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A low flow ionization technique to integrate quantitative and qualitative small molecule bioanalysis

Ragu Ramanathan*, Nirmala Raghavan, S. Nilgun Comezoglu, W. Griffith Humphreys

Department of Biotransformation, Bristol-Myers Squibb, Princeton, NJ 08540, United States

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ABSTRACT

Mass spectrometry-based assays are used in drug discovery and development to detect, characterize and quantify drugs, metabolites, impurities and degradants. Recently, high resolution-based mass spectrometers have begun to emerge as a platform with potential for performing integrated qualitative and quantitative assays in order to streamline the drug discovery and development process. However, the widely different LC–MS response observed for a drug and its metabolites limit the direct use of LC–MS responses for relative quantitative determination of metabolites. This in turn limits the use of conventional LC–ESI-MS methods, in the absence of reference standards, as an integrated technique for detection, characterization and quantification of drugs and metabolites. The goal of this study was to explore the use of LC–captive spray ionization (CSI)-mass spectrometry for detection, characterization and quantification of drugs and metabolites. CSI allows the use of conventional HPLC or uHPLC columns and flow rates of 0.35–0.6 mL/min (before post-column flow splitting) and can be considered as a technique which can function as a nanospray or microspray. Also, in comparison to conventional nanospray ionization (NSI) techniques, setup and maintenance of CSI do not require: (1) *X*, *Y*, and *Z* positioning or cameras to guide the spray positioning, (2) difficult to control splitters to deliver nano-flow ratios and difficult to maintain nanospray nozzles. Evaluations using equimolar mixture of buspirone and four monoxy metabolites present in human plasma show that LC–CSI-MS is a highly sensitive technique that gives a near equimolar response for the compounds used in this example. Comparisons of LC–ESI-MS data with that obtained using LC–CSI-MS show that reasonable quantification of metabolites may be achievable without using reference standards or administration of radiolabeled drugs.

1. Introduction

Despite the significant efforts to find a highly sensitive universal detector that gives a near equimolar response independent of the compound, LC–MS or LC–MS/MS quantification of structurally related compounds (metabolites, degradants, etc.) in the absence of reference standards remains a major challenge during drug discovery and development. In traditional pharmaceutical settings, separate groups are often established for handling qualitative and quantitative work streams. Qualitative work streams, which are usually low throughput, may involve full-scan LC–MS analysis for detecting metabolites followed by detailed LC–MS/MS analysis for structural elucidation. Typically for quantification, reference standards of analyte are required so that LC–MS/MS responses from unknown quantities of the analyte can be related back to LC–MS/MS responses obtained using known quantities of the analyte. Standard curve of a drug can be used to quantify its metabolite(s), however, there is a chance for significant over or under estimation of quantity because structural alterations by metabolism or biotransformation lead to unpredicted changes in ionization efficiencies.

A highly sensitive universal detector, that gives a near equimolar response independent of the compound, would facilitate the integration of qualitative and quantitative workflows and reduce the overall cost of developing drugs. The concept of using integrated qualitative and quantitative assay to get maximum information from a single injection has been around since the 1990s [1,2]. Due to limitations associated with scan speed and sensitivity, conventional triple quadrupole mass spectrometer-based integrated quantitative and qualitative approaches failed to provide complete information necessary for reliable decision making.

Recent MS developments, such as the Q-Trap 5500 and Q-TOF, offer fast scan speeds without sacrificing resolution. These developments have reopened the door for utilizing hybrid mass spectrometers for obtaining metabolite information while performing quantification of drugs. To highlight the utility of the Q-Trap mass spectrometer, which has both triple quadrupole and

* Corresponding author at: Department of Biotransformation, Bristol-Myers Squibb, P.O. Box 4000, Princeton, NJ 08540, United States. Tel.: +1 609 252 4730.
E-mail addresses: ragu.ramanathan@bms.com, ragu_ramanathan@yahoo.com (R. Ramanathan).

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ion trap functionalities, for obtaining quantitative and qualitative information, several investigators [3–9] collected full-scan data and showed that information about circulating metabolites, dosing vehicle, interfering matrix components, and co-eluting metabolites can be obtained simultaneously by using Q-Trap instruments. Traditionally, time-of-flight (TOF) mass spectrometers have been used to a lesser extent for quantitation due to limitations in dynamic range. However, over the last decade, TOF mass spectrometers have gone through tremendous improvements and have been used in integrated qualitative and quantitative assays to exploit their efficient duty cycle and higher resolution capabilities over conventional triple quadrupole mass spectrometers. Nagele and Fandino [10], Pelander et al. [11], Tiller et al. [12] and Kammerer et al. [13] have reported the utility of TOF mass spectrometers for such applications. Most recently, the Orbitrap-based systems have emerged as the platform of choice for performing integrated qualitative and quantitative assays [14,15].

One factor that can influence the success of integrated qualitative and quantitative assay by high resolution mass spectrometry (HRMS) is the effective scan rate of the system. For quantitation, quadrupole instruments typically "dwell" on a specific m/z or transition for MS/MS. In contrast, TOFs, ion traps and Orbitrap mass analyzers do not dwell on a specific target, but rather acquire scan data across the m/z range. A critical component is the speed with which either the dwell or scan can be accomplished or how scan speed affects mass resolution and mass accuracy as well as determining the number of points obtained from a single chromatographic peak. In some of the newly designed quadrupole mass analyzers (API 5000, TSQ Vantage, etc.), dwell times can easily be shorter than 50 ms, as can TOF scan times. This permits acquisition of numerous data points across a chromatographic peak, which is critical for accurate and precise quantitation. Mass resolution is unaffected by changes in dwell time/scan rate, though signal:noise ratio usually decreases with faster scanning. However, the resolution on Fourier transform-based mass analyzers is linked to scan rate.

