoylecgonine obtained with (top panel) and without (bottom panel) response normalizing (RN) HPLC flow.

improve quantitative estimates for metabolite levels in exploratory and early clinical drug metabolism studies when radiolabeled drugs are typically not available or when impractical to administer to humans. Other applications may include determination of impurity levels in drug substance/products from manufacturing processes and reaction byproducts during chemical synthesis.

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References

- Jemal, M.; Ouyang, Z.; Powell, M. L. A Strategy for a Post-Method-Validation Use of Incurred Biological Samples for Establishing the Acceptability of a Liquid Chromatography/Tandem Mass-Spectrometric Method for Quantitation of Drugs in Biological Samples. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 1538–1547.
- Jemal, M. High-Throughput Quantitative Bioanalysis by LC/MS/MS. *Biomed. Chromatogr.* **2000**, *14*(6), 422–429.
- Onorato, J. M.; Henion, J. D.; Lefebvre, P. M.; Kiplinger, J. P. Selected Reaction Monitoring LC-MS Determination of Idoxifene and Its Pyrrolidinone Metabolite in Human Plasma Using Robotic High-Throughput, Sequential Sample Injection. *Anal. Chem.* **2001**, *73*(1), 119–125.
- Poquette, M. A.; Lensmeyer, G. L.; Doran, T. C. Effective Use of Liquid Chromatography-Mass Spectrometry (LC/MS) in the Routine Clinical Laboratory for Monitoring Sirolimus, Tacrolimus, and Cyclosporine. *Ther. Drug Monit.* **2005**, *27*(2), 144–150.
- Srinivas, N. R. Applicability of Bioanalysis of Multiple Analytes in Drug Discovery and Development: Review of Select Case Studies Including Assay Development Considerations. *Biomed. Chromatogr.* **2006**, *20*(5), 383–414.
- Vengurlekar, S. S.; Heitkamp, J.; McCush, F.; Velagaleti, P. R.; Brisson, J. H.; Bramer, S. L. A Sensitive LC-MS/MS Assay for the Determination of Dextromethorphan and Metabolites in Human Urine—Application for Drug Interaction Studies Assessing Potential CYP3A and CYP2D6 Inhibition. *J. Pharm. Biomed. Anal.* **2002**, *30*(1), 113–124.
- Chowdhury, S. K. Early Assessment of Human Metabolism: Why, How, Challenges, and Opportunities. *Proceedings of the ASMS Conference on Mass Spectrometry and Allied Topics*; Indianapolis, IN, June, 2007.
- Wilm, M.; Mann, M. Analytical Properties of the Nano-electrospray Ion Source. *Anal. Chem.* **1996**, *68*(1), 1–8.
- Gatlin, C. L.; Eng, J. K.; Cross, S. T.; Detter, J. C.; Yates, I. J. R. Automated Identification of Amino Acid Sequence Variations in Proteins by HPLC/Microspray Tandem Mass Spectrometry. *Anal. Chem.* **2000**, *72*(4), 757–763.
- Figgeys, D.; Aebersold, R. Nanoflow Solvent Gradient Delivery from a Microfabricated Device for Protein Identifications by Electrospray Ionization Mass Spectrometry. *Anal. Chem.* **1998**, *70*(18), 3721–3727.
- Figgeys, D.; Ning, Y.; Aebersold, R. A. Microfabricated Device for Rapid Protein Identification by Microelectrospray Ion Trap Mass Spectrometry. *Anal. Chem.* **1997**, *69*(16), 3153–3160.
- Zhang, S.; Van Pelt, C. K.; Henion, J. D. Automated Chip-Based Nano-electrospray-Mass Spectrometry for Rapid Identification of Proteins Separated by Two-Dimensional Gel Electrophoresis. *Electrophoresis*. **2003**, *24*(21), 3620–3632.
- Henion, J.; Pace, E.; Howe, K.; Zhang, S.; Van Pelt, C. Chip-based electrospray mass spectrometry applied to large and small molecules. *Abstracts, 35th Central Regional Meeting of the American Chemical Society*; Pittsburgh, PA, October, 2003, p 9.
- Borts, D. J.; Cooks, S. T.; Bowers, G. D.; O'Mare, M. J.; Quinn, K. E. Use of Automated Nano-electrospray for Drug Metabolite Structure Elucidation. *Proceedings of the ASMS Conference on Mass Spectrometry and Allied Topics*; Nashville, TN, May, 2004.
- Balimane, P. V.; Pace, E.; Chong, S.; Zhu, M.; Jemal, M.; Van Pelt, C. K. A. Novel High-Throughput Automated Chip-Based Nano-electrospray Tandem Mass Spectrometric Method for PAMPA Sample Analysis. *J. Pharm. Biomed. Anal.* **2005**, *39*(1/2), 8–16.
