Automated Nanospray Using Chip-Based Emitters for the Quantitative Analysis of Pharmaceutical Compounds

Leonard J. Corkery* and Henrianna Pang
Eli Lilly Canada Inc., Toronto, Ontario, Canada

Bradley B. Schneider and Thomas R. Covey
MDS SCIEX, Concord, Ontario, Canada

K. W. Michael Siu
Department of Chemistry and Center for Research in Mass Spectrometry, York University, Toronto, Ontario, Canada

An automated nanospray system based on chip technology (the NanoMate) was successfully interfaced to a modified Particle Discriminator Interface on a triple quadrupole mass spectrometer. A number of the interface parameters were optimized to improve the sampling efficiency for ions from the chip-based system. Analytical performance was assessed using a number of biochemicals as well as via a methodology for a pharmaceutical that passed validation as required by Good Laboratory Practices. Infusion analyses in flow rates <1 μL/min provided advantages in terms of throughput and sample consumption when compared to other methodologies based on liquid chromatography. (J Am Soc Mass Spectrom 2005, 16, 363–369) © 2004 American Society for Mass Spectrometry

In the pharmaceutical industry, there is a need to develop methods capable of quantification of low ng/mL levels of analyte in plasma-extracted samples. Liquid chromatography/tandem mass spectrometry (LC/MS/MS) methods, with atmospheric-pressure chemical ionization (APCI) or electrospray ionization (ESI) with pneumatic assistance, the latter commercially available as IonSpray, are frequently used for quantitative determination of drug candidates in biological matrices [1]. The main attractions of LC/MS/MS are the attributes of selectivity, sensitivity, and reproducibility [2]. However, LC/MS/MS can suffer from drawbacks such as long analysis time, protracted method development [3, 4], and ion suppression due to matrix effects [5].

Nanoelectrospray (nanoESI) technology [6] offers the possibility of increased sensitivity and lower sample consumption over conventional ESI, but historically has had drawbacks such as low sample throughput, difficult set up, poor signal stability, and poor tip-to-tip reproducibility [7]. To overcome these problems, nanoESI initiated by microfluidic devices or microchips fabricated on glass, quartz, or plastic substrates has been explored [8–10]. However, these systems have not been commercially available. Recently, a nanoESI chip-based system, the NanoMate, has been introduced [11]. This system contains an array of 100 individual ESI nozzles on a single chip and a robotic arm capable of delivering samples from a 96-well plate. Major advantages of this system include multiple-sample capability with minimal need for sample-to-sample reoptimization, no sample carryover, high sample throughput, and low sample consumption. Qualitative [12] and quantitative [13, 14] applications of this technology have been carried out in both the small molecule and large molecule arena, but no methodology has gone through the rigor of validation according to Good Laboratory Practices (GLP) guidelines [15]. Any methodology adopted by the pharmaceutical industry must meet GLP requirements.

The sensitivity of nanoESI can be enhanced if more of the sprayed plume is sampled into the mass spectrometer. Recently, an interface designed especially for nanoESI, the particle discriminator interface (PDI), has been developed for coupling to ESI with flow rates of 1 μL/min or lower [16]. Salient features of the PDI include improved sensitivity due to more efficient ion sampling, improved desolvation/declustering of ions, and improved stability. The geometric and low-flow characteristics of the NanoMate necessitate physical proximity between the spray tip and the ion inlet of the

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Address reprint requests to Dr. K. W. M. Siu, Department of Chemistry, York University, 4700 Keele Street, Toronto, ON M3J 1P3, Canada. E-mail: kwmsiu@yorku.ca

* Also at the Department of Chemistry and Center for Research in Mass Spectrometry, York University, Toronto, Ontario, Canada.

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mass spectrometer. The standard PDI can be used; however, preliminary investigations have shown that its dimension precludes optimal placement of the Nanomate and optimal sampling of the nanoESI plume. Modification to the standard PDI is necessary for optimal use with the Nanomate.

In certain circumstances, electrochemical oxidation of analytes can occur with the use of nanoESI, if the contact time of the sample with the electrode is exceedingly long [17, 18]. There are a number of ways to eliminate these reactions, some of which are described in this report.

