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Mixed-mechanism ionization to enhance sensitivity in atmospheric pressure ionization LC/MS

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Abstract

A novel dual-mechanism ionization technique for LC/MS/MS has been observed, characterized and applied to the quantitation of a tertiary amine-containing drug compound in dog plasma. This mixed-mechanism ionization approach can improve the sensitivity of the pneumatically assisted electrospray experiment. Under conditions of higher than normal chromatographic flow and lower electrospray voltage, approximately a 4-fold increase in sensitivity was realized. A detection limit of 16 pg (45 fmol) on-column, and inter-day imprecision and inaccuracy of <11 and $<15\%$, respectively, were obtained. A trade-off in concentration sensitivity in favor of ease of sample preparation was made to increase sample throughput. Although results strongly suggest that mixed-mechanism ionization is in operation, and that pneumatically assisted electrospray is a partial contributor to the overall ionization process, the exact nature of the second mechanism of ionization is unclear at this time.

Keywords: Electrospray; Atmospheric pressure chemical ionization; Biological fluids; Quantitation

1. Introduction

In recent years, liquid-chromatography tandem-mass spectrometry (LC/MS/MS) has become a standard and widely used technique for the determination of drug substances in complex, biological matrices, such as blood plasma. The strengths of the approach include high selectivity, rapid method development, good sensitivity and applicability for quantifying a variety of molecules [1,2]. An important requirement for the practical utility of LC/MS/MS has been the ability to interface the high-pressure domain of analytical scale HPLC with the high-vacuum domain of mass spectrometry. This complex transition utilizes several interlaced processes including desolvation, nebulization, ion formation and ion introduction. Collectively, these processes have been described as atmospheric-pressure ionization (API).

Numerous variations of API have been devised. Some of those in routine use include atmospheric pressure chemical ionization (APCI) [3–5], elec-

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trospray ionization (ESI) [6], and pneumatically assisted electrospray ionization (Ionspray) [7]. Although there are subtle differences in approach, the ionization mechanisms for these techniques can be generally characterized as either APCI or electrospray. In APCI, ionization occurs through ion–molecule reactions induced by a corona discharge, whereas electrospray relies on ion ejection from the accompanying solvent, in combination with coulombic explosion of microdroplets. To some extent, these mechanisms have been studied and are understood well enough to provide reliable routine sample introduction for a wide number of applications [8]. Many aspects of the ionization mechanization such as cluster size [9] or the effects of the electric field [10] are still poorly understood and continue to be the focus of investigations. With this in mind, it is important to recognize that although API continues to provide an important tool for determining drugs in biological samples, the ion sources introduced thus far are still less than optimum for many compounds and applications.

With this understanding, the work reported here demonstrates practical application of mixed-mechanism ionization for quantifying selected analytes in mammalian blood plasma matrix by LC/MS/MS. This ionization approach uses a pneumatically assisted electrospray apparatus operating at electrospray voltages that are lower than normally used for electrospray operation. Supplemental ionization for the analyte was achieved predominantly by an alternate mechanism that is yet to be determined. This alternate ionization was achieved under relatively high flow conditions, by positioning the sprayer tip in closer than normal proximity to the orifice. Under the favorable conditions of high chromatographic flow and low capillary voltage, we have observed that this type of ionization will discriminate against the matrix background in favor of the analytes, while maintaining approximately the same ionization efficiency for analyte molecules. The feasibility of using this mixed-mode ionization for routine quantitation of a readily ionizable analyte in a biological matrix is assessed.

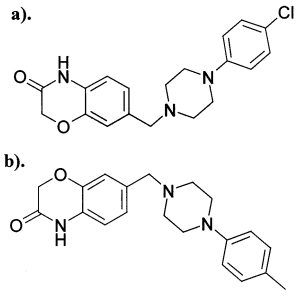

Fig. 1. Chemical structures for (a) compound I and (b) compound II (internal standard).

2. Experimental section

2.1. Materials

Test compounds were synthesized by Parke–Davis Pharmaceutical Research (Ann Arbor, MI). The structures for these are given in Fig. 1. They are designated as compound I (analyte) and compound II (internal standard). Liquid nitrogen provided curtain, drying and nebulizing gas for the mass spectrometer ion source and was purchased from AGA, (Maumee, OH). HPLC grade methanol and acetonitrile, and reagent grade ammonium acetate were obtained from EM Science (Gibbstown, NJ) and were used as received. Reagent grade water was prepared from in-house reversed osmosis, using a Milli-Q system (Millipore, Millford, MA). Dog plasma (heparinized) was prepared in-house using whole blood collected from Beagle dogs.

