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Short Communication

Influence of ionization source design on matrix effects during LC–ESI-MS/MS analysis

Chinmoy Ghosh[a,b,*], Chandrakant P. Shinde[b], Bhaswat S. Chakraborty[a]

[a] Bio-Analytical Department, Cadila Pharmaceuticals Limited, 1389-Trasad Road, Dholka, Gujarat, India
[b] School of Studies in Chemistry, Jiwaji University, Gwalior, M.P., India

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ABSTRACT

Glycerophosphocholines (GPChos) are known to cause matrix ionization effects during the analysis of biological samples (i.e. plasma, urine, etc.) in LC–MS/MS. In general, such matrix effect is directly related to an insufficient sample clean-up of the biofluids. In addition to GPCho; design of ionization source and/or LC also plays a very important role in matrix effects. In this research paper, different types of matrix effects, i.e. ion suppression or enhancement were observed in differently designed ion sources coupled with different LCs, from the same molecule, acamprosate (ACM), under the same chromatographic conditions. ACM was analyzed in a negative polarity in electrospray ionization interface using Z-spray and orthogonal spray ion source design. The analyte showed almost complete ion suppression in the Z-spray ionization source coupled with UPLC/HPLC, whereas there was very little ion enhancement in the orthogonal spray ionization source coupled with HPLC. In both the cases different GPChos were responsible, as evident from the presence of *m/z* 815.4 in Z-spray ion source and *m/z* 759.0 in orthogonal spray ion source. Hence, this approach can be used to evaluate the matrix effects in plasma samples during development and validation of LC–MS/MS method of drugs and their metabolites in different biological matrixes.

1. Introduction

Mass spectrometry (MS) is a powerful qualitative and quantitative analytical technique that has been used for most clinical and research laboratories for the last three decades. In clinical laboratories, mass spectrometers are used to measure a wide range of clinically relevant analytes. When applied to biological samples, the power of MS lies in its selectivity toward the identification and quantification of compounds [1].

There is, however, one limitation associated with the LC–MS analysis, i.e. susceptibility to the matrix effects (ME) [2,3]. Matrix effect is defined as the effect of co-eluting residual matrix components on the ionization of the target analyte. Typically, suppression or enhancement of analyte response is accompanied by diminished precision and accuracy of subsequent measurements [4–6]. Matrix effects may thus limit the utility of LC–MS for quantitative analysis [7] which includes suppression or enhancement of ions, decreased or increased sensitivity of analyte over time, increased baseline, imprecision of results, retention time drift and chromatographic peak tailing.

Evaluation of matrix effects and chromatography/MS efficiency is critical to the quality of LC/MS method development. Therefore, it is now becoming an essential part of method validation, further optimization and method transfer across platforms to other MS. Moreover, developing a satisfactory and almost matrix effects free bioanalytical method is a preliminary step for any pharmacokinetic study.

Matrix effect does not solely relate to the ionization process of LC–MS/MS [8–10]. Extraction technique, chromatographic procedure including mobile phase constitution, flow rate, analytical column, etc. should also be considered while developing the methods, where precise, accurate and reproducible analysis are required. But there is hardly any paper describing the role and influence of differently designed ionization sources of ESI interface [11] on matrix effects. So in the present research work, ACM [Fig. 1] was analyzed by using two different LC–ESI-MS/MS instruments having differently designed ionization sources coupled with LC, to find out the consequence on matrix effects.

Two procedures are reportedly available for an evaluation of matrix effects. The first is a qualitative evaluation and the second one is a quantitative evaluation. By quantitative evaluation, the exact degree of ion suppression/enhancement can be determined which is useful information for any analytical method.

* Corresponding author at: Contract Research Organization, Cadila Pharmaceuticals Limited, 1389, Trasad Road, Dholka-387 810, Ahmedabad, Gujarat, India. Tel.: +91 2714 221481/83/84x237, fax: +91 2714 221848.
E-mail address: chinmoy_ghosh@yahoo.com (C. Ghosh).

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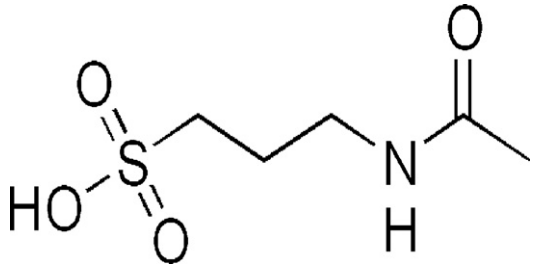

Fig. 1. Structure of ACM.

2. Experimental

The qualitative evaluation procedure is based on the post column infusion of analyte in a chromatographic run along with an extract of a blank matrix. In the presence of matrix effects, i.e. ion suppression/enhancement the signal shows downward/upward directions from its original baseline, otherwise there is no change in baseline response.

