Effect of Mobile Phase pH on the Electrospray Ionization Efficiency and Qualitative Analysis of Pharmaceuticals in ESI + LC-MS/MS

Husam I.S. Kafeenah1, Rozita Osman2 and N. K. A. Bakar1,*

1Department of Chemistry, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia and 2Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia

*Author to whom correspondence should be addressed. Email: kartini@um.edu.my

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Abstract

The effect of mobile phase pH on positive ionization process and retention time of nine pharmaceuticals on ultra-performance liquid chromatography-electrospray–tandem mass spectrometry (LC-MS/MS) was discussed. The effective use of high and low mobile phase pH in LC-MS/MS qualitative analyses method was also evaluated by comparing the instrument detection limit, quantification limit, precision, linearity and signal to noise (S/N) under low and high mobile phase pH. In this work, six mobile phase pH that ranged between pH 2 and pH 10 were used to evaluate the effect of the mobile phase pH changes on the ionization process in electrospray ionization. Results revealed that high mobile phase pH ionized more pharmaceuticals molecules and gave a higher signal than low mobile phase pH in positive ionization mode. The results proved that ammonium ion was better as a proton donor in high pH mobile phase than the hydronium ion in acidic mobile phase. The results revealed that the qualitative LC-MS/MS analyses method by using high mobile phase pH has better performance for most analytes in terms of sensitivity, precision, linearity and S/N than the low mobile phase pH.

Introduction

Recently liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become one of the most important techniques in clinical laboratory analysis due to high sensitivity (femtogram-to-picogram), robust, low detection limit and ability to analyze thermally labile and non-volatile bio-molecules (1–6). Moreover, LC-MS/MS can be used for analyzing small and large molecules with various polarities in complex matrix samples (7, 8). Electrospray ionization (ESI) is one of the most popular ionization techniques that use LC-MS/MS (3, 9, 10). ESI system is utilized to connect high-performance liquid chromatography (HPLC) with the MS instrument by forming ions in a gaseous phase. The gaseous phase is from the solution extracted from HPLC by implementing electrical energy to assist the transition process (11, 12). The key to successful LC-MS/MS analysis and an optimum signal response is the successful ionization process. Several factors should be considered to achieve maximum ionization of the analytes, such as the molecular structure of analyte, pKa value of analyte, pH of the mobile phase, HPLC flow rates, capillary (sprayer) voltage, sprayer position, nebulizing gas, and drying gas settings (13–15). The pH value of mobile phase and pKa value of the analyte are highly dependent parameters for the formation of ions in ESI. Choosing a mobile phase for LC-MS/MS analysis differs from the HPLC mobile phase selection. In HPLC, chromatographic separation is the main target of choosing a suitable mobile phase and the buffers, while for LC-MS/MS the main target is to promote ionization without losing separations and asymmetric peaks (13, 16, 17). For example, in reversed-phase chromatography, the analytes are preferred to be non-ionized in the liquid phase to obtain the best resolution and retention factor in HPLC. This is by increasing the interaction between the analytes and the stationary phase. On the other hand, the desirable form of the analytes is to be ionized in the liquid phase.
that will help to produce more ions and increase the ESI-LC-MS/MS sensitivity.

Generally, the desirable pH value should be 2 units less than the pka value of the basic analyte, such as amines for the selection of mobile phase pH value in ESI-LC-MS/MS analysis. Therefore, the analyte will be protonated and ionized in the positive mode, indicating that acidic mobile phase is preferred to increase the sensitivity of positive ionization mode by adding acetic acid, formic acid or trifluoroacetic acid. Meanwhile for acidic analytes such as carboxylic acid and phenol, the preferable pH value is 2 units above pka value of the analyte in the negative mode so the analyte will be deprotonated to produce negative ions (18). In positive ionization mode that use an acidic mobile phase, the retention and the peak symmetric shape will be lost, making the high pH mobile phase the best option for good separation. However, high pH mobile phase theoretically is supposed to suppress ionization in the positive mode by forming the unionized state of these compounds (19–21). This theory led most of the previous studies to use the low pH mobile phase for basic compound analysis in the positive mode even with the possibility of losing the good retention and peak symmetric shape.