2.2. Apparatus

A Model API-365 LC/MS/MS system (PE Sciex, Concord, Ont., Canada), outfitted with a quaternary solvent delivery system and autosampler (series 200, Perkin–Elmer, Norwalk, CT) was used for all LC/MS/MS experiments. The ion source for all experiments was the Turbo Ionspray, capable of operating at pneumatically assisted electrospray flow rates (100–500 μl min^{-1}). A positive ionization mode was utilized. Typical instrument conditions were as follows: nebulization gas 15; curtain gas 12; collision activated dissociation gas 5; temperature 450°C; orifice 15

V; ring 150 V; Q0 −5.0 V; IQ1 −6.0; ST −10.0 V; RO1 −5.5 V; IQ2 −30.0 V; RO2 −65 V; IQ3 −100 V; RO3 −150 V; DF −225 V and CEM 2100. Ion reaction transitions of 358→162 and 338→162 were used for compounds I and II, respectively, in multiple-reaction monitoring (MRM) experiments. Detection was accomplished by channel electron multiplier, using dwell times of 300 ms, with a 40 ms interchannel delay for each of the two ions of interest. To induce or eliminate the mixed mechanism ionization in the source during experiments, the ionsprayer was positioned between indices of 15 and 3 mm, relative to the curtain plate.

The analytical column was octadecyl-silica (Genesis, 4-μ particle size, p/n FL25960E2, 250 × 3.0 mm, Jones Chromatography, Lakewood, CO), operating at ambient room temperature. The mobile phase was composed of 30:70 ammonium acetate (0.1 mM, pH 4.0): acetonitrile at a flow rate of 0.50 ml min^{-1}. The ammonium acetate was an essential component for the mobile phase in that it was thought to provide the correct pH environment for amine protonation.

Mass and voltage profiling experiments for compounds I and II were accomplished by direct infusion of 100 ng ml^{-1} solutions at flow rates of 100–500 μl min^{-1} using a Harvard apparatus (model 55-1111, Harvard Instruments, South Natick, MA).

2.3. Standards and quality controls

From a stock solution containing 50.0 μg ml^{-1} of compound I dissolved in methanol, a 5.00 μg ml^{-1} working solution was prepared volumetrically. These solutions were used to volumetrically prepare standard solutions containing between 5 and 250 ng of I in dog plasma. Quality controls were prepared at 5, 10, 50, and 200 ng ml^{-1} of I in dog plasma.

2.4. Sample preparation procedure

A plasma protein precipitant solution, containing 20 ng ml^{-1} of compound II (internal standard) in mobile phase, was added to a conical, glass autosampler vial containing 30 μl of plasma sample, standard or quality control. After placing the individual vials on a 96-deep well format plastic rack, the racks containing vials were vortex mixed (1 min) and centrifuged at 4000 rpm for 10 min. The 96-well containers were placed in an autosampler tray and samples (20 μl) were injected by inserting and positioning the needle so that it would avoid the protein pellets at the bottom of the vials. In this way, phase separation was not performed on prepared samples. This minimized the number of transfer steps and considerably reduced the overall work associated with sample preparation.

3. Results and discussion

3.1. Effect of electrospray voltage on MS response at different sprayer positions

Fig. 2a depicts the ion current intensity for I and II, respectively, as a function of electrospray voltage at a sprayer position of 10 mm, relative to the curtain plate. The analytes were delivered directly into the source at an infusion rate of 350 μl min^{-1} and a source temperature of 450°C. These ion current/capillary voltage profiles agree with results previously obtained in our labs for pneumatically assisted electrospray experiments involving easily ionizable groups such as amines. At the highest electrospray voltages, some roll-off in ion intensity was observed, possibly due to ion-suppression effects caused by discharge phenomena. The overall profiles for these ionization functions suggest that a single-mechanism ionization is in operation over the capillary voltage range from 0 to 5300 V [6].