Herein we demonstrate that it is possible to successfully analyze samples in the ng/mL regime using infusion nanoESI-MS with a modified PDI interface in methodologies that meet GLP standards, which serves as evidence for good analytical performance of the technology. We show that a modified PDI with a 2-cm laminar flow chamber (as opposed to the standard 1-cm one) provides optimal sensitivity and stability for use with the Nanomate. Evaluation includes assessment of accuracy and precision of the determinations, within batch and between batch reproducibility, as well as limits of detection and quantification. We used for the purpose of illustration two compounds of pharmaceutical interest, a growth hormone secretagogue (GHS) and gemcitabine, an oncolytic agent.

Experimental

Chip-Based nanoESI Emitters

NanoESI infusion analysis was achieved using a NanoMate 100 (Advion BioSciences, Ithaca, NY) [11]. This system holds a 96-well plate, a rack of 96 disposable conductive pipette tips, and a nanoESI chip. During infusion analysis, the system sequentially picks up a pipette tip, aspirates 1–5 μL of sample from the 96-well plate, and then delivers the sample to the inlet side of the ESI chip. The ESI chip is a fully integrated monolithic nanoelectrospray device that consists of a 10 $\times$ 10 array of nozzles etched from the planar surface of a silicon wafer. In our study, 5 μL of sample solution was delivered to the back plane of the nanoESI chip. The electrospray process was initiated by applying 1.6 kV and approximately 0.4 psi nitrogen head pressure to the sample in the pipette tip to ensure constant sample flow to the chip. These settings gave an approximate sample flow rate of 150–200 nL/min for samples prepared in 50/50 aqueous/organic solvents. The nanoESI chip was positioned directly in front of the aperture in the curtain plate. Samples were analyzed in approximately 60 s intervals using ChipSoft v. 4.7.1 software (Advion Biosciences), which controlled the automated nanoESI system. Data were averaged using Excel 2000 (Microsoft).

Mass Spectrometer

The NanoMate was custom mounted onto an MDS SCIEX API 3000 triple quadrupole mass spectrometer equipped with a prototype PDI [16]. The interface retaining ring was modified to permit close placement of the chip. The mass spectrometer was tuned to give unit mass resolution (peak width: 0.7 Th, full width at half maximum) for the third quadrupole, Q3. All MS/MS data were recorded in multiple-reaction monitoring (MRM) mode under optimal conditions.

PDI Interface

The PDI interface is designed to improve performance by providing two stages of unwanted particle and solvent removal, and two stages of desolvation. For these experiments, the length of the laminar flow chamber on the PDI source was extended to shorten the distance between the spray tip of the nanoESI chip and the entrance of the PDI laminar flow chamber. A schematic diagram of the modified interface is shown in Figure 1. It was operated at room temperature in this study. Typical nitrogen backpressure was 0.4 psi; the applied potential 1.5–1.6 kV; and the curtain gas was set to 0.2–0.8 L/min.

Reagents and Consumables

All chemicals were of analytical grade and were used without further purification. Acetonitrile (ACN) and formic acid were purchased from EM Science (Merck, Darmstadt, Germany). Methanol (MeOH) was purchased from Burdick and Jackson (Muskegon, MI). Dimethyl sulfoxide (DMSO) was purchased from Aldrich, (Milwaukee, WI). Deionized water was from a Milli-Q system (Millipore, Molsheim, France). Taurocholic acid, reserpine, and bradykinin were purchased from Sigma (St. Louis, MO). Dog plasma was purchased from Harlan Bioproducts for Science (Indianapolis, IN). All analytical and internal standards (IS) were obtained from Lilly Research Laboratories (Indianapolis, IN). Gemcitabine was an oncolytic agent [19]. GHS was a derivative of growth hormone [20]. The internal standard was an isotopically labelled GHS. Polypropylene 96-well plates (2 mL) were purchased from VWR (Mississauga, Ontario, Canada). Polypropylene vials (Micro tube 2 mL PP) were purchased from Sarstedt (Montreal, Quebec, Canada). Solid-phase extraction (SPE) plates were 3M Empore high-performance extraction disk plates (Universal Resin) purchased from VWR (Mississauga, Ontario, Canada).