In a recent investigation, Bateman et al. [14,15] systematically investigated the performance of an Orbitrap mass spectrometer, in the full-scan integrated qualitative and quantitative mode, for analysis of samples from metabolic stability and in vivo studies. High resolution (20,000) and stability of mass accuracy for every peak in every scan are needed for the effective use of full-scan data for quantitative determinations. With full-scan data it is possible to obtain acquisition without the need for SRM method development, dynamic range extension by using isotope data, metabolite identification, and analysis of endogenous compounds.

Over the last 5 years, several groups have evaluated low flow ESI techniques for achieving normalized LC–MS response for drugs and metabolites. Among them, Hop et al. [16,17] compared ionization efficiencies of drugs and metabolites obtained using conventional flow LC–MS with that from a microchip-based infusion nanospray ionization (NSI)-MS and showed that LC–MS responses, between drugs and metabolites, varied much less with the microchip-based NSI-MS. The presence of a large excess of protons to smaller droplets through the generation of a much higher electric field around the microchip nozzles was attributed to similar ionization efficiencies observed in the NSI based system [16,17]. In one study, 25 compounds that were from six structurally different classes were studied to compare the responses from LC–MS and NSI-MS. The LC–MS data showed that the responses varied about 21-fold across all compounds, whereas the variation in responses from the infusion nano-ESI was only 2.2-fold. Valaskovic et al. [18] used a pulled capillary based infusion NSI-MS to analyze common pharmaceutical drugs and their metabolites to show that a more uniform equimolar response between a drug and its metabolite is achievable by lowering the infusion flow rates to10 nL/min or lower. In that study, responses between six structurally distinct compounds and their metabolites spiked into rat plasma were compared using different flow rates (from the flow rate used in conventional ESI at 5 μL/min to <4 nL/min for nanospray). Nanospray showed a clear response advantage over conventional ESI by generating a semi-equimolar response for compounds and their metabolites. In general, using the lowest practical flow rate for analysis was advantageous. There was an increasing trend toward equimolar response as flow rate was decreased from ca. 20 nL/min (normal) to <10 nL/min. They achieved an MS response ratio of 1:0.5 for an equimolar mixture of cocaine and benzoylecgonine with a NSI flow rate of 3.7 nL/min. In the case of an equimolar mixture of morphine and its metabolite, morphine glucuronide, a flow rate of ≤10 nL/min generated almost an equimolar response.

Most recently, Ramanathan et al. [19] took the NSI-MS approach further and showed the utility of NSI-MS in conjunction with HPLC for analysis of plasma, bile and urine. In this novel approach, the solvent composition in which a compound is introduced into the NSI source was normalized by post-column addition of an equivalent flow rate solvent composition in an opposite gradient. Addition of the second opposite gradient normalized the mobile phase composition and minimized the influence of the mobile phase composition on ionization efficiencies and further limited the variability in LC–NSI-MS response of structurally diverse molecules. In their study, the addition of the second opposite gradient normalized the response ratio for equimolar mixture of compounds and ranged from1:0.85 to 1:1.2 for tolbutamide:hydroxyl-tolbutamide and cocaine:benzoylecgonine mixtures, respectively.

Overall, the NSI-MS has been a gold standard technique in proteomics research but the technique has not made inroads into the drug metabolism arena due mainly to difficulties associated with setup, maintenance and lack of robustness and reproducibility. The majority of NSI sources require X, Y, Z positioning and cameras to monitor the spray. In addition, tapered tips and microchip-based spray nozzles are known to clog due to matrix related materials and particulates present in in vitro incubations, plasma, urine and bile.

In this study, we have sought to take advantage of the commercially available advanced captive spray ionization (CSI) source for detection, characterization and quantification of small molecule drug metabolites. CSI (250 nL/min to 50 μL/min) bridges the gap between NSI (<500 nL/min) and conventional flow ESI (50–1500 μL/min). Use of the CSI technique in metabolite characterization and quantification has been presented in some national meetings [20], but not published before. To demonstrate the utility of CSI for quantifying metabolites in the absence of reference standards, an equimolar mixture containing buspirone and four of its monooxy metabolites (M3, M6, M7 and M8) (Fig. 1) was analyzed using LC–UV–ESI-MS and LC–UV–CSI-MS.

2. Experimental

HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Water was purified using the Millipore Milli-Q_{plus} water purification system (Bedford, MA, USA). Trifluroacetic acid was from Sigma–Aldrich (St. Louis, MO, USA). Buspirone and reference standards for buspirone metabolites (Fig. 1) were all prepared at Bristol-Myers Squibb (Princeton, NJ, USA). Stock solutions were prepared in acetonitrile (30 μM). All other reagents and solvents were obtained from commercial sources. To check the recovery of buspirone, M3, M6, M7 and M8 from human plasma, control human plasma was extracted with and without enriching with buspirone, M3, M6, M7 and M8. Extraction recovery was checked using 100 and 1000 ng/mL samples. Samples were extracted by the addition of 4 mL of acetonitrile to 2.0 mL of