The quantitative evaluation of matrix effects, on the other hand, as described by Matuszewski et al. is based on the injection of two sets of samples [12,13]. Set A consists of neat standard solutions (eventually a calibration line), resulting in the reference peak area(s). For set B extracts of 6 different blank matrices are supplemented (after the extraction) with the same amount of standards as used for set A (which then results in peak areas B). The matrix effects in terms of matrix factor (MF) can then be calculated using the peak area(s) obtained for the set of samples spiked after extraction (B) divided by the peak area(s) obtained for the neat standards (A) times 100, where 100% indicates absence of any matrix effects, whereas <100% means suppression and values >100% indicates enhancement of the ionization process. The formula is given below:

$$\mathrm{MF} = \frac{B}{A} \times 100 \quad (1)$$

The experiments described in this manuscript have been conducted to reduce or minimize the matrix effects during LC–ESI-MS/MS analysis of ACM in plasma samples by changing the ion source design and/or LC hardware. Two different experiments were taken into consideration for this purpose. Experiment-I was performed by using the Z-spray ion source design coupled with Acquity UPLC/HPLC, whereas, experiment-II, was conducted using the orthogonal spray ion source design attached with HPLC. In order to evaluate and determine the matrix effects in differently designed ionization source of the ESI interface, two different LC–MS/MS instruments were used.

2.1. Chemicals and reagents

ACM (99.8%) was procured from IND-SWIFT Laboratories Limited, Mohali, Punjab, India. All chemicals were of analytical reagent grade unless stated otherwise. The water used for the preparation of mobile phase and other solutions was collected from a Milli Q_{PS} (Milli Pore, USA). HPLC-grade methanol, acetonitrile and acetic acid were supplied by J.T. Baker, USA and Finar Chemicals Limited, Ahmedabad, India, respectively. Ammonium acetate was purchased from Qualigens fine Chemicals, Mumbai, India. Drug free human plasma treated with K_2EDTA used during analysis was supplied by Clinical unit of Cadila Pharmaceuticals Limited, Ahmedabad, India. The plasma was stored at $-30 \pm 5\,^{\circ}$C before sample preparation and analysis.

2.2. Experiment 1

2.2.1. Instrumentation

Matrix effect was quantified using Waters Quattero Premier XE MS/MS system attached separately with UPLC and HPLC (Waters Corporation, Milford, USA), equipped with an ESI interface used to generate negative ions $[M-H]^-$. The compounds, i.e. phospholipids were separated on a reversed phase column (Hypersil BDS C18, 150 × 4.6 mm ID, particle size 5 μm, Thermo Electron Corporation, UK), with an isocratic mobile phase consisting of 10 mM ammonium acetate (pH: 5.50 ± 0.05) in milli-q water and acetonitrile at a ratio of 15:85 (v/v). The mobile phase was eluted at 0.80 mL/min. The auto sampler rinsing volume was 500 μL and injection volume was 5 μL. The column and auto sampler temperature were maintained at 35 °C and 4 °C, respectively.

The mass transitions used for ACM was m/z 179.90→79.90. Quadrupoles Q1 and Q3 were set on a unit resolution. The analytical data were processed by Masslynx software. Ion source design was based on Z-spray with negative polarity. The detailed mass parameters set to the instrument were as follows: capillary, cone and extractor voltages were adjusted to 3.5 kV, 26 V and 3 V, respectively. The source temperature and desolvation temperature were set to 100 °C and 300 °C, respectively. Cone gas and desolvation gas flow were set to 50 L/h and 650 L/h. Ion energy and collision energy were fixed to 1 V and 23 V, respectively. LM1, LM2, HM1 and HM2 resolutions were set to 15.5.

2.2.2. Sample treatment

Protein precipitation method was used to extract ACM. Only 250 μL plasma sample was transferred to a ria vial for analysis. Add 1000 μL of acetonitrile to precipitate protein and vortex it for 1.5 min followed by centrifuge at 10,000 RPM for 5 min. The supernatant was then directly injected (5 μL) to LC–MS/MS.

2.3. Experiment 2

2.3.1. Instrumentation

Here, matrix effects were quantified using SCIEX API 4000 MS/MS system (MDS Sciex, Canada) attached with HPLC (Shimadzu, Japan), equipped with an ESI interface used to generate negative ions $[M-H]^-$. All other chromatographic parameters were same as experiment-1.

The optimized ion spray voltage and temperature were set at −4500 V and 450 °C. The typical ion source parameters, viz., declustering potential, collision energy, entrance potential and collision cell exit potential were −50, −10, −32 and −5 V. Nitrogen gas was used as nebulizer gas, curtain gas and collision-activated dissociation gas, which were set at 40, 12 and 7 psi, respectively. Quantification was performed by multiple reaction monitoring of the deprotonated precursor ion and the related product ion for ACM. The mass transitions used for ACM was m/z 179.90→79.90. Quadrupoles Q1 and Q3 were set on a unit resolution. The analytical data were processed by Analyst software (Version 1.4.2; Applied Biosystems). Ion source design was based on orthogonal spray with negative ionization mode.

2.3.2. Sample treatment

Sample preparation technique was also same as experiment-1. Same lots of plasma were also used during sample treatment.