Fig. 2b also depicts ion current intensity for compounds I and II, respectively, as functions of electrospray voltage. In this experiment, however, the sprayer was repositioned to index 5. Under these conditions, the ion intensities reach their primary maxima at much lower voltages ($\approx$ 2300 V) than would be expected from the purely pneumatically assisted electrospray experiment. A second maximum, occurring at higher voltages was consistent with pneumatically assisted electrospray. Comparisons of these two profiles suggest

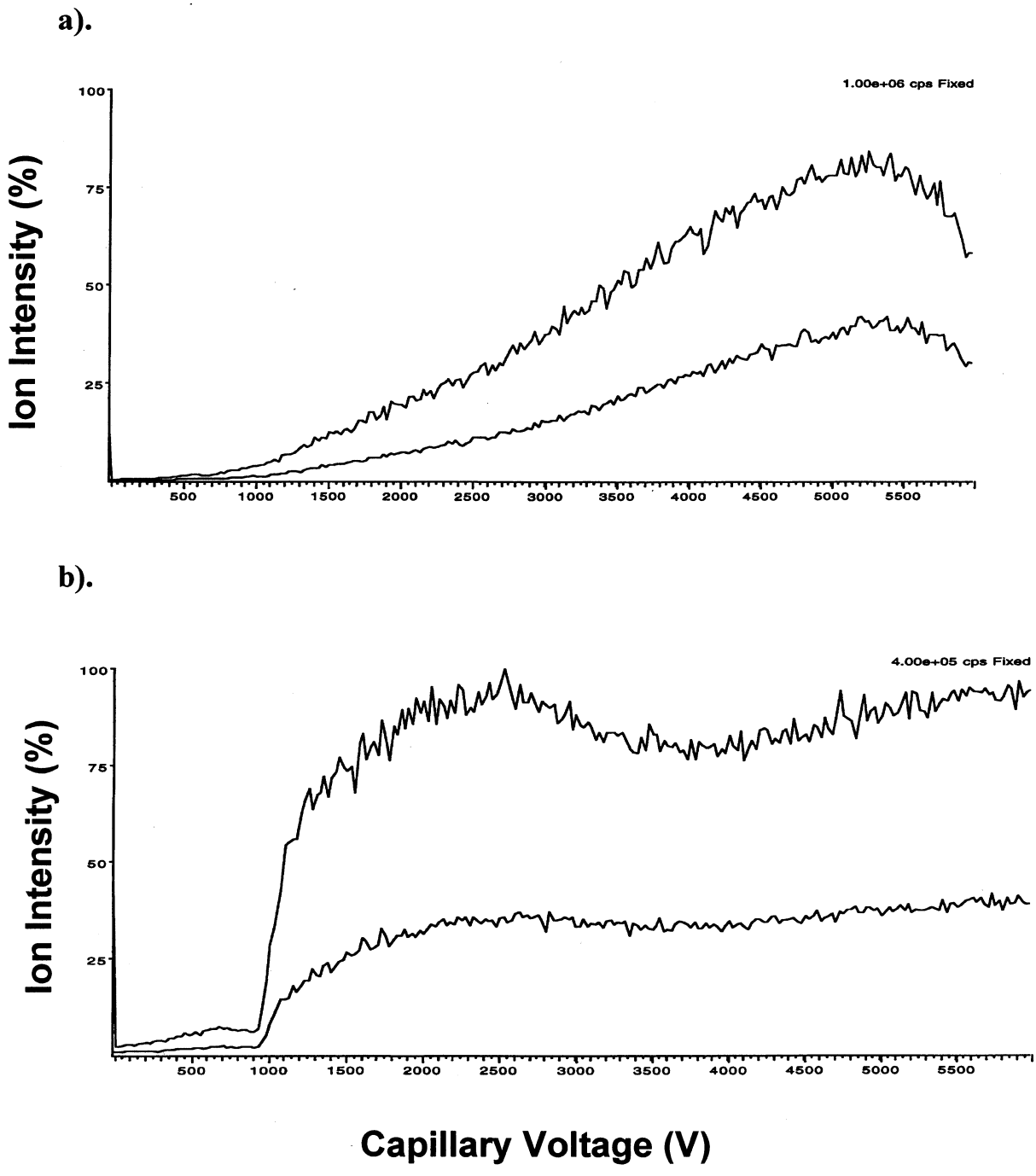

Fig. 2. Precursor ion intensity as a function of capillary voltage, for compounds I (upper trace) and II (lower trace) under ion spray (a) and mixed-mechanism ionization (b) conditions.