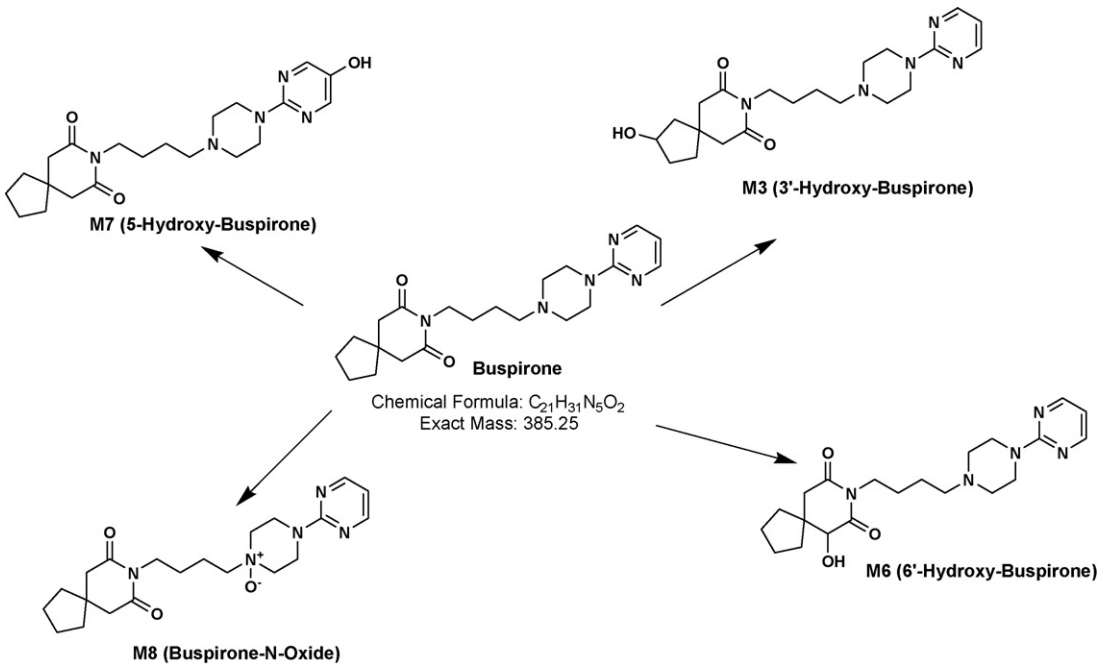

Fig. 1. Buspirone and monooxy metabolites evaluated for establishing LC–CSI-MS method.

plasma while the sample was mixed on a vortex mixer. After centrifugation at 2000 × *g* for 10 min, the supernatant was transferred to another tube. The supernatant was diluted with 1:1 acetonitrile:methanol before injection. The blank plasma was spiked with buspirone, M3, M6, M7 and M8 after completing the extraction step. Aliquots (25 μL) from spiked and unspiked samples were injected into HPLC, and LC–UV and LC–ESI-MS or LC–CSI-MS responses compared for semi-quantitative estimation of extraction recoveries for buspirone, M3, M6, M7 and M8.

2.1. LC–ESI-MS

All LC–ESI-MS experiments were performed using a LTQ-Orbitrap Discovery mass spectrometer (Thermo Electron Corp., San Jose, CA) equipped with a manufacture-supplied source operated in the positive ionization mode. Vendor-provided Xcalibur (v.2.07) software in combination with LTQ-Orbitrap Discovery instrument control software (v2.4), with no additional modifications, was used for all HPLC and MS functions as well as for processing data. Vendor-supplied calibration mixture containing caffeine, MRFA, and Ultramark was used to calibrate the mass spectrometer. Upon acceptance of calibration, no additional tuning or calibration were performed during the data collection. Generic ESI source and MS conditions were used to avoid preferential MS conditions for any one of the metabolites or drugs. The mass spectrometer was nominally operated at 30,000 FWHM resolving power at 600 Da with the conditions listed in Table 1. For all experiments, the Orbitrap mass spectrometer was scanned from 100 to 1100 Da. For selected samples mass defect filter and background subtraction techniques were applied, post-acquisition, to discern drug derived material from background ions [21,22].

All LC–MS experiments were performed using an Accela UHPLC system and associated PDA and autosampler (Thermo Electron Corp., San Jose, CA). When using a 4.6 mm × 250 mm column and 1 mL/min flow rate, the column effluent was split such that most (~75%) of the effluent was analyzed by a Model 5 β-RAM radioactivity detector (IN/US Systems, Inc., Tampa, FL) (data not discussed in this paper) or diverted to waste. For ESI-MS experiments using a 2.1 mm × 150 mm column, 100% of the column effluent was delivered to the API source at a flow rate of 350 μL/min. Peak areas of the metabolites and the corresponding parent drug were integrated and then normalized to the peak area of the major component (most often the parent drug).

2.2. LC–captive spray ionization (CSI)-MS

All LC–CSI experiments were performed using a commercially purchased Axial Desolvation Vacuum Assisted Nano Capillary Electrospray (ADVANCED) ionization source or captive spray ionization source (Michrom Bioresources, Inc., Auburn, CA) controlled and manipulated by Xcalibur (v.2.07) software. When changing the instrument configuration from ESI-MS to CSI-MS, the ion transfer tube temperature was reduced to 25 °C and the conventional LTQ-Orbitrap ion transfer was replaced with commercially purchased CSI-MS ion transfer tube. As shown in Fig. 2, the CSI-MS ion transfer tube is designed such that the ion transfer entrance fits into the spray assembly and allows the ion spray emitter to position close to the entrance of the ion transfer tube. The seal between the ion spray emitter and the ion transfer tube creates a vacuum seal to allow nitrogen flow generated through sheath and auxiliary gas supplies to focus 100% of the spray from the emitter into the ion transfer tube. The entire process, from the removal of the ESI-MS source and ion transfer tube to installation of CSI-MS ion transfer tube and mounting of the CSI-MS source, took approximately 30 min. All other operating parameters used with CSI source are listed in Table 1. Additional details about CSI-MS operating conditions are provided by Raghavan et al. [20] and Nugent et al. [23]. For all LC–CSI-MS experiments, the LC conditions, described for LC–ESI-MS, were used and the column effluent was split, post-PDA detector, such that most (~98%) of the effluent was directed into the waste, off-line fraction collector or online radioactivity detector (only when using radioactive samples). Approximately 2% of the effluent, sent to the CSI source, corresponded to a flow rate of approximately 7 μL/min. All split ratios were adjusted by varying the internal diameter (ID) and lengths of fused silica capillary connected to the captive spray tip assembly (Fig. 2). Split ratios were verified by injecting radioactive samples, collecting effluent and counting aliquots using liquid scintillation spectrometry (LSS). All LC–CSI-MS data were processed and integrated using the Xcalibur software.