Recently, some research works used high pH of the mobile phase in positive mode for ESI-LC-MS/MS analysis of some basic compounds. Some of these studies reported the ionization efficiency regardless of the mobile phase pH, and no relation between ion producing in electro-sprayed and the equilibrium concentration of the ion in the mobile phase (22–24). Whereas, others reported that the signals of some basic compounds were equal for ammonium hydroxide and acetic acid (25–27). Indeed there were a series of published papers about the effect of high pH mobile phase on the ionization of basic pharmaceuticals at a positive mode (28). To the best of knowledge, none of these studies tried to use high pH mobile phase to develop quantitative ESI-LC-MS/MS analyses method.

This study aims to investigate the effect of the mobile phase pH on ionization and separation of both basic and acidic pharmaceuticals by using the positive ionization mode in ESI-LC-MS/MS. Pharmacuticals of different chemical characteristics were selected as shown in Table 1. In addition the effectiveness of high mobile phase pH usage in developed LC-MS/MS quantitative method was investigated by comparing the performance of instruments, such as instrument detection limit (IDL), limit of quantitation, precision, linearity and signal to noise of LC-MS/MS screening method in acid (pH 4) and basic (pH 9) mobile phases.

Materials and Methods

Chemicals and reagents

Acetaminophen was purchased from Sigma-Aldrich (St. Louis, MO, USA). Other pharmaceutical standards were obtained from Sigma-Aldrich (Schnelldorf, Germany). HPLC-grade methanol, ammonium hydroxide, formic acid, ammonium bicarbonate and ammonium format were supplied by Merck (Darmstadt, Germany). Ultra-pure water was prepared from a Milli-Q water purification system (MA, USA).

Preparation of standard solutions

A mixture of standard solutions of pharmaceuticals 1 mg L\(^{-1}\) was prepared from the stock solutions with a concentration of 1000 mg L\(^{-1}\). All standard solutions were prepared in methanol and stored in a clean 20 mL amber glass in a freezer at \(-10^\circ\text{C}\). Six mobile phases with different pH were prepared (pH 2, pH 4, pH 6, pH 8, pH 9 and pH 10). The low pH mobile phases (pH 2, pH 4 and pH 6) were prepared from 10 mM ammonium formate solution in HPLC-grade water. Then concentrated formic acid was added and adjusted to the desired pH. The high pH mobile phase (pH 8, pH 9 and pH 10) consisted of ammonium bicarbonate (10 mM) in HPLC-grade water, followed by the use of ammonium hydroxide solution to adjust the required pH.

UPLC-ESI-MS/MS analysis

Agilent 1290 Infinity system (Agilent Technologies, Germany) was utilized for chromatographic separation. The system consisted of binary bump with solvent degasser, thermostated column oven, and auto sampler. The column temperature was 35 \(\circ\\text{C}\) and the flow rate was 0.1–0.2 mL min\(^{-1}\). The injection volume was 3 \(\mu\text{L}\) in each experiment. Chromatographic separation was achieved by using the Accucore Polar Premium LC column (100 mm \(\times\) 2.1 mm, particle size 2.6 \(\mu\)m) supplied by Thermo Scientific (Loughborough, UK). The same column was used for all experiments throughout the experimental period without any change in the column performance.