3. Results

3.1. Experiment 1

During method development, a neat ACM sample was infused through an infusion pump to tune the parent molecule in both the

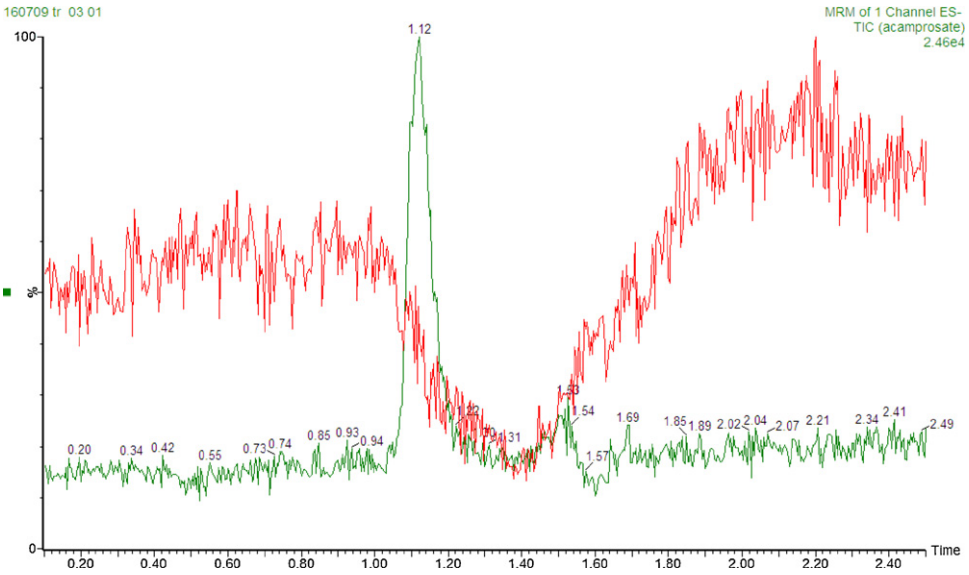

Fig. 2. Matrix effects of ACM in Quattro Premier XE coupled with UPLC (ion suppression).

polarities. But stable parent ion (m/z 179.9) was observed only at negative polarity. The neat analyte was infused along with an injection of extracted plasma blank in HPLC and UPLC; both showed the complete ion suppression. Fig. 2 evidenced the fact in UPLC system and Fig. 3 for HPLC system. Furthermore, to determine the degree of matrix effects, MF was estimated by using six different plasma lots, which also revealed the complete ion suppression in both HPLC and UPLC, as the MF value was zero. Hence, further investigation was confined into UPLC system only to find out whether LC has any role on ME for this molecule.

So, to find out the phospholipids which were causing the matrix effects in UPLC, pre-cursor ion scan at m/z 184.0 was performed, in positive polarity, keeping all other LC–MS parameters constant as mentioned in experiment 1, because most phospholipids have the common product ion at m/z 184.0 in positive ionization polarity [13].

Fig. 4 represents the precursor ions scan at the retention time of ACM. From the spectra it was observed that one lyso-phosphotidylcholine at m/z 527.3 and three other phosphotidylcholines at m/z 762, 789.1 and 815.4 (most intense) and its isotopes were present. Reportedly all these phosphotidylcholines may be responsible for the matrix effects, i.e. ion suppression in instrument 1. Moreover, the Q1 scan of the extracted plasma blank was also performed in the negative ionization mode and ions were extracted at the retention time of ACM. Among all the extracted ions there was a major response at m/z 255.1[Fig. 5], possibly a fragmented ion of glycerophospholipid, which may also be responsible for matrix effects, i.e. ion suppression in instrument 1. So, different ions were observed at the retention window of ACM, which were supposed to be responsible for such ionization behavior.

3.2. Experiment 2

The neat ACM sample was infused through Harvard infusion pump and the parent molecule was tuned in MS mode in negative polarity. It showed a stable parent ion, i.e. m/z 179.9. The neat

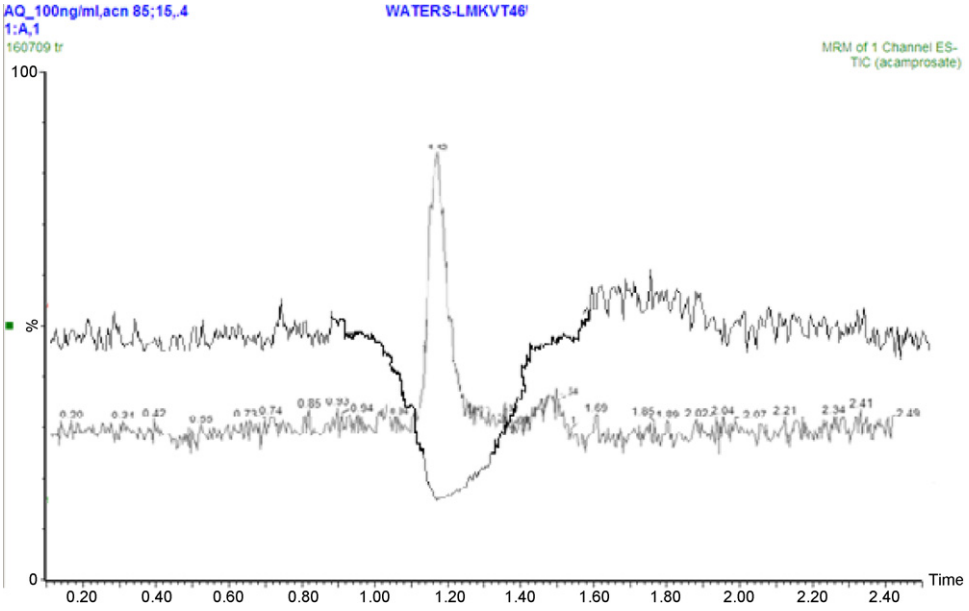

Fig. 3. Matrix effects of ACM in Quattro Premier XE coupled with HPLC (ion suppression).