Table 1
LC–ESI-MS and LC–CSI-MS conditions used for detection, characterization and quantification of buspirone and its monooxy metabolites.

Parameter		Settings	
Ionization source		ESI	CSI
Ionization mode		Positive	Positive
Spray voltage (kV)		4.0	1.75
Heated capillary temp. (°C)		275	175
Sheath gas (arbitrary unit)		30	5
Auxiliary gas (arbitrary unit)		15	2
HPLC flow (μL/min)		1000 or 350	350
HPLC effluent to MS (μL/min)		250–350	7
HPLC effluent to waste or in-line radioactivity detector (μL/min)		750	343
HPLC column dimensions		2.1 mm × 150 mm or 4.6 mm × 250 mm	2.1 mm × 150 mm
HPLC column		Zorbax Rx C8; 5 μm; 100 A	
	Time	0.01% TFA in Water	Acetonitrile
HPLC Gradient	0	92	8
	8	92	8
	30	60	40
	35	10	90
	40	10	90
	41	92	8
	45	92	8

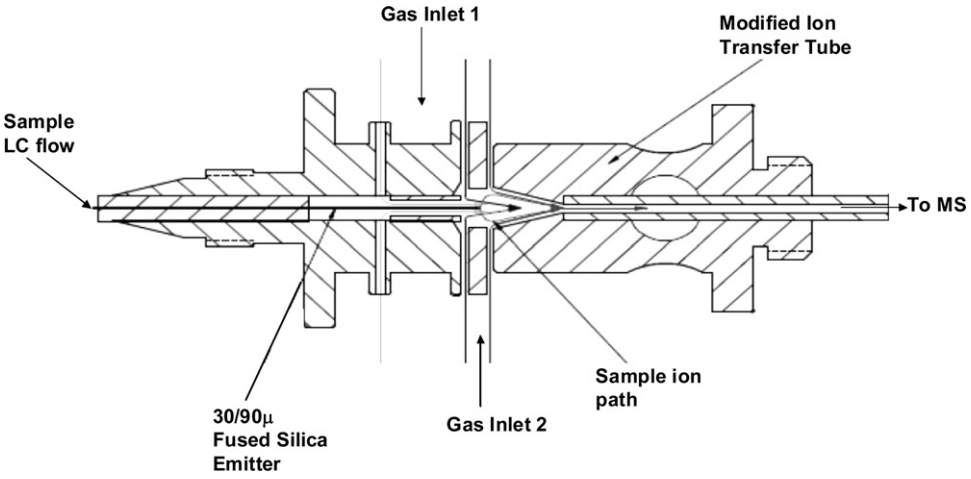

Fig. 2. Schematic of axial desolvation vacuum assisted nano capillary electrospray (ADVANCE) ionization source or captive spray ionization (CSI) source used with LTQ-Orbitrap mass spectrometer. (From: www.michrom.com).

3. Results and discussion

LC–CSI-MS was evaluated for obtaining quantitative and qualitative information on drug and its metabolites without the use of reference standards. To demonstrate the utility of the method, equimolar amounts of buspirone and its four monooxy metabolites were spiked into control human plasma and extracted. Extraction recoveries for all five components were above 95%.

The first set of experiments involved LC–PDA-MS analysis of an equimolar mixture containing buspirone and four of its monooxy metabolites (M3, M6, M7 and M8) (Fig. 1). As shown in Fig. 3, comparison, of LC–UV spectra at various wavelengths, suggests that the ratio of M7 to buspirone changed dramatically at the three wavelengths. The peak areas for buspirone, M3, and M8 vary with varying wavelengths, but retain a fairly constant ratio relative to the parent. With the high variability in measurement for a given compound due to poor signal:noise ratio, LC–UV response variation increased and integration of the peaks became difficult as the concentration of the equimolar mixture was decreased to either 15 or 5 μM. In a recent study, Vishwanathan et al. [24] evaluated equimolar LC–UV and LC–MS responses of four sets of drugs and their metabolites present at two concentrations (1 and 10 μg/mL). While the LC–UV responses for compounds and metabolites from sets 1–3 were similar (parent drug's UV maxima was used) and varied only ±30% from respective parent compound's LC–UV responses, responses of compound 4 and its metabolites showed a significant (>400%) differences in the LC–UV responses. These demonstrated differences show that biotransformation reactions can result in significant changes in the UV-absorbing characteristics of a parent drug and its metabolites, and it is difficult to use UV to normalize LC–MS response differences between two compounds when the concentrations are low. Overall, it is important to evaluate the UV profiles of the metabolites with respect to the parent drug and assess the suitability of UV-based semi-quantitative approaches for estimating metabolite concentrations.

A comparison of LC–ESI-MS and LC–CSI-MS responses with those from LC–UV, following the analysis of an equimolar mixture containing buspirone and its four monooxy metabolites, is shown in Fig. 4. Experimental data from multiple injections are summarized in Table 2. All LC–MS data were acquired using full-scan HRMS and data were processed post-acquisition using a ±5 ppm window. While the LC–UV responses of the metabolites present at equimolar amounts ranged from 86 to 143% at 220 nm (80–236% at 254 nm), LC–ESI-MS responses varied between 67 and 77%. Using the LC–ESI-MS data directly, concentrations of M3, M6, M7, and M8 would have been under estimated, on average by 27%.