The gradient elution mode was performed in chromatographic separation for both LC-MS/MS qualitative method at pH 9 and pH 4 mobile phases. For low mobile phase pH (pH 4), the gradient was adjusted to ensure a satisfied retention, which started with 15% methanol at a flow rate of 0.1 mL min\(^{-1}\) for 1 min. Increase with a linear gradient from 15% to 80% of methanol at a flow rate of 0.2 mL min\(^{-1}\) for 6 min, then to 100% methanol at a flow rate 0.1 mL min\(^{-1}\) over the next 7 min. Finally, the percentage of methanol was reduced to 15% in 1 min at a flow rate of 0.1 mL min\(^{-1}\). For high mobile phase pH (pH 9), the gradient was adjusted to assure a timely elution, which started with 10% methanol at a flow rate of 0.2 mL min\(^{-1}\). Then, the percentage of methanol was increased from 10% to 80% within the next 6 min at a flow rate of 0.2 mL min\(^{-1}\). After 4 min, methanol was increased with a linear gradient to reach 100% (hold 1 min) with the same flow rate. Finally, the methanol was reduced to 10%. The column was re-equilibrated for 4 min after each run for each pH of the studied mobile phase.

An Agilent technologies 6490 triple-quadrupole mass spectrometer interfaced with UPLC via Agilent Jet Stream system AJS ESI as an ionization source (Agilent Technologies, Singapore) was used for the detection in this work. The ESI source was operated in positive ion modes with multiple reaction monitoring (MRM) by using the following operation parameters, as shown in Table I. The dwell time was set at 0.2 s. Nitrogen gas was used for both desolvation and nebulizing gases at a flow rate of 14 L min\(^{-1}\) and a temperature of 225 \(\circ\text{C}\). The temperature of the ion source was set at 450 \(\circ\text{C}\). Capillary voltage was 2 kV; nebulizer pressure was 45 psi for both modes. Agilent Mass hunter software was used for data acquisition and processing. The precursor and product ion transitions and the retention times for each compound were used to identify the analytes of interest. Peak area was used for quantification.

IDL, linearity and the precision of the qualitative methods

IDL of the UPLC-MS/MS was estimated from a standard deviation (SD) of 10 replicate injections of a mixture of standard solution (0.016 ng L\(^{-1}\)) that contained 0.05 pg, of each compound. Then, Equation (1) was used to calculate IDL (23, 29, 30).

\[
\text{IDL} = t \times \left( \frac{\text{RSD (100\%)} \times \text{amount measured}}{1} \right)
\]
Results

In this study, pharmaceuticals of basic and acidic compounds that have the ability to ionize in positive mode (including acidic pharmaceuticals) were selected. The peak areas of the MRM chromatogram and the retention time for each pharmaceutical compound were used to compare the efficiency of the ionization and retention time of the six mobile phase pH. Accucore Polar Premium LC column was used. The column was suitable for a wide range of pH of the mobile phase (pH 2 to pH 10).

pH of the mobile phase

Six different pH of mobile phases that ranged from 2 to 10 were tested to study the effect of the pH of mobile phase on the ionization and retention time of acidic and basic pharmaceuticals operated in positive mode. The ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS) parameters of the method were set at pH 4. In addition, the gradient method of pH 4 mobile phase was used in the analyses of the different pH mobile phases (low and high) for the ionization and the retention time evaluation to avoid any confusion in the results due to the difference in organic percentage at the time of elution between the low and high pH mobile phase gradients. Figure 1 shows the effect of the mobile phase pH on the ionization of acidic, basic, polar and nonpolar compounds. The peaks for all analytes were 10 times larger in the basic mobile phase (pH 9) than the acidic mobile phase (pH 2). Except for metformin and salbutamol, the signal was not significantly increased as the other compounds due to the loss of symmetry of peaks and loss of some retained analytes in the column after the retention time increased at pH 9 and pH 10.

Figure 2 illustrates the relation between the mobile phase pH and the ionization intensity. It was observed that the response signal increased when pH value was increased, indicating an increase in the ionization rate, despite the expectation of an increase in the protonation when the mobile phase pH was decreased (32–34).