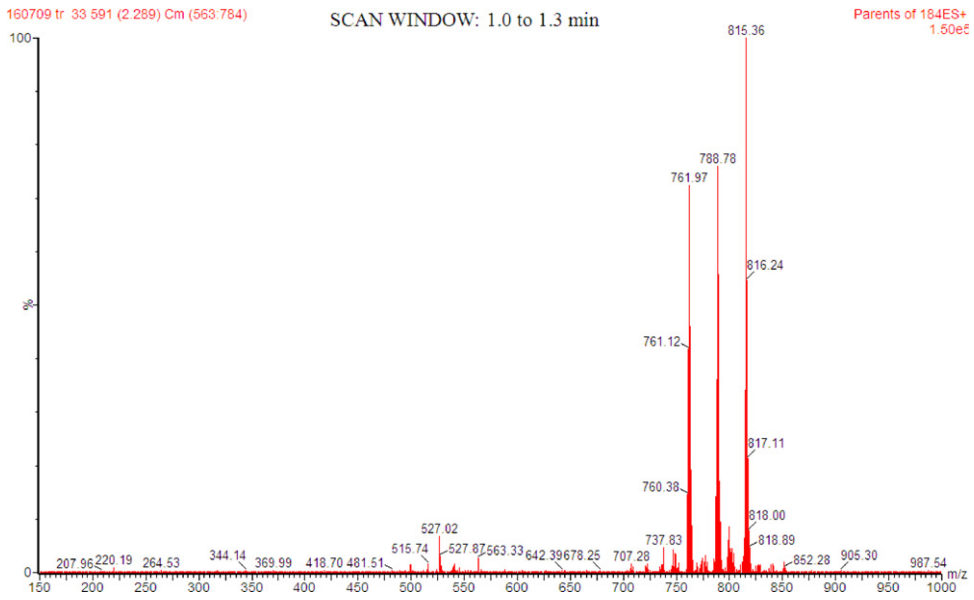

Fig. 4. Pre-cursor ions scan of *m*/*z* 184 in Waters Quattro Premier XE in positive mode.

analyte was infused along with an injection of extracted plasma blank, but it showed very little ion enhancement [Fig. 6]. Furthermore, to determine the degree of matrix effects, matrix factor was estimated from six different plasma lots which were used in experiment 1. The average matrix factor at low quality control (LQC) level was 0.9005, the %CV was 7.11 and the average MF at high quality control (HQC) level was 1.116 with %CV was 8.87. The overall MF was 1.008, which indicates that there was very little or no ion enhancement.

Previous research [13] has described the *m*/*z* 184.0 as a common pre-cursor ion for phospholipids in positive ion polarity. To identify the phospholipids, we performed the pre-cursor ion scan at *m*/*z* 184.0, in positive polarity, keeping all other LC–MS parameters same as mentioned in experiment 2.

The pre-cursor ions scan at the retention time of ACM obtained from experiment 2 is presented in Fig. 7. From the spectra, it was observed that two lyso-phosphotidylcholines at *m*/*z* 497 and 525 and six phosphotidylcholines at *m*/*z* 704, 759, 761, 785, 787 and 811 were present. Among all these, *m*/*z* at 759 (most intense), 761 and 787 were the major phosphotidylcholines, in instrument 2. Again, Q1 full scan of the extracted plasma blank was also performed in the negative ionization mode and ions were extracted at the retention time of ACM, which showed a major response at *m*/*z* 215.0 [Fig. 8], a fragmented ion of diacylated glycerophospholipid [14]. These phospholipids may be responsible for the ion enhancement in instrument 2.

4. Possible causes of different ionization in different ion source design of ESI-MS/MS

The ESI technique involves a number of steps, including the formation of charged droplets, desolvation, ion generation, declustering and ion sampling. As the name suggests, the basis of ESI technique lies in using a strong electric field to create an excess