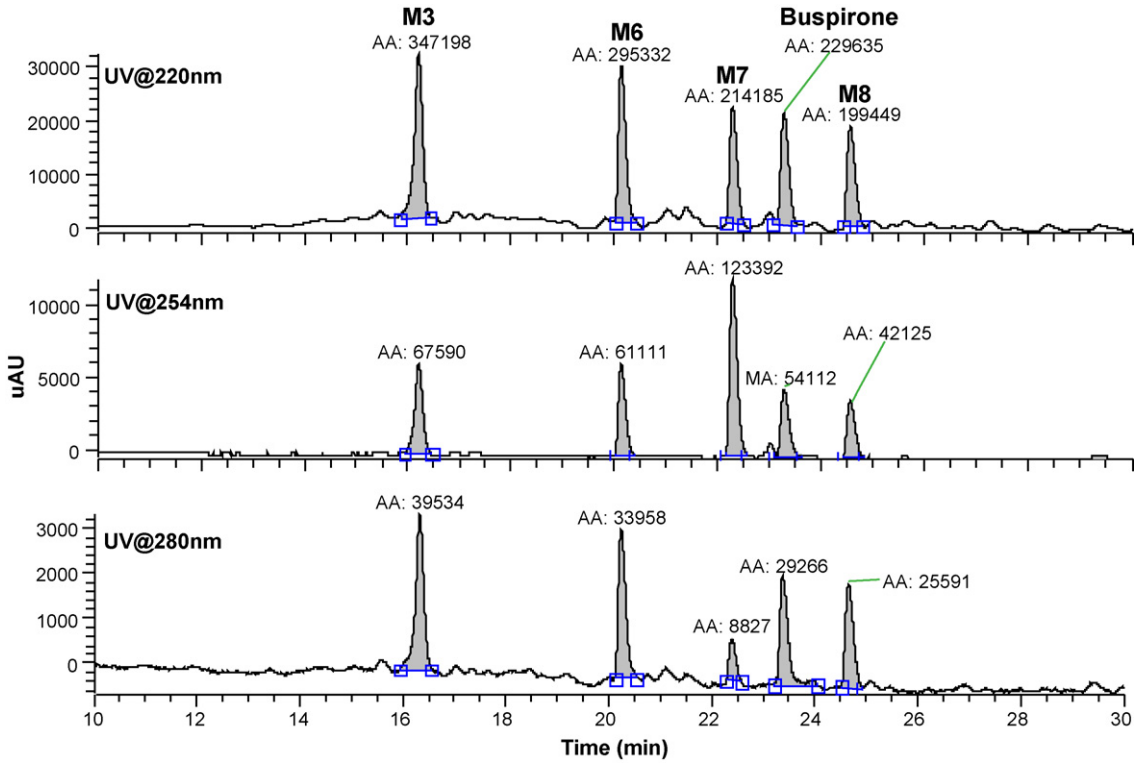

Fig. 3. LC–UV spectra at 280 nm (bottom panel), 254 nm (middle panel) and 220 nm (top panel) of an equimolar (30 μM) mixture containing buspirone and four monooxy metabolites in human plasma.

Overall advantages of low flow nanospray ionization techniques over conventional flow ESI include decreased sample consumption and increased sensitivity [19,25]. The concept of achieving equimolar responses with NSI has also been demonstrated by several research groups [16–18] and the technique has been utilized in drug metabolism and pharmacokinetics (DMPK) studies [25]. However, NSI techniques failed to become the ionization technique of choice for DMPK studies due to difficulties associated with maintenance, operation and reproducibility.