Procedure

Calibration curves were prepared by fortifying dog plasma with the GLP validation compound. Appropriate volumes of standard solutions in plasma were diluted with 50/50 ACN/H_2O to produce standard

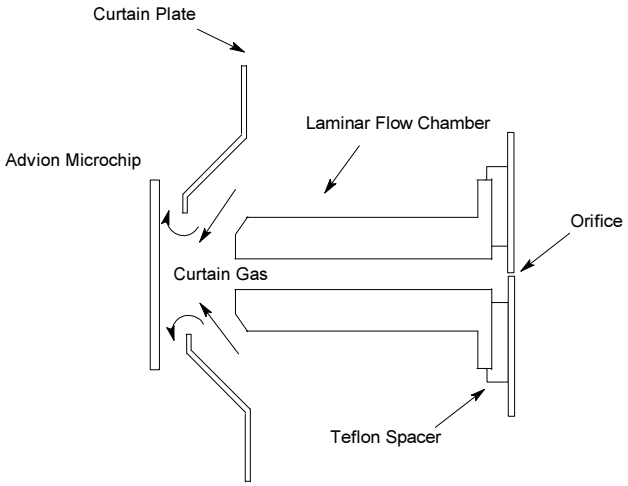

Figure 1. Modified PDI interface showing the position of the ESI chip relative to the curtain plate.

samples with the following concentrations: 5, 10, 20, 50, 100, 500, 1000, and 2500 ng/mL in a diluted plasma matrix. Quality control (QC) samples were prepared at 5, 1250 and 2500 ng/mL.

All plasma samples were vortexed for approximately 30 s. The samples were then centrifuged to remove particulate matter at ~3000 rpm for 5 min. A MultiProbe (Perkin Elmer, MultiPROBE II EX, Meriden, CT) was used to transfer 50 μL of blank plasma, standard samples and QC samples onto a 96-well plate. This was followed by the addition of 150 μL of 1% formic acid to the plasma blank. The plate was then transferred to a Tomtec Quadra 96 station (Tomtec, Model 320, Hamden, CT) and 150 μL of 20 ng/mL internal standard in 1% formic acid was added to the standard and QC samples. All the samples were then mixed by cycles of aspiration and dispensing. An SPE plate was conditioned with 200 μL of MeOH and then 200 μL of deionized water as recommended by the manufacturer. The samples (approximately 200 μL) were transferred into an SPE plate located in a vacuum manifold. The plate was subsequently washed with 500 μL of water and dried for ~30 s. The plate was then washed with 500 μL of 95/5 water/MeOH and briefly dried. A 96-well polypropylene collection plate containing 250 μL water was placed under the SPE plate and samples were eluted with 250 μL of acetonitrile. The plate was then evaporated to dryness and capped. Immediately prior to analysis, the samples were reconstituted with 500 μL of 50:50 ACN/H_2O and mixed for approximately 30 min. This results in a ten-fold dilution from the original plasma concentration to the final extraction (working solution) concentration. The final internal standard concentration was 6 ng/mL. The samples were centrifuged at 14,000 *g* for approximately 10 min prior to analysis.

Results and Discussion

Interface Optimization

For this application, the PDI interface was modified to enable closer placement of the nanoESI chip to the PDI. This entailed lengthening of the laminar flow chamber of the PDI and modification of the curtain plate (see Figure 1). With the standard 1-cm chamber, space limitations imposed by the NanoMate chip and the mass spectrometer entrance flange set a lower limit of 10.5 mm spacing between the chip and the entrance to the laminar flow chamber of the PDI. With the 2- and 3-cm long chambers, it was possible to shorten the spacing to 5 mm. However, previous experiments had shown that PDI interfaces with extended chambers tend to have lower ion transmission because of more extensive neutralization on the conducting wall [16]. In order to determine the optimum spacing, various compounds were run in both positive and negative ion detection modes using a 2-cm laminar flow chamber. The separation between the curtain plate and the PDI entrance was kept constant at 3 mm, and the separation between the NanoMate chip and the curtain plate was varied. Figure 2 shows an example of data acquired for a sample of taurocholic acid prepared in 50/50 ACN/H_2O with 2 mM ammonium acetate. The count rate improved as the chip was brought closer to the curtain plate from a spacing of 9 mm to 2 mm. The optimum spacing was 2 – 3 mm from the plate. The count rate for deprotonated taurocholic acid was attenuated by slightly more than a factor of two when the spacing between the chip and the curtain plate was 6.5 mm, as was necessary with a standard 1-cm chamber because of geometric requirements.