The effect of the mobile phase pH value on the retention time

The mobile phase pH effect was not only limited to changes of the signal response, but also altered the retention time of certain analytes. A small change in retention time can be observed on acetaminophen, caffeine, simvastatin and nifedipine (Figure 3). These compounds belong to unionized polar and nonpolar compounds, where the mobile phase pH has small or no effect on the retention time due to no change that happened on the analyte statuses, such as ionized or deionized with pH changing (35).

For acidic compounds in this study, such as mefenamic acid, diclofenac and perindopril in Figure 3, changes in retention time could be notified when mobile phase pH changed from pH 2 to pH 10. pKa value of these compounds was above pH 2, which meant they existed in deionized form at pH 2. When the mobile phase pH raised to pH 10 the pKa value of these compounds became lower than the mobile phase pH, this ionized the analytes and formed an anion that preferred the aqueous mobile phase than the stationary phase. Therefore, the retention time for these compounds was reduced. For the basic analytes (metformin and salbutamol) as shown in Figure 3, the retention time was increased due to the increase in the mobile phase pH to above pKa value of the analytes. This condition that produced a deionized form of the analytes preferred the stationary phase of the column on the aqueous mobile phase. In general, basic and acidic compounds were better retained in deionized form (uncharged) (36–38). Broadened and tailing peaks shapes were
obtained when the mobile phase pH was changed from pH 6 to pH 10 due to potential ionization of silanols on the silica surface at high pH. This negatively charged silanols acted as ion exchange stationary phase which increased the interaction with the basic ions, and ended with a broadening peak and tailing peak (32).

**LC-MS/MS quantitative analyses method at high and low pH mobile phases**

For the comparative study of the qualitative LC-MS/MS method performances at high and low mobile phase pH, two LC-MS/MS methods were optimized at pH 4 and pH 9 mobile phases. IDL, LOQ, instrument precision, signal to noise (S/N), accuracy and linearity for the two LC-MS/MS screening methods were compared.

The results obtained are summarized in Table II, which indicated the high sensitivity and precision for the LC-MS/MS analyses under pH 9. The IDL and LOQ were as low as 0.01 fg, and 10 ng L$^{-1}$, respectively, which were lower than the values found at pH 4 of the mobile phase. Only metformin and salbutamol gave lower IOD and LOQ at high mobile phase pH due to the loss of the peak shape and symmetry. Most of the analytes showed better linearity in high mobile phase pH than low mobile phase pH. Instrument precision was calculated as the RSD of 10 replicates of 100 ng L$^{-1}$ analysis (23, 25, 39). The instrument precision for the analytes at high mobile phase pH was ranged from 1% to 11.4% (except for Salbutamol and Metformin). In general, the method was more precise under high pH of the mobile phase.

In this study, S/N for high and low mobile phase pH at very low concentrations of analytes (1 ng L$^{-1}$) were compared. Apart from metformin and salbutamol, all the targeted compounds had higher S/N at high pH which explained the better sensitivity and detection limit of those compounds in high pH mobile phase. Increase in the S/N can be explained by two reasons. First, an increase in the response signal of the analytes. The second reason was due to elution of some
Effect of mobile phase pH on the electrospray

Table II. A Comparison of the Performance of the Screening Methods under Acidic and Basic Mobile Phase