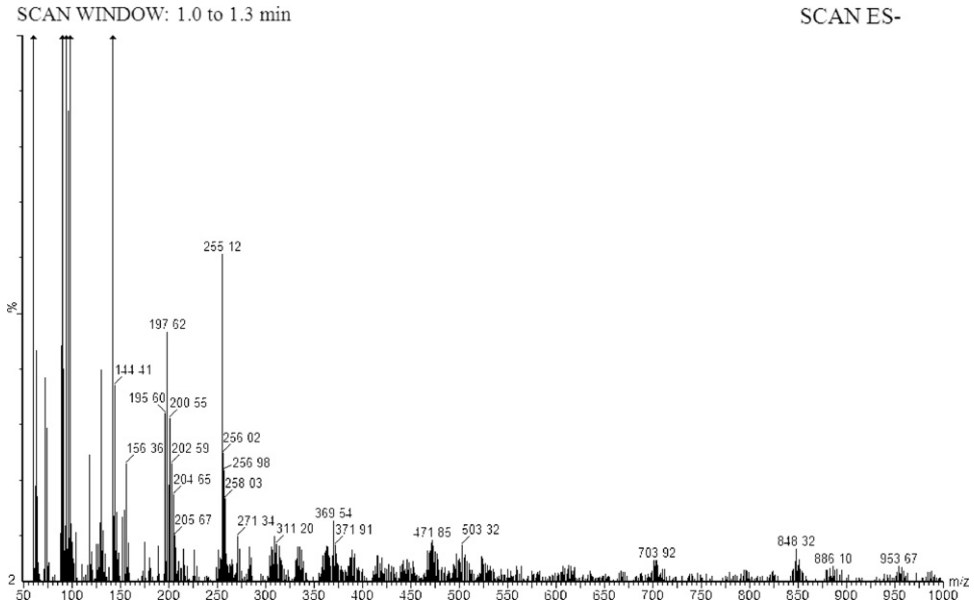

Fig. 5. Ions at the retention time of CAM in Waters QPXE (Q1 scan).

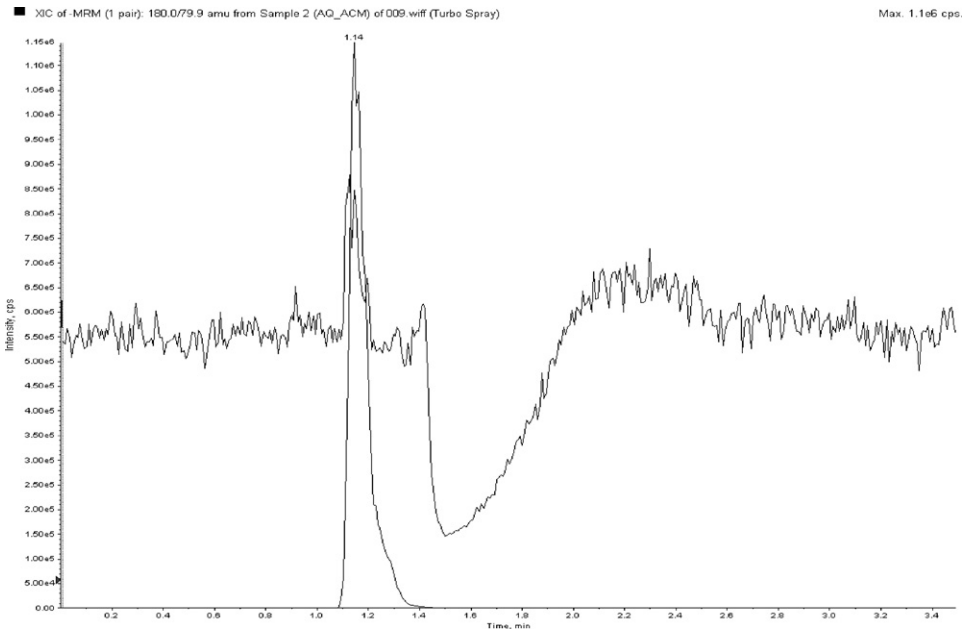

Fig. 6. Matrix effects of ACM in API-4000 (ion enhancement).

of charge at the tip of a capillary containing the analyte solution [15–17]. Charged droplets exit the capillary as a spray and travel at atmospheric pressure down an electrical gradient to the gas conductance limiting orifice or tube. Gas phase ions are then transported through different vacuum stages to the mass analyzer and ultimately the detector. In order to direct charged species into the mass spectrometer a series of counter electrodes is used in order of decreasing potential. Typically, the principal counter electrode is the curtain plate. This counter electrode is a plate with an orifice which passes the ions to the mass spectrometric sampling system, i.e. toward the vacuum interface of MS. The pressurized nitrogen gas forces the liquid through the capillary. The value of the electric field (E_C) at the capillary tip opposite to a planar counter electrode can be calculated by the proposed equation of Lobe et al. [18].

$$E_C = \frac{2V_C}{r_C \ \ln(4d/r_C)} \tag{2}$$

where V_C is the applied potential, i.e. ISV, r_C is the capillary outer diameter, d is distance from capillary tip to the counter electrode, i.e. orifice plate. For example, if V_C is 3000 V, r_C is 10^{-4} m and d is