A series of experiments were conducted to compare the performance of this system with 1-cm, 2-cm, and 3-cm laminar flow chambers. Physical limitation necessitated the use of a flat curtain plate with the 1-cm chamber PDI, whereas the longer chamber PDIs used curtain plate shapes as shown in Figure 1. In order to maintain similar field distributions within the regions between the chip, curtain plate, and laminar flow chamber, a flat profile was designed on the front of the modified curtain plates. Performance, in terms of sensitivity and stability, was independent of the shape of curtain plate used, provided that the physical spacings between the chip, curtain plate, and chamber were constant. This was verified by experiments with a 2-cm chamber using a flat curtain plate shimmed outward by 1 cm and a curtain plate with the general shape shown in Figure 1. To assess differences in performance using the Nanomate with various length laminar flow chambers, a solution of 10^{-6} M bradykinin in 59.5%/39.5%/1% H_2O/MeOH/CH_3COOH was infused. The chip-to-curtain-plate spacings were 6.5 mm, 2.5 mm, and 2.5 mm for the 1-cm, 2-cm, and 3-cm chambers, respectively. The corresponding count rates for doubly protonated bradykinin were 2.23×10^5 ($\pm$ 0.12×10^5)

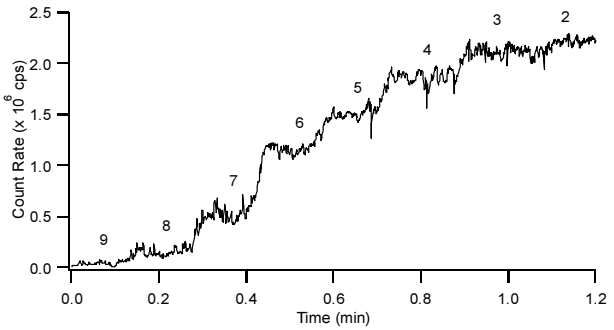

Figure 2. Count rate for deprotonated taurocholic acid as the spacing between the chip and the curtain plate varied from 9 mm to 2 mm.

cps, 3.30×10^5 ($\pm$ 0.05×10^5) cps, and 2.41×10^5 ($\pm$ 0.07×10^5) cps, respectively. It is evident that the ability to place the NanoMate chip closer to the laminar flow chamber in the 2-cm and 3-cm PDI offset the decrease in ion transmission as the chamber length was increased. In addition, the signal was more stable with reduced spacing between the chip and the PDI (5.4% RSD for the 1-cm chamber PDI; 1.5% RSD for the 2-cm chamber PDI). Similar stability and performance improvements were also observed in the negative ion mode for samples of taurocholic acid. The 2-cm chamber configuration was used for all of the subsequent experiments in this study.

Electrochemistry

In low flow-rate ESI systems, it is known that electrochemical reactions can lead to substantial analyte degradation due to extended residence times for analytes at the electrode surface [17, 18]. This leads to a gradual reduction in ion current for the analyte and a concomitant increase in ion current for oxidative products. The use of metal-coated tips [21] or the addition of a low concentration (<1 mM) of an easily oxidized compound can mitigate this degradation [22]. In this study in which conductive carbon-impregnated pipette tips were used as the electrode, the addition of hydroquinone to standard solutions of GHS was investigated and found to stabilize the signal. Experiments with reserpine in 50/50 MeOH/water, which is very sensitive to oxidation, were used to demonstrate this stabilization effect by two different methods (Figure 3). Rapid oxidation occurs with the use of a highly conductive carbon-impregnated pipette tip as the electrode, as shown in Trace A with the conversion of m/z 609 to 607. The addition of hydroquinone to the sample infused through this tip inhibited the electrochemical oxidation of reserpine by acting as a sacrificial reactant (Trace B). If direct contact of the sample with the surface of this electrode is prevented, oxidation of the analyte is also eliminated (Trace C). These modified tips were provided by Advion.