- Dethy, J. M.; Ackermann, B. L.; Delatour, C.; Henion, J. D.; Schultz, G. A. Demonstration of Direct Bioanalysis of Drugs in Plasma Using Nano-electrospray Infusion from a Silicon Chip Coupled with Tandem Mass Spectrometry. *Anal. Chem.* **2003**, *75*(4), 805–811.
- Van Pelt, C. K.; Zhang, S.; Fung, E.; Chu, I.; Liu, T.; Li, C.; Korfmacher, W. A.; Henion, J. A Fully Automated Nano-electrospray Tandem Mass Spectrometric Method for Analysis of Caco-2 Samples. *Rapid Commun. Mass Spectrom.* **2003**, *17*(14), 1573–1578.
- Zhang, S.; Van Pelt, C. K.; Wilson, D. B. Quantitative Determination of Noncovalent Binding Interactions Using Automated Nano-electrospray Mass Spectrometry. *Anal. Chem.* **2003**, *75*(13), 3010–3018.
- Froesch, M.; Bindila, L. M.; Baykut, G.; Allen, M.; Peter-Katalinic, J.; Zamfir, A. D. Coupling of Fully Automated Chip Electrospray to Fourier Transform Ion Cyclotron Resonance Mass Spectrometry for

- High-Performance Glycoscreening and Sequencing. *Rapid Commun. Mass Spectrom.* **2004**, 18(24), 3084–3092.
20. Zamfir, A.; Vakhrushev, S.; Sterling, A.; Niebel, H. J.; Allen, M.; Peter-Katalinic, J. Fully Automated Chip-Based Mass Spectrometry for Complex Carbohydrate System Analysis. *Anal. Chem.* **2004**, 76(7), 2046–2054.
 21. Zhang, L.; Laycock, J. D.; Miller, K. J. Quantitative Small Molecule Bioanalysis Using Chip-Based NanoESI-MS/MS. *JALA.* **2004**, 9(3), 109–114.
 22. Ackermann, B. L.; Dethy, J.-M. Understanding the Role and Potential of Infusion Nanoelectrospray Ionization for Pharmaceutical Bioanalysis. In *Using Mass Spectrometry for Drug Metabolism Studies*, Korfmacher W., ed. CRC Press, Boca Raton, FL, **2005**, pp. 329–356.
 23. Corkery, L. J.; Pang, H.; Schneider, B. B.; Covey, T. R.; Siu, K. W. M. Automated Nanospray Using Chip-Based Emitters for the Quantitative Analysis of Pharmaceutical Compounds. *J. Am. Soc. Mass Spectrom.* **2005**, 16(5), 798.
 24. Yu, L. J.; Chen, Y.; DeNinno, M. P.; O'Connell, T. N.; Hop, C. E. C. A. Identification of a Novel Glutathione Adduct of Diclofenac, 4'-Hydroxy-2'-Glutathione-Deschlorodiclofenac Upon Incubation with Human Liver Microsomes. *Drug Metab. Dispos.* **2005**, 33(4), 484–488.
 25. Corso, T. N.; Li, J.; Van Pelt, C. K. Ultra-low Volume Fraction Analysis by Chip-based Nanoelectrospray for Increased Peptide Coverage. *Abstracts, 35th Northeast Regional Meeting of the American Chemical Society*; Binghamton, NY, October 2006, NRM-474.
 26. Tomkins, B. A.; Van Berkel, G. J.; Jenkins, R. A.; Counts, R. W. Quantitation of Cotinine in Nonsmoker Saliva Using Chip-Based Nanoelectrospray Tandem Mass Spectrometry. *J. Anal. Toxicol.* **2006**, 30(3), 178–186.
 27. Wickremesinha, E. R.; Ackermann, B. L.; Chaudhary, A. K. Validating Regulatory-Compliant Wide Dynamic Range Bioanalytical Assays Using Chip-Based Nanoelectrospray Tandem Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2005**, 19(1), 47–56.
 28. Wickremesinha, E. R.; Singh, G.; Ackermann, B. L.; Gillespie, T. A.; Chaudhary, A. K. A Review of Nanoelectrospray Ionization Applications for Drug Metabolism and Pharmacokinetics. *Curr. Drug Metab.* **2006**, 7(8), 913–928.
 29. Valaskovic, G. A.; Utley, L.; Lee, M. S.; Wu, J. T. Ultra-Low Flow Nanospray for the Normalization of Conventional Liquid Chromatography/Mass Spectrometry Through Equimolar Response: Standard Free Quantitative Estimation of Metabolite Levels in Drug Discovery. *Rapid Commun. Mass Spectrom.* **2006**, 20(7), 1087–96.
 30. Valaskovic, G. A.; Murphy, J. P., III; Lee, M. S. Automated Orthogonal Control System for Electrospray Ionization. *J. Am. Soc. Mass Spectrom.* **2004**, 15(8), 1201–15.