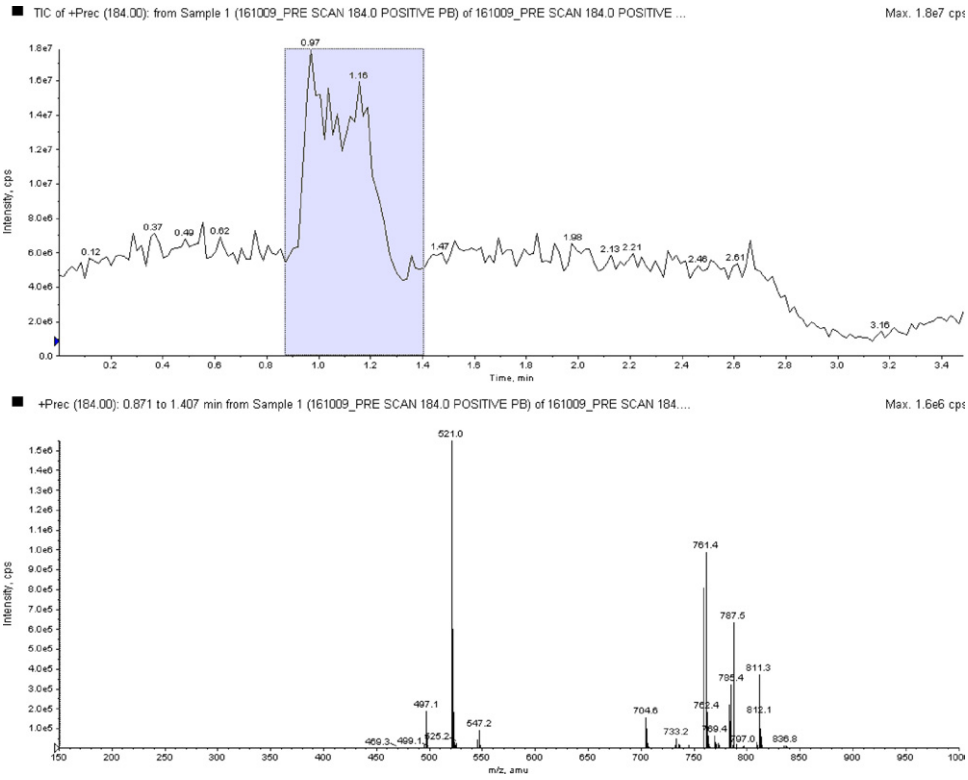

Fig. 7. Pre-cursor ions scan at m/z 184 in plasma blank in positive polarity in API-4000.

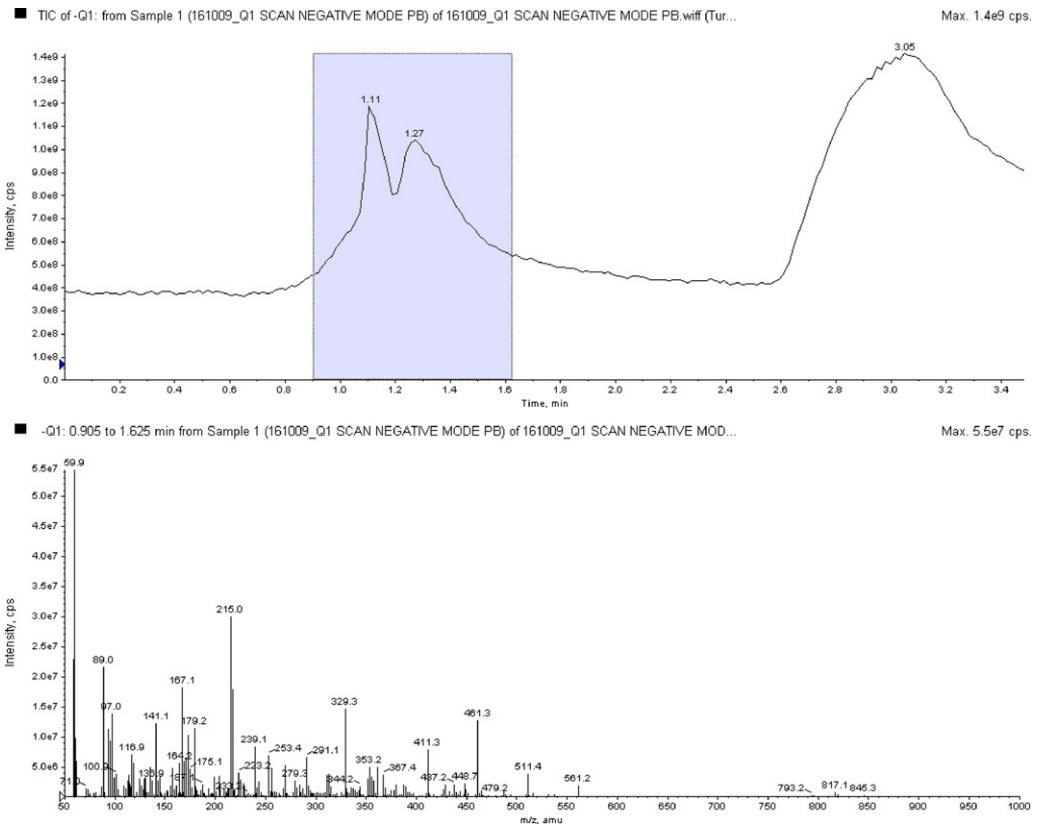

Fig. 8. Q1 spectrum of plasma blank in negative polarity in API-4000.

0.02 m, E_C has a value of 9×106 V/m. E_C is directly proportional to V_C, and inversely proportional to r_C and d, but decrease very slowly with d due to logarithmic dependence on d.