Preliminary Experiments

Initial experiments were carried out for both gemcitabine and GHS with the NanoMate and standard IonSpray sources, and comparisons were drawn between the two. These results were contrasted to conventional LC/APCI methods previously developed for these compounds in industry [23] and similar compounds in the literature [19, 20, 24]. Standards in plasma were prepared by SPE from blank plasma matrices. Standard solutions without plasma were prepared by direct addition to 50:50 organic:aqueous solvents. A series of solutions ranging from 0.5 to 250 ng/mL were prepared and calibration curves were constructed. From these plots, limits of detection (LOD) and quantification (LOQ), and coefficients of determination (R^2) were determined. The LOQ for gemcitabine was 0.5 ng/mL and 0.3 ng/mL using the NanoMate and ESI sources, respectively. Overall, the nanoESI performance of the Nanomate was similar to that of ESI (within a factor of two) based on the experiments with gemcitabine. Previous literature methods for gemcitabine gave similar LOQs of 0.3 ng/mL with LC/APCI [23] and 0.2 ng/mL with IonSpray [22]. The Nanomate performance for GHS was compared to literature data obtained using LC/APCI. The LOQ for GHS was 0.1 ng/mL with the Nanomate method, and 0.5 ng/mL with an LC/APCI method [23]. In this particular example, the performance of nano ESI was five times better. However, APCI has been shown to give LOQs of 0.1 ng/mL for structurally similar GHS compounds [20]. The analysis time was 4 min or longer using LC/APCI methods versus approximately 15 s using the NanoMate. As a result, the sample throughput was a lot higher with the Nanomate than LC/APCI.

GLP Validation

Validation was carried out for the bioanalysis of GHS by nanoESI with the NanoMate to better evaluate its performance. As per GLP validation guidelines, three batches of samples were prepared and each batch was

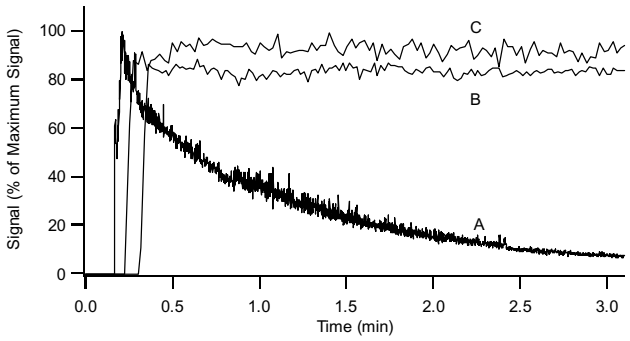

Figure 3. Signal from the molecular ion of a 100 pg/uL reserpine solution infused at 200 nL/min. Trace A, carbon-impregnated polypropylene tip; B, addition of hydroquinone to A; and C, modified tip without hydroquinone.

Table 1. Figures of merit

Sample	Ratio of background/ IS response[a]	Standard deviation (s)	Slope (m)	LOD (3σ/m) (ng/mL)	LOQ (10σ/m) (ng/mL)
1	0.013369	0.005718	0.1964	0.087	0.291
2	0.009785	0.002554	0.2554	0.030	0.100
3	0.008107	0.003621	0.2554	0.043	0.233
4	0.007343	0.001396	0.2594	0.016	0.053
5	0.015119	0.004084	0.2594	0.047	0.157
Mean				0.045	0.169
n[b]				5	5

[a]Average of n = 15 runs on 15 independent nozzles.
[b]Sixth sample not analyzed.

run on a separate day. Each batch had a duplicate standard curve and QC samples from two different lots of blank plasma. Each sample was infused a minimum of five times each with a different nozzle, and the resulting ion current profiles for the MRM transitions of the analyte and its internal standard were analyzed.

Samples were prepared by SPE from plasma extracts. Standard solutions were prepared, in duplicate, ranging from approximately 0.5 to 250 ng/mL (final extraction concentration). Quality control (QC) samples were prepared at the lower level of quantification (LLOQ), mid-level of the calibration range and upper level of quantification (ULOQ), corresponding to 0.5, 125, and 250 ng/mL, respectively. Six replicates of QC samples were prepared for each concentration. The internal standard was added to the standard and QC samples at approximately 6 ng/mL. Zero samples, containing internal standard only, were prepared in duplicate. Blank samples, containing no analyte or internal standard, were also prepared in duplicate.