 31. Schultz, G. A.; Corso, T. N.; Prosser, S. J.; Zhang, S. A Fully Integrated Monolithic Microchip Electrospray Device for Mass Spectrometry. *Anal. Chem.* **2000**, 72(17), 4058–4063.
 32. Corso, T. N.; Van Pelt, C. K.; Zhang, S.; Prosser, S. J.; Schultz, G. A. Integrated Microchip-Based Nanoelectrospray Device for High-Throughput Mass Spectrometry. *Proceedings of SPIE-The International Society for Optical Engineering*; New York, May 2001, p. 4265. (Biomedical Instrumentation based on Micro- and Nanotechnology), pp. 81–90.
 33. Henion, J. Chip-Based Automated Nanoelectrospray LC/MS for the Efficient Qualitative and Quantitative Determination of Metabolites and Biomarkers. *Abstracts, 38th Middle Atlantic Regional Meeting of the American Chemical Society*; Hershey, PA, June, 2006; p. MRM-269.
 34. Alfazema, L. N.; Richards, D. S.; Gelebart, S.; Mitchell, J. C.; Snowden, M. J. Rapid, Accurate, and Precise Quantitative Drug Analysis: Comparing Liquid Chromatography Tandem Mass Spectrometry and Chip-Based Nanoelectrospray Ionization Mass Spectrometry. *Eur. J. Mass Spectrom.* **2005**, 11(4), 393–402.
 35. Schmidt, A.; Karas, M.; Dulcks, T. Effect of Different Solution Flow Rates on Analyte Ion Signals in Nano-ESI MS, or: When Does ESI Turn Into Nano-ESI? *J. Am. Soc. Mass Spectrom.* **2003**, 14(5), 492–500.
 36. Hop, C. E.; Chen, Y.; Yu, L. J. Uniformity of Ionization Response of Structurally Diverse Analytes Using a Chip-Based Nanoelectrospray Ionization Source. *Rapid Commun. Mass Spectrom.* **2005**, 19(21), 3139–42.
 37. Gorecki, T.; Lynen, F.; Szucs, R.; Sandra, P. Universal Response in Liquid Chromatography Using Charged Aerosol Detection. *Anal. Chem.* **2006**, 78(9), 3186–3192.
 38. Ramanathan, R.; Zhong, R.; Alvarez, N.; Kennedy, C.; Grotz, D.; Rindgen, D.; Cox, K.; Chowdhury, S.; Wirth, M.; Alton, K. Comparative Metabolism and Excretion of a Novel CCR5 Receptor Antagonist, SCH 417690 (Vicriviroc), in Human, Monkey, and Rat. *Drug Metab. Rev.* **2005**, 37(4), 725–726.
 39. Ramanathan, R.; Ghosal, A.; Miller, M. W.; Chowdhury, S. K.; Alton, K. B. Piperazine Derivatives and Their Preparation, Pharmaceutical Compositions, and Use as CCR5 Antagonists for Treatment of Human Immunodeficiency Virus or Inflammatory Diseases. US Patent Application 2006105964, May, 2006.
 40. Marozsan, A. J.; Kuhmann, S. E.; Morgan, T.; Herrera, C.; Rivera-Troche, E.; Xu, S.; Baroudy, B. M.; Strizki, J.; Moore, J. P. Generation and Properties of a Human Immunodeficiency Virus Type 1 Isolate Resistant to the Small Molecule CCR5 Inhibitor, SCH-417690 (SCH-D). *Virology.* **2005**, 338(1), 182–199.
 41. Strizki, J. M.; Tremblay, C.; Xu, S.; Wojcik, L.; Wagner, N.; Gonsiorek, W.; Hipkin, R. W.; Chou, C.-C.; Pugliese-Sivo, C.; Xiao, Y.; Tagat, J. R.; Cox, K.; Priestley, T.; Sorota, S.; Huang, W.; Hirsch, M.; Reyes, G. R.; Baroudy, B. M. Discovery and Characterization of vicriviroc (SCH 417690), a CCR5 Antagonist with Potent Activity Against Human Immunodeficiency Virus Type 1. *Antimicrob. Agents Chemother.* **2005**, 49(12), 4911–4919.
 42. Ramanathan, R.; Su, A. D.; Alvarez, N.; Feng, W.; Chowdhury, S. K.; Stauber, K.; Reyderman, L.; Alton, K. B.; Wirth, M.; Patrick, J. E. Metabolism and Excretion of Desloratadine (Clarinet) in Mice, Rats, Monkeys, and Humans. *Drug Metab. Rev.* **2006**, 38(4), 725–726.
 43. Ramanathan, R.; Alvarez, N.; Su, A. D.; Chowdhury, S.; Alton, K.; Stauber, K.; Patrick, J. Metabolism and Excretion of Loratadine in Male and Female Mice, Rats, and Monkeys. *Xenobiotica.* **2005**, 35(2), 155–189.