The required ISV, i.e. V_C can also be calculated. It depends on the surface tension of mobile phase/solvent (γ), radius of the capillary (r_C), permittivity of the vacuum (ε_0), distance of the capillary tip from the orifice (d) and θ the half angle of the Taylor cone.

$$V_C \approx \left(\frac{r_C \gamma \cos \theta}{2\varepsilon_0}\right)^{1/2} \ln\left(\frac{4d}{r_C}\right) \quad (3)$$

The above equation was experimentally verified by many researchers [19–21]. From the experiments it was observed that for stable ES spray, V_C should be few hundred volts more than the calculated one.

The optimal potential difference between the sprayer and the principal counter electrode depends on experimental parameters, such as the charged state of the analyte, the solution flow rate, the solvent composition and the distance between the tip and the counter electrode. In the presence of an electric field liquid emerges from the tip of the capillary in the shape of a cone, also known as "Taylor Cone" [21] [Fig. 9]. When the electrostatic repulsion between charged molecules at the surface of the Taylor Cone approaches the surface tension of the solution – known as reaching the Rayleigh limit – charged droplets are expelled from the tip. The droplet containing the excess charge generally follows the electric field lines at the atmospheric pressure toward the counter electrode. However, trajectories will also be affected by space charge and gas flow.

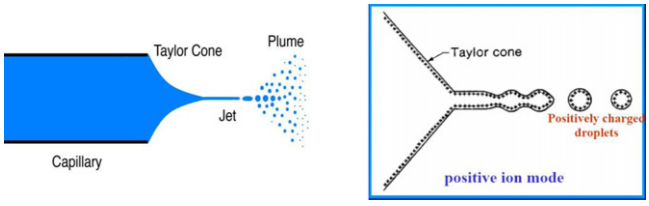

Fig. 9. Taylor cone.

The mechanism of forming the Taylor Cone is not clearly understood, but it is known that under certain conditions, the morphology of the spray emitted from the capillary tip can change [22]. The various spray modes strongly depend on the capillary voltage and are related to pulsation phenomenon observed in the capillary current.

Gomez and Tang [22] have shown that the ligament exited from the Taylor cone persisted for a short distance, roughly 2 mm and then broke up into droplets. This droplet formation showed a bimodal distribution of droplets, which consists of a primary distribution of large droplets and a satellite distribution of smaller droplets. The satellite droplets were produced at break-up and were displaced radially by small disturbance and/or space charge effects.

Once airborne, the liquid droplets' structural integrity becomes dependent upon the struggle of surface tension with the electrostatic repulsion that results from the solvated ions. Up to a point, known as the Rayleigh limit, surface tension will hold the repulsive forces in check and prevent droplet fragmentation. Due to evaporation, however, continuous shrinkage in droplet size gradually brings the charges closer together, increasing repulsion proportionally. Eventually, the Rayleigh limit is overcome and the droplet undergoes Coulombic explosion, splitting into progeny droplets in which the process is reset (Fig. 10). The amount of charge, q_R, at which the Rayleigh limit is exceeded and fission occurs has been described by the mathematical relationship [23].

$$q_R = 8\pi(\varepsilon_0 \gamma R^3)^{1/2} \quad (4)$$

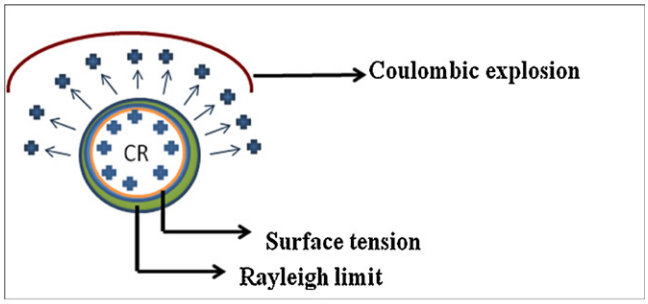

Fig. 10. Ion formation mechanism in ESI interface.

where q_R is the charge on the droplet, γ the surface tension of the solvent/mobile phase, R is the radius of the droplet and ε_0 is the electrical permittivity.

Juraschek and Rollgen showed that liquid flow rate, capillary diameter and electrolyte concentration can all impact the spray mode. Controlling the spray mode is thus crucial in achieving a stable spray and an optima signal.

The size of the spray droplet released from the Taylor Cone, highly dependent on the flow rate and capillary diameter, is critical to the efficient ionization of the analyte. Since a small droplet contains less solvent, desolvation and ionization can be more efficient. Because less fission is required to produce ions. The salt concentration in the final off spring droplet may be lower compared to the droplet that has undergone more evaporation fission cycle. As a result, the background noise in the mass spectrum may be reduced [24]. In addition with smaller droplets, analytes that are not surface active will have a greater chance of being transferred to the gas phase rather than being lost in the bulk of the parent droplet residue.

Kebarle concluded that charging a single protein in the evaporating droplet is due to small ions found at the surface of the droplet [25]. The mechanism of forming small analyte ions is still not clear. Charging analyte molecules can occur through more than one process [26]. Charge separation (in the ESI source), adduct formation, gas phase molecular reactions, and electrochemical reactions may also contribute to ionization during the electrospray process [27].