Ratios of the average peak heights for the analyte ion current profile to the internal standard were calculated and used for regression analysis. The calibration curves were linear over the range tested (0.5 to 250 ng/mL) with $R^2 \geq 0.998$. Regarding accuracy and precision, the standards and QC samples were within 20% of the theoretical values at the LLOQ, and 15% at all other levels in the calibration range (94% of all results are within criteria). The intra-batch (within batch) accuracy ranged from approximately ±2% to ±14% at the LLOQ and the ULOQ, respectively. The inter-batch (between batch) precision ranged from 5% to 9% while the intra-batch precision ranged from 2% to 10%. Nozzle-to-nozzle variability (% relative standard deviation [RSD]) was calculated for each sample concentration (n = 5) for all samples analyzed based on the analyte/IS ratio. The average nozzle-to-nozzle variability (%RSD) was approximately 5%. Zero samples were used to calculate the background signal for the system by calculating the ratio of background signal to internal standard. Two lots of plasma zero samples, six samples in total, were evaluated across three batches. Approximately fifteen replicates per sample were analyzed utilizing separate nozzles for each replicate. The standard deviation of this ratio was determined, and from this, the LOD and LOQ were calculated: LOD = 3σ/m, LOQ = 10σ/m, where σ = standard deviation of zero sample and m = slope of calibration curve. Table 1 contains the calculated LOD and LOQ values. The average LOD and LOQ values were 0.045 and 0.169 ng/mL, respectively. The stability (%RSD) of the MRM trace at the LLOQ level ranged from 8.5 to 14.0%.

True blank samples (no IS or analyte) were used to validate that there was no interference in signal from the plasma matrix. Two plasma lots, six samples in total, were evaluated. Table 2 contains the signal response in blank versus the signal response in the LLOQ sample. There were no significant interferences observed. Competition for ESI by matrix components can suppress the ion signal for an analyte [5]. To evaluate the matrix effect, neat solutions (non-matrix) were prepared by spiking known concentrations of analyte, ranging from 0.05 to 250 ng/mL, into 50:50 organic/aqueous solutions. Spiked samples in matrix (plasma) were then prepared by spiking the same neat solutions into dried down blank plasma extracts. The loss of signal due to matrix effect was determined from:

$$\text{Loss of Signal} = \left(\frac{S_n - S_m}{S_n}\right) \times 100\% \quad (1)$$

where S_m is the signal for sample in matrix and S_n is the signal for sample without the matrix.

The average loss of signal due to the matrix effect was 51% (a factor of two suppression). Even though suppression was observed, the analyte and internal standard were suppressed to a similar extent, and there was no significant change in the variance of the blank (background), leading to little change in the LOQ.

Conclusions

NanoESI in the chip format (NanoMate) has been successfully coupled to a modified particle discriminator interface. This technology is sufficiently accurate, precise, and reproducible to meet GLP validation as demonstrated with one particular pharmaceutical compound. The NanoMate/PDI-based technology demon-

Table 2. Data in two plasma lots (six samples)

Component	Blank source	Response in blank (cps)	Response in LLOQ sample (cps)	Blank response (% of LLOQ)
GHS	Lot 1–1	4.9062	270.1782	
	Lot 1–2	8.3694	303.3936	
	Lot 2–1	18.4464	424.8148	
	Lot 2–2	29.7598	437.1323	
	Lot 1–3	20.4321	699.3169	
	Lot 1–4	16.2664	507.7227	
n		6	6	
Mean Response		16.3634	440.4264	
Mean % of Response				3.7
Internal Std	Lot 1–1	17.3160	2601.1636	
	Lot 1–2	8.0808	2904.3693	
	Lot 2–1	13.9821	3832.6123	
	Lot 2–2	9.5196	3906.2371	
	Lot 1–3	13.8860	5813.5586	
	Lot 1–4	4.9018	4141.1725	
n		6	6	
Mean response		11.2810	3866.5189	
Mean % of response				0.3

strated three times lower LOQ than LC/APCI/MS/MS for GHS. In addition, the analysis time and sample consumption of the former are much reduced in comparison with those of the latter. Oxidation of redox-sensitive analytes, exacerbated by long residence time at electrode surfaces, can be easily eliminated by a variety of means, some of which are demonstrated in this study.

Acknowledgments

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