that two ionization mechanisms are at work. At the distal sprayer position, the solvent mediated proton transfer and coulombic explosion of the microdroplets, normally associated with electrospray, dominates. At the proximal sprayer position, a different ionization mechanism, possibly employing electrical discharge is also in operation. Under these conditions, it is possible that the driving force for the ionization is a static charge, possibly derived from the instrument curtain plate. Interestingly, however, no sprayer curtain plate arcing was observed during the ionization process.

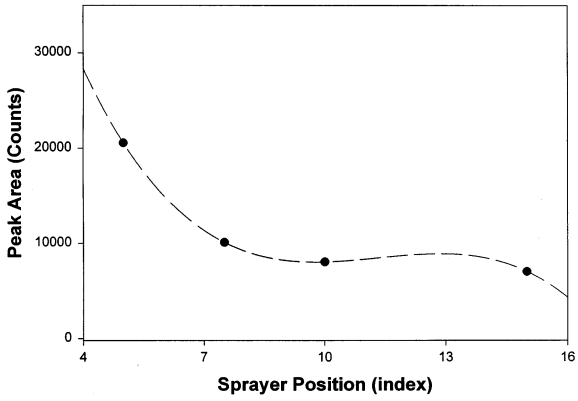

Fig. 3. Response as a function of sprayer position for compound I under conditions of mixed-mechanism ionization.

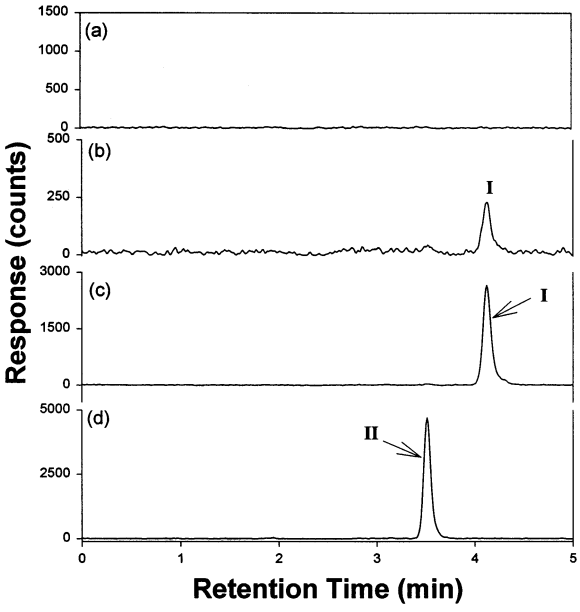

Fig. 4. Chromatograms representing (a) plasma blank, (b) 5 ng ml^{-1} standard, (c) a sample from a dog dose with I (8.6 μg ml^{-1} of I diluted 100 × prior to sample preparation), and (d) internal standard (compound II).

Regardless of the exact mechanism of ionization at the lower capillary voltage, it has been observed that the analyte ionization is strongly dependent on the proximity of the sprayer to the curtain plate. This relationship is depicted in Fig. 3, where approximately a 3-fold increase in response of I, based on peak area, between distal and proximal positions is observed. This observation that the analyte ionization is dependent on the proximity of the sprayer provides some evidence for the hypothesis that this new ionization mechanism is linked to the voltage on the curtain plate.

3.2. Chromatographic characterization

Representative chromatograms showing plasma blank, a low standard of I in prepared dog plasma (5 ng ml^{-1}), and a sample from a dog dosed with I are shown in Fig. 4a–c, respectively. A representative chromatogram for II, the internal standard, is given in Fig. 4d. Chromatographic peak shapes were generally excellent, and no matrix interference was detected during the course of the work.