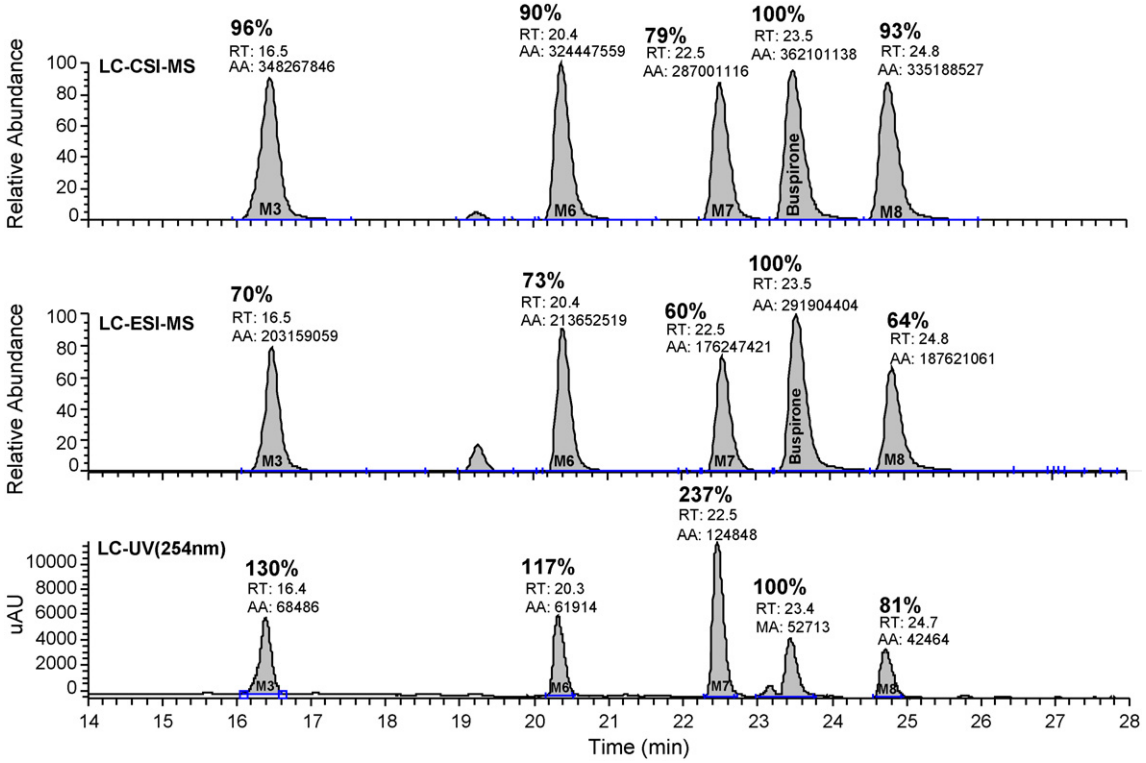

Fig. 4. Comparison of LC–MS extracted ion chromatograms (HRMS with 10 ppm window) and LC–UV (254 nm) of buspirone and monooxy buspirone present in human plasma.

Table 2
Comparison of LC–ESI-MS, LC–CSI-MS and LC–UV responses from a mixture containing equimolar buspirone and four monooxy metabolites in human plasma.

Parent/metabolite	LC–ESI-MS response ($N = 3$)	LC–CSI-MS response ($N = 3$)	LC–UV response ($N = 6$)	
			254 nm	220 nm
Buspirone (m/z 386)	255240833 (100%)	339061580 (100%)	55238 (100%)	242861 (100%)
M3 (m/z 402)	206822886 (73%)	308689103 (91%)	68725 (124%)	346842 (143%)
M7 (m/z 402)	171998321 (67%)	292361412 (86%)	130485 (236%)	225017 (93%)
M6 (m/z 402)	192225175 (77%)	317631965 (94%)	60856 (110%)	291088 (120%)
M8 (m/z 402)	192225175 (75%)	335659891 (99%)	44247 (80%)	209556 (86%)

Uniformity in ionization response for structurally diverse compounds or equimolar response, under infusion NSI-MS conditions, was also reported by Valaskovic et al. [18] and Hop et al. [16,17]. Explanations by Valaskovic et al. [18] involve minimization of ion suppression due to reduction of initial droplet size, which in turn affects the total available surface area and reduces the time required for solvated molecules to migrate to the surface and decrease the number of columbic explosions required for the formation of ions. Reduction of the number of columbic explosions before ionization decreases the matrix effects because preferential molecule or matrix enrichment during columbic cycles are minimized. Explanations by Valaskovic et al. [18] were further supported by the observation of improved normalized MS response for various compounds with reduced NSI flow rates. At a flow rate of about 10 nL/min most compounds showed similar ionization efficiencies and demonstrated equimolar MS response.

Similar encouraging results were also reported by Hop et al. [16,17], Schmidt et al. [26], Gangl et al. [27], Li et al. [28] and Juraschek et al. [29]. These direct infusion NSI-MS experiments were conducted using a silicon wafer based microchip consisting of a 20×20 array of non-reusable nozzles fabricated by proprietary etching techniques. Each nozzle had an ID of 5.5 μm and the nozzle design allowed flow rates between 200 and 500 nL/min. A chip based NSI source further benefits from the smaller initial droplets due to a much higher electric field around the nozzles, which in turn supplies a large excess of protons to the smaller droplets and further eliminates the differences in the ionization efficiencies of molecules.

The encouraging results reported by Valaskovic et al. [18] and Hop et al. [16,17] were all obtained by direct infusion of drugs and their metabolites into NSI-MS systems. However, infusion NSI-MS systems are not suitable for analyzing drugs and metabolites present in complex biological samples such as plasma, urine and bile. Ramanathan et al. [19] utilized a microchip-based NSI-MS system coupled to a HPLC and demonstrated that normalized response for metabolites and drugs is achievable using LC–NSI-MS and even further demonstrated that normalized response for drugs and their metabolites improved when the solvent composition is normalized throughout the LC run time. The setup utilized by Ramanathan et al. [19] achieved normalized solvent composition throughout the LC run time by using two HPLC systems and the approach deemed impractical for routine 24/7 operation and required careful optimization and maintenance. Some of the important findings from Ramanathan et al. [19] included the impact of solvent composition and flow rates. Equimolar responses for drugs and metabolites were more evident when the mobile phase composition reaching the NSI source contained 50/50 organic/aqueous and such equimolar response was evident only when the flow reaching the NSI was maintained at around 500 nL/min or below.

Recently, captive spray ionization (CSI) was introduced as a promising ionization technique for analysis of peptides and proteins [23]. CSI (250 nL/min to 50 μL/min), in many ways, bridges the gap between NSI (<500 nL/min) and conventional flow ESI (50–1500 μL/min). ESI-MS uses high sheath gas to desolvate ions, which in turn dilutes the sample ions resulting in a small percentage (<15%) of the sample entering the MS. CSI uses the vacuum of MS to pull in gas around the spray tip, desolvating and funneling all the sample ions into the MS, resulting in sensitivity comparable to NSI at higher LC flow rates. This source provides ESI robustness and NSI sensitivity. At early stages of drug development, detecting, characterizing and quantifying represent significant challenges due to the unavailability of synthetic standards or radiolabeled drugs. Here we have demonstrated the sensitivity and robustness of CSI technology for quantitation of the metabolites but also for detecting and characterizing drug derived material when utilizing integrated quantitative and qualitative assays.