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IOD (fg)</th>
<th>LOQ (ng/L)</th>
<th>Precision ( % )</th>
<th>S/N</th>
<th>Retention time min</th>
<th>R²</th>
<th>Range (μg/L)</th>
<th>Accuracy ( pH 9 ) %</th>
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<tbody>
<tr>
<td></td>
<td>pH 9</td>
<td>pH 4</td>
<td>pH 9</td>
<td>pH 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Acetaminophen</td>
<td>0.1</td>
<td>29.1</td>
<td>500</td>
<td>5 × 10³</td>
<td>1.2</td>
<td>10.6</td>
<td>128.0</td>
<td>23.0</td>
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<td>3.9</td>
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<td>5–200</td>
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<td>Caffeine</td>
<td>0.1</td>
<td>2.8</td>
<td>10</td>
<td>500</td>
<td>1.0</td>
<td>10.2</td>
<td>399.0</td>
<td>68.0</td>
</tr>
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<td></td>
<td>4.4</td>
<td>4.4</td>
<td>0.99</td>
<td>0.99</td>
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<td></td>
<td></td>
<td>0.01–200</td>
<td>0.5–200</td>
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<td>Diclofenac</td>
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<td>0.3</td>
<td>10</td>
<td>50</td>
<td>1.5</td>
<td>2.1</td>
<td>635.0</td>
<td>161</td>
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<td></td>
<td></td>
<td>4.4</td>
<td>8.5</td>
<td>0.99</td>
<td>0.99</td>
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<td></td>
<td></td>
<td></td>
<td>0.01–200</td>
<td>0.05–200</td>
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<tr>
<td>Mefenamic acid</td>
<td>0.1</td>
<td>0.3</td>
<td>10</td>
<td>50</td>
<td>1.5</td>
<td>1.4</td>
<td>101.0</td>
<td>54.0</td>
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<td>4.7</td>
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<td></td>
<td>0.01–200</td>
<td>0.05–200</td>
</tr>
<tr>
<td>Metformin</td>
<td>50.5</td>
<td>2.9</td>
<td>1 × 10⁴</td>
<td>50</td>
<td>21.3</td>
<td>11.3</td>
<td>11.0</td>
<td>92.0</td>
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<td></td>
<td>8.5</td>
<td>1.4</td>
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<td></td>
<td>10–200</td>
<td>0.05–200</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>0.1</td>
<td>0.4</td>
<td>50</td>
<td>500</td>
<td>1.5</td>
<td>5.6</td>
<td>693.0</td>
<td>191.0</td>
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<td></td>
<td>7.3</td>
<td>7.3</td>
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<td></td>
<td></td>
<td>0.05–200</td>
<td>0.5–200</td>
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<tr>
<td>Perindopril</td>
<td>0.1</td>
<td>0.3</td>
<td>50</td>
<td>500</td>
<td>11.4</td>
<td>3.8</td>
<td>2335.0</td>
<td>419.0</td>
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<td>3.9</td>
<td>6.4</td>
<td>0.99</td>
<td>0.99</td>
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<td></td>
<td>0.05–200</td>
<td>0.5–200</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>26.3</td>
<td>0.3</td>
<td>1 × 10³</td>
<td>50</td>
<td>29.1</td>
<td>2.6</td>
<td>35.0</td>
<td>400.0</td>
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<td></td>
<td>pH 4</td>
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<td></td>
<td></td>
<td>10.8</td>
<td>3.4</td>
<td>0.92</td>
<td>0.99</td>
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<td></td>
<td></td>
<td>1–200</td>
<td>0.05–200</td>
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<tr>
<td>Simvastatin</td>
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<td>3.8</td>
<td>100</td>
<td>2500</td>
<td>3.2</td>
<td>2.5</td>
<td>246.0</td>
<td>74.0</td>
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<td>0.1–200</td>
<td>2.5–200</td>
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</tbody>
</table>

Figure 2. Comparison of LC-MS/MS responses in acidic and basic mobile phase.

Discussion

Many theories can explain the unexpected increase in the signal when high pH mobile phase is used. According to one of the theories it may be because of some compounds eluting in high organic mobile phases, such as metformin and salbutamol due to the increase in retention time, which probably will help to increase the ionization by increasing the evaporation speed of the droplets in the ESI (26).

However, this theory could not explain the increase in the signal for the analytes eluted early in low organic mobile phase percentages, such as diclofenac and mfenamic acid. This means the increase in the signal is not due to the increase in organic percentage in the mobile phase but due to the increase in the pH and presence of ammonium hydroxide in the mobile phase.