3.3. Quantitation limits and sensitivity

The quantitation limit for I using this approach was 16 pg (45 fmol) on-column, with a signal-to-noise ratio (S/N) of 12. This quantitation limit was ascertained by determining the lowest standard concentration that gave consistent, acceptable results when included as part of a standard curve. When numerical smoothing (moving average) was applied to these chromatograms the S/N increases to approximately 30. In practical terms the quantitation limit for samples was 5 ng ml^{-1} using a 20-μl injection volume, with an associated uncertainty of 4% relative standard deviation. The higher sensitivity of this approach allowed the use of a streamlined sample preparation with higher sample dilution. This sample preparation approach, in turn allowed for the improved efficiency

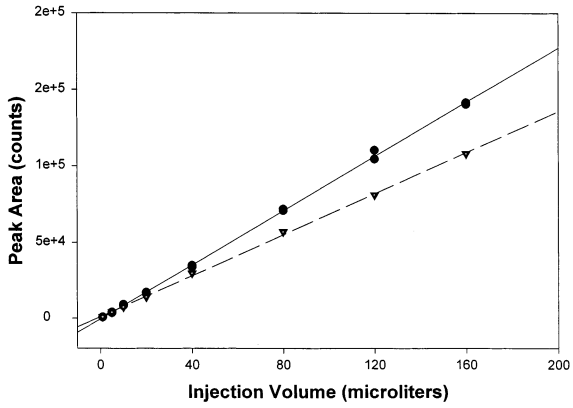

Fig. 5. Response as a function of injection volume for a solution containing 10 ng ml^{-1} of compound I at sprayer positions of 5 (—) and 10 (- - - -).

Table 1
Within- and between-day accuracy and precision for I at four quality-control levels

	Quality control level (ng ml^{-1})			
	5.0	10.0	50.0	200
Day 1				
% RSD	–	18.2	5.8	4.7
% RE	–	0.7	10.1	1.3
Day 2				
% RSD	3.1	3.1	4.4	2.2
% RE	−12.4	1.2	10.1	4.3
Day 3				
% RSD	2.9	11.0	1.5	4.2
% RE	−16.7	−3.4	12.1	7.1
Inter-day				
% RSD	3.9	11.0	3.8	4.1
% RE	−14.6	−0.5	10.8	4.2

in the form of decreased sample preparation time. A much lower concentration quantitation limit could have been achieved had the sample utilization been higher.

Fig. 5 demonstrates that a linear increase in response was obtained as the injection volume increased, indicating no obvious ion suppression. There was a practical limit to this approach, however, because the sample preparation involved a simple protein precipitation, without any separation of the supernatant from the pellet. Increasing the injection volume also increases the amount of undesired matrix components introduced into the system and could lead to premature column failure. For this reason extremely large injection volumes were not pursued. A trade-off in detection limit was made in favor of ease of sample preparation, and injection volume was limited to 20 μl for routine work. It is interesting to note that the results obtained in Fig. 5 demonstrate mass dependence response rather than the expected concentration dependence. Because it was independent of sprayer position, it appears that this mass dependent response is the result of chromatographic focusing rather than mixed mechanism ionization.

3.4. Linearity, precision and accuracy of the technique

This technique exhibited detection linearity from 5 to 250 ng ml, based on six-point calibration curves. Relative errors in back-calculated values for standards ranged from −7.8 to 6.1% for calibration curves generated on 3 separate days. These data suggest linearity consistent with typical instrument performance.

Intra-day precision for four levels of quality controls ($n = 3$ replicates) (Table 1) ranged from 1.5 to 18.2% RSD intra-day, and was typically less that 6%. Inter-day precision ranged from 3.8 to 11.0%. Intra-day relative error estimates ranged from −3.4 to 12.1%, except at the lowest control level, where they ranged from −12.4 to −16.7%. Inter-day relative error ranged from −0.5 to 10.8%, except at the limit of quantitation, where they were −14.6%. These values suggest acceptable performance for a quantitative bioanalytical method [11].

4. Conclusions

These results demonstrate the utility of a mixed-mechanism ionization approach to improve the sensitivity of the pneumatically assisted electrospray experiment. Under conditions of higher than normal chromatographic flow and

lower electrospray voltage, approximately a 4-fold increase in sensitivity was obtained for a tertiary amine-containing drug compound in a mam malian plasma matrix. Although results strongly suggest that mixed-mechanism ionization is in effect, and that pneumatically assisted electrospray is a minor contributor to the overall ionization process, the exact nature of the second mechanism of ionization is unclear at this time. This technique demonstrated mass dependence response rather than concentration dependence. The quantitative performance of the resulting assay method was acceptable and allowed minimal sample preparation effort.

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