Extracted ion chromatogram from LC–CSI-MS analysis of a mixture containing buspirone and four of its monooxy metabolites is shown in Fig. 4. LC–CSI-MS responses for all five equimolar components ranged from 79 to 96% and demonstrated equimolar response. Average concentrations for M3, M6, M7, and M8 from three LC–CSI-MS analysis show the range to be between 86 and 99%. The average LC–CSI-MS data show clear quantitative improvements over LC–UV or LC–ESI-MS estimated concentrations. To achieve near equal MS response from an equimolar mixture of tolbutamide (TOL) and hydroxy-tolbutamide (OH-TOL), Valaskovic et al. [18] reduced the NSI flow rate to 7 nL/min. Even at 7 nL/min, 100:80 MS response ratio was observed for equimolar amounts of TOL:OH-TOL. With mobile phase composition normalization and NanoMate flow rate of about 500 nL/min, Ramanathan et al. [19] achieved 100:90 LC–MS response ratio or better for equimolar mixture of TOL:OH-TOL. In contrast to experimental evidence from Valaskovic et al. [18] and Ramanathan et al. [19], observations of LC–CSI-MS normalized response for buspirone and its monooxy metabolites at 2–7 μL/min were very encouraging because routine operations, at ultra low flow rates required to achieve equimolar response, were often impractical and could potentially lead to more errors.

In our experiment, to achieve equimolar response, it was necessary to minimize the changes in solvent composition over the LC time range where the drugs and metabolites were eluting. In our experimental setup, buspirone, M3, M6, M7 and M8 eluted between 16 and 26 min of the 45 min-gradient and the HPLC mobile phase composition during the 10 min period varied from 25 to 35% organic. The same sample was analyzed by using isocratic conditions but improvements were marginal and resulted in difficulties in integration of the peak areas due to overlapping peaks. When the HPLC gradient was changed to deliver one of the metabolites at 10% organic rather than at 25% organic, the earlier eluting metabolite was underestimated even when the flow rate was dropped as low as practically possible for LC–CSI-MS experiments.

The success of equimolar LC–CSI-MS response for buspirone and its metabolites, formed via diverse biotransformation pathways, suggests that LC–CSI-MS can be used simultaneously to quantify a drug as well as characterize and quantify its metabolites without the use of metabolite standard curves. Results reported here show a direct bearing on the ability to estimate levels of drug metabolites from early discovery studies and first-in-human clinical studies. Contrary to prior published procedures for achieving equimolar response, which required either infusion of samples or operations at ultra low flow rates, LC–CSI-MS is capable of providing equimolar

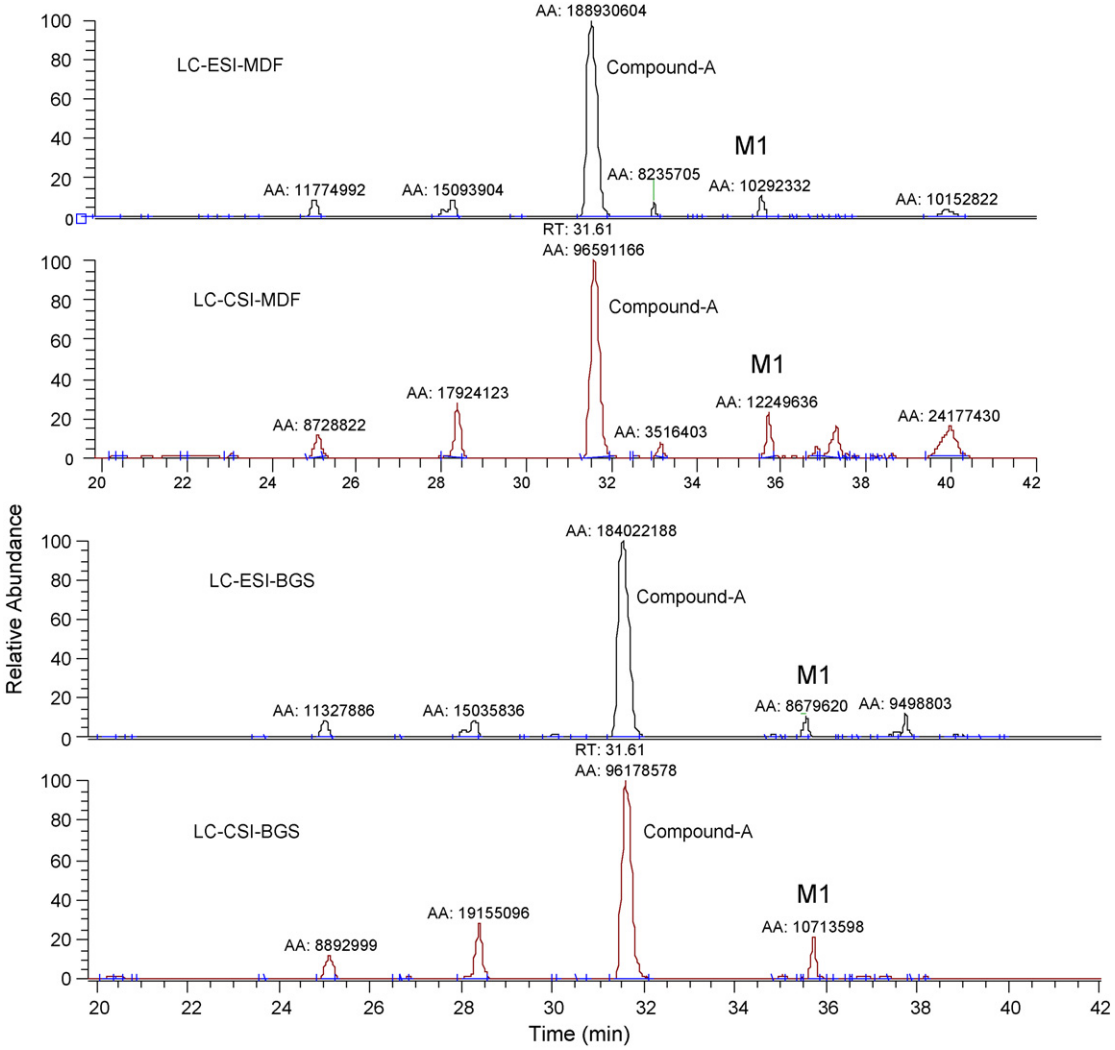

Fig. 5. Comparison of LC–ESI-MS and LC–CSI-MS plasma chromatograms following administration of compound A to subjects.

response for drug and its metabolites with flow rates of 2–7 μL/min. Equimolar response at low μL/min flow rates, however, requires minimal change in mobile phase solvent composition across the drugs and metabolites that are being quantified.