In Figure 2, it is obvious that the response signal increased slightly from pH 2 to pH 6, but when ammonium hydroxide was added to the mobile phase to increase the pH to above pH 6, the response signal increased up to 10 times in most analytes. This indicated that the potential role of ammonium hydroxide in increasing the ionization rate at high pH. Generally, positive ions generated as positive adducts of the analytes, such as H⁺, NH₄⁺, Na⁺, and K⁺ in the low mobile phase pH hydronium ion as the source of the positive ion adducts (35) as in Equation (2):

\[ M + H_3O^+ \rightarrow MH^+ \]
The presence of the hydronium ion in the mobile phase as positive ion adducts dissociated the analytes in the mobile phase that were supposed to enhance the signal in LC-ESI in positive mode. This observation was reported generally for the basic compounds \(29, 47, 48\). Increasing the pH decreased the concentration of the hydronium ion while at the same time the ammonium ion concentration was increased. Observed signal was increasing in mobile phase rich on ammonium hydroxide, indicating the main role of ammoniated adducts in the positive ionization mode at high pH. This directly found another pathway for the ionization mechanism in the positive mode where the ammonium hydroxide will replace the hydronium ion to produce the positive ions \(49\). Several theories were proposed to explain the ammonium hydroxide rule in the new ionization pathway, such as the ammonium ions are attached to the amides in the analytes. Then the amide loses the ammonium to form a positive ion in ESI gas phase as the following Equation \(3\) \(26, 50, 51\):

\[
M + \text{NH}_4^+ \rightarrow [M + \text{H}]^+ + \text{NH}_3 \tag{3}
\]

This theory was proven by Hua and Jenke \(49\) by studying the ammonium adduct intermediate mechanism, where the experiment succeeded in detecting the ammonium adduct as base ion accompanied by the protonated analytes in high pH mobile phase.

Another explanation assumed that a protonation could happen in the liquid phase due to the evaporation of ammonia from the liquid droplets which leave an excess of the protons in the solution Equation \(4\) \(51\):

\[
M_{(l)} + \text{NH}_4^{+}_{(l)} \rightarrow \text{NH}_3(g) + M_{(l)} + \text{H}^+_{(l)} \rightarrow [M + \text{H}]^+ \tag{4}
\]

In another theory, positive ions forming due to the collision-induced dissociation between clusters formed by the analyte and an ammonium ion which occur in the gas phase Equation \(5\) \(26\):

\[
(M + \text{NH}_4^+) \rightarrow [M + \text{H}] + \text{NH}_3 \tag{5}
\]
However, regardless of the real ionization mechanism, the previous result proved that ammonium ion is better as a proton donor in high pH mobile phase than the hydronium ion in acidic mobile phase. Moreover, the high pH mobile phase does not suppress the ionization in the positive mode but on the contrary it enhances the ionization and increases the signal.

Conclusion

A series of six mobile phase pH were examined to evaluate the effect of pH of mobile phase on ionization in ESI-MS. The response signal was increased when the eluent pH increased. All the pharmaceuticals were successfully quantitated by ESI + LC-MS/MS under high pH mobile phase. The mobile phase that contained ammonium ions was better as an additive material than formic acid in improving the sensitivity in positive ionization mode. Changes on the retention time of targeted pharmaceuticals in reversed-phase UPLC by changing mobile phase pH from acidic to basic were investigated. The acidic compounds were retained in a shorter time in basic mobile phase, where the basic compound retention time was longer in basic mobile phase. For the weak basic compounds, no change was noted in the retention time. The effectiveness of using high and low mobile phase pH in LC-MS/MS quantitative analysis method was evaluated by comparing the IDL, LOQ, instrument precision, accuracy, linearity and S/N. Results showed better performance for most of the analytes in terms of sensitivity, precision, linearity and S/N when ESI + LC-MS/MS analysis under high mobile phase pH was used. The use of high mobile phase pH was not suppressing the ionization in ESI + LC-MS/MS, on the contrary, it enhanced the ionization and the signal response.

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References