Efficient transport of ions and charged droplets from the sprayer into the mass spectrometer is challenging and depends on parameters such as interference arrangement and gas throughout into the instrument [20]. As gas and ions are transported from atmospheric pressure into vacuum, strong cooling of the mixture occurs during expansion. Under these conditions, polar neutral molecules make cluster with analyte ions and it is therefore very important to achieve efficient desolvation within the atmospheric region.

If the flow rate is in the range 0.05–3 mL/min, sensitivity can be an issue, due to the decrease in the ionization efficiency resulting from large size droplets.

From the above discussion it is observed that the formation of ions depend on multiple parameters, e.g. capillary diameter, distance from capillary tip to the counter electrode, radius of the droplets, electrolyte concentration, etc. In this manuscript ACM was analyzed by using two different ion source designs of two different instruments, hence the capillary diameter was different. This may cause the formation of different ions (e.g. phospholipids) in API 4000 and QPXE instruments. Moreover, in API 4000, nitrogen gas was used as CAD gas, whereas, in QPXE, argon gas, which is heavier than nitrogen gas, is used as CAD gas. This may also lead to different ion formation, which was observed during the actual experiment of ACM in two different instruments, e.g. API 4000 and QPXE.

These may be the possible causes of formation of different ions in different ion source design of ESI-MS/MS. There may be many other causes, which may require more detailed research and investigation.

5. Discussion

Different endogenous phospholipids have been established to be the major cause of matrix effects while using ESI interface. Phospholipids are present in extremely high concentrations in biological matrices and can vary greatly between subject samples and experimental time points in pharmacokinetic and related studies. Some research has shown [28,29] that even if phospholipids do not co-elute with drugs, the presence of phospholipids in extracted samples can result in retention time shifts, elevated baselines, and divergent curves, thus influencing assay performance and ruggedness. Thus, it is preferable to remove phospholipids during sample preparation by chromatographic techniques using various column switching configurations to avoid these matrix effects.

Previous reports describing the matrix effects in LC–MS/MS analysis [28–41], addresses this important aspect superficially. Very few of these reports actually deal with matrix effects in depth. So it is essential to describe matrix effects in all LC–MS/MS bioanalysis. Additionally, this information could be useful for any further research work or advancement in this field.

In this research work, in experiment 1, where analysis was performed by using ESI-MS/MS system having Z-spray ion source design coupled with two separate LC systems, i.e. UPLC and HPLC separately, showed matrix effects in the form of complete ion suppression. On the other hand, in experiment 2, where orthogonal spray ion source design with HPLC–ESI-MS/MS system was used during sample analysis, showed little ion enhancement.

It was the phosphotidylcholines that were supposed to be responsible for this matrix effects. It is observed that different phospholipids were responsible for this different behavior. In orthogonal spray design, phospholipids at m/z 215, 759, 761 and 787 were observed, whereas, in Z-spray design phospholipids at m/z 255, 762, 789 and 815 were identified. This may be because different phospholipids were ionized when the different ion source designs were used.

Thus, from the experiments it was observed that except the ion source design and design of LC, all other experimental conditions including chromatographic condition and sample extraction technique were the same. So, not only the chromatographic conditions, extraction techniques or co-extracted anti-coagulant, but also the hardware design of LC and ionization source may also play a very important role in matrix effects.

In this experimental design, Z-spray coupled with HPLC or UPLC, showed complete ion suppression (MF = 0), whereas, orthogonal spray coupled with HPLC, showed very little ion enhancement (MF = 1.008) or almost no matrix effects. Hence, changing the design of ionization source will affect matrix effect outcomes, though; it is not always true that Z-spray will show more matrix effects in comparison with orthogonal spray ion source design, as this is molecule dependent. The information presented in this manuscript can greatly improve the method development approaches using LC–MS/MS and make the analytical method more reliable.

6. Conclusion

The effect of co-eluting compounds arising from the matrix can cause matrix effects in terms of signal suppression/enhancement in LC–MS/MS analysis. The charge competition between the matrix ions and analyte ions inside the ionization source, causing the ion suppression or enhancement, may affect the reproducibility and

accuracy of the results. So during method development matrix effects should be evaluated, and it should be eliminated or minimized. In the present research work a cause of matrix effect was studied, which revealed that the design of ionization source may cause matrix effects. As seen in the experiment the Z-spray ion source coupled with UPLC and HPLC showed complete suppression of ions, whereas orthogonal spray design of the ion source attached with HPLC showed very less ion enhancement, though the other chromatographic conditions and sample extraction technique were same for both the experiments. Moreover, different phospholipids were identified as responsible for this outcome. Though this experiment was performed only with one molecule, i.e. ACM, but this experiment clearly indicates that design of ionization source plays a significant role in matrix effects. It is not always true that Z-spray is prone to matrix effects and orthogonal spray design will free from any matrix effects related issues, but ionization source design will have influences on matrix effects as observed with this experiment. Thus, during method development, matrix effects can be minimized or eliminated by changing the ion source design of MS/MS system. So, the presented manuscript gives an insightful idea regarding the role of ion source design on matrix effects. Hence, there is a great scope of further extensive research works on this area.

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