Once the CSI-MS method was established, in a second set of experiments, the established technique was applied to detect, characterize and quantify a metabolite present in human plasma samples following administration of drug A. Initially, drug A (compound A) and its monooxy metabolite M1 were quantified using a validated LC–ESI-MS/MS method. LC–ESI-MS/MS quantification method was validated using a triple quadrupole mass spectrometer, reference standards for drug A and metabolite M1 and previously established validation criteria [30,31]. Samples from each time point and each subject were separately analyzed and AUC_{0-24} was determined for the parent compound and M1. AUC of metabolite was determined to be 7% of the parent drug's AUC. In a separate experiment, plasma from each subject was pooled using the "AUC pooling" or "time-proportional pooling" method. This type of pooling has been described in detail by Hop et al. [32] and Hamilton et al. [33]. AUC pooling, allows time-proportional integration of individual plasma time points from a subject into one sample rather than analyzing individual time points to generate a concentration–time curve (described above for the validated method). Once samples are generated for each subject, then equal volume of plasma from all drug dosed subjects can be pooled to generate a composite sample. Similarly, equal volume of plasma from all placebo dosed subjects can be pooled to generate a composite placebo sample. This pooling method, not only streamlines the metabolite profiling workflow, allows metabolite AUC (once LC–MS responses differences are addressed and LC–MS extracted ion chromatographic peaks are integrated) to be readily estimated based on the parent AUC measured using a quantitative bioanalytical method.

Both drug dosed and placebo dosed AUC pooled samples were analyzed using full-scan HRMS and subjected to mass defect filtering (MDF) and background subtraction (BGS) approaches to detect metabolites and other drug derived material [21,22]. Fig. 5 compares the MDF and BGS data from LC–ESI-MS and LC–CSI-MS. With about 2% of the HPLC flow, sensitivity achievable with CSI-MS is clearly superior in comparison to what is achieved with 100% or 25% of HPLC flow for conventional flow ESI-MS experiments. As shown in Table 3, using LC–CSI-MS method, the AUC of M1 was estimated to be ~7% of the parent NCE's AUC and closely agreed with that obtained using a validated assay. Since the concentrations of M1 were not sufficient to get a clear UV response, UV correction factor was derived from another higher dose study. Without any response corrections, LC–ESI-MS estimated exposure of M1 was about 2.6% of the parent drug's AUC. Upon application of UV correction, which would have been impossible without the higher dose cohort, the exposure of M1 was estimated to be 6%. Unavailability

Table 3
Comparison of AUC of metabolite M1 obtained using validated LC–MS/MS, semi-quantitative LC–ESI-MS and semi-quantitative LC–CSI-MS.

Parent/metabolite	LC–ESI-MS–MS (%) validated assay	LC–ESI-MS (%) (not corrected)	LC–ESI-MS (%) (UV correction)	LC–CSI-MS (%) (not corrected)
Compound A	100	100	100	100
M1 (Day 1)	7.0	2.6	6.0	7.0
Equimolar mixture of compound A and metabolite M1 gave 1:1 LC–UV response at 254 nm				

of UV correction factors at lower drug concentrations and time and resource intensive quantitative assays, clearly demonstrates LC–CSI-MS as a means to obtain integrated qualitative and quantitative data to streamline the drug discovery and development process. Overall, LC–CSI-MS data can potentially be used to decide whether a metabolite reference standard needs to be synthesized for definitive quantification of metabolites as suggested in the FDA's metabolites in safety testing (MIST) guidance [34].

4. Conclusions

High resolution-based mass spectrometers have begun to emerge as a platform of choice for performing integrated qualitative and quantitative assays for streamlining the drug discovery and development process. These high resolution based assays are fast and sensitive and capable of quantifying drugs when reference standards are available. One of the difficulties of such assays is getting relative quantitative determination of metabolites or other drug derived material because of the inherent difficulties associated with achieving equimolar response when using conventional flow LC–ESI-MS. LC–CSI-MS has been demonstrated as a sensitive technique capable of detection, characterization and quantification of drugs and metabolites when employed with HRMS as part of integrated quantitative and qualitative assays. In comparison to other NSI techniques, CSI requires no X, Y, Z positioning or cameras to guide the spray positioning and delivers robust performance 24/7. Operational CSI flow rates of 250 nL/min to 50 μL/min provide the comparative simplicity in interfacing CSI source with micro-LC pumps rather than nano-LC pumps. Evaluations using equimolar mixture of buspirone and four monooxy metabolites present in human plasma show that LC–CSI-MS gives an equimolar response independent of the compound. However, this needs to be evaluated further using compounds with different structures including conjugated metabolites (glucuronides, sulfates, etc.). Comparison of LC–ESI-MS data with that obtained using LC–CSI-MS show that quantification of metabolites may be achievable without using a reference standard or administration of a radiolabeled drug. This in turn allows LC–MS quantitative and qualitative assessment of metabolites in discovery and first-in-human studies for early screening of disproportionate drug metabolites and human specific metabolites to improve patient safety and to assist in avoiding costs associated with late stage drug development failures.

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