Development and Validation of a Capillary Electrophoresis Method for the Determination of Sulfate in Indinavir Sulfate Raw Material

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In this work, a simple and fast capillary electrophoresis method using indirect UV detection (230 nm) for the quantitative determination of sulfate in the indinavir sulfate raw material was described. A running electrolyte consisting of 10 mmol L⁻¹ ammonium molybdate containing 0.15 mmol L⁻¹ cetyltrimethylammonium bromide (pH 7.5) was used. Samples were injected hydrodynamically by applying 5000 Pa pressure during 3 s. The response was linear from 10.1 μg mL⁻¹ to 79.8 μg mL⁻¹. Limits of detection and quantification were 0.34 and 1.13 μg mL⁻¹, respectively. The intra-day precision expressed as relative standard deviation was 2.8% for 10 independent sample preparations. Recoveries varying from 96.9 to 102.4% at three concentration levels were obtained. The methodology was successfully applied for real samples. The concentration of sulfate in indinavir sulfate raw material varied from 13.3 to 13.5% (m/m calculated as anhydrous base).

Keywords: indinavir sulfate, capillary electrophoresis, sulfate, validation, HIV

Introduction

Indinavir sulfate was approved by the FDA (Food and Drugs Administration) on March 13, 1996, for use in combination with other protease inhibitors, nucleoside analogues or reverse transcriptase inhibitors for the treatment against acquired immune deficiency syndrome (AIDS). Indinavir is prescribed as a sulfate salt due to superior gastrointestinal solubility and absorption when compared with the free base.

When drugs are manufactured as salts, an analytical technique is required to confirm that correct levels of counter ion are present. The counter ions of drugs with ionic groups are of importance because the physico-chemical properties, stability and bioavailability of the drugs depend on their salt forms. An analytical technique such as ion chromatography (IC) and titrimetry is often used for the analysis of counter ion in pharmaceutical drug substances. However, these methods are laborious and time consuming, besides the high maintenance cost of IC technique.

Capillary electrophoresis (CE) has been successfully applied to the analysis of a variety of anionic solutes in several complex samples. CE has shown to be a powerful analytical tool for the determination of ions in the various segments of chemical, pharmaceutical and food industry, as well as in the characterization of samples of clinical and environmental interest. Various metal, inorganic, and organic counter ions have been determined by CE in pharmaceutical formulation. This work describes the first report of a CE validated method for quantitative determination of sulfate in indinavir sulfate raw material.
Experimental

Reagents and solutions

All reagents were of analytical grade and used with no further purification. Potassium sulfate and sodium salts of chloride, nitrate, perchlorate (internal standard), carbonate and cetyltrimethylammonium bromide (CTAB) were obtained from Aldrich/Sigma (St. Louis, MO, USA). Ammonium molybdate was obtained from Dinâmica (São Paulo, Brazil). Indinavir sulfate raw material was kindly donated by the Instituto de Tecnologia de Fármacos – Fundação Oswaldo Cruz (Rio de Janeiro, Brazil).

Stock solutions of chloride, nitrate and carbonate (1000 μg mL⁻¹), sulfate (844.0 μg mL⁻¹), perchlorate (5000 μg mL⁻¹) and indinavir sulfate (269.9 μg mL⁻¹ of sulfate ion) were prepared in deionized water. Working standard solutions were prepared fresh daily by diluting appropriately the stock solutions with deionized water.

Calibration curve

Appropriate aliquots of the standard stock solutions of sulfate and a fixed aliquot of perchlorate (internal standard) were transferred into separate 10 mL volumetric flasks. The volume was completed with deionized water. Concentration range from 10.1 to 79.8 μg mL⁻¹ of sulfate, and 20 μg mL⁻¹ of perchlorate were obtained.

Accuracy

To determine the method accuracy, recovery tests were performed according to procedures endorsed by AOAC.¹⁴ Commercial raw material solution was spiked with known quantities of sulfate standard solution and analyzed by the proposed method, according to the procedure depicted in Table 1.

Apparatus

All experiments were conducted in a capillary electrophoresis system (Agilent Technologies, model HP 3D CE, Palo Alto, CA, USA), equipped with a diode array detector, temperature control device maintained at 29 °C and data acquisition and treatment software (HP ChemStation, rev A.06.01). Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with dimensions 48.5 cm total length (40 cm effective length) and 75 μm i.d. x 375 μm o.d. were used. Samples were injected hydrodynamically, with a 5000 Pa for 3 s and detected indirectly at 230 nm. The applied voltage was set at -10 kV. At the beginning of each day, the fused-silica capillary was conditioned by flushing 1 mol L⁻¹ NaOH solution (5 min), followed by a 5 min flush of deionized water and electrolyte solution (40 min). Before each run, the capillary was just rinsed with fresh electrolyte solution (3 min).

Results and Discussion

Since most inorganic anions have low or no absorbance in the UV-Vis range of the spectrum, the indirect UV detection is often employed in their determination. This technique uses a UV-absorbing ion, known as the probe ion, in the background electrolyte. Displacement of the probe ion by a migrating sample anion results in a quantifiable decrease in the background absorbance.⁴ In this work, molybdate was selected as the electrolyte anion because its mobility is similar to the mobility of the analytes of interest, it presents high molar absorptivity (5,650 cm⁻¹ L mol⁻¹) and its solutions are stable upon storage and continuous analysis.¹⁵

The separation mechanism in CE is based on differences between the electrophoretic mobilities of charged species in the presence of an electric field. In a conventional CE system the electroosmotic flow (EOF) is towards the cathode, anions with an electrophoretic mobility higher than the electroosmotic mobility of the bulk electrolyte cannot reach the detector and the polarity of the potential applied must be reversed in order to detect these anions. However, under these conditions anions with mobilities lower than electroosmotic mobility would never reach the detector. Therefore, for most anion separations it is necessary to use a modifier to reverse the direction of

Table 1. Procedure for the recovery study (standard solution of sulfate added to indinavir sulfate raw material solution)

<table>
<thead>
<tr>
<th>Commercial sample solution (269.9 μg mL⁻¹)</th>
<th>Sulfate standard solution (844.0 μg mL⁻¹)</th>
<th>Internal standard (5000 μg mL⁻¹)</th>
<th>Final concentration (μg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>40.0</td>
<td>20.0</td>
<td>13.5</td>
</tr>
<tr>
<td>500</td>
<td>120</td>
<td>40.0</td>
<td>26.6</td>
</tr>
<tr>
<td>500</td>
<td>240</td>
<td>40.0</td>
<td>33.7</td>
</tr>
<tr>
<td>500</td>
<td>350</td>
<td>40.0</td>
<td>43.0</td>
</tr>
</tbody>
</table>

¹ Solution of indinavir sulfate raw material; ² To 10 mL volumetric flasks; ³ Internal standard: perchlorate.
the EOF. Alternatively, rapid and highly efficient separation of anions can be achieved by CE in the absence of any flow modifier using the short-end injection technique.

In order to obtain the best separation of the anions, i.e., short analysis time and good resolution, both separation modes, with and without flow reversal, were studied in this work using a molybdate solution as electrolyte.

Separation of inorganic anions without flow reversal

The pH is an important parameter in CE separations when fused-silica capillaries are used because it governs the EOF. The pH also governs the ionization of weak acids or bases, but for most inorganic anions this effect is not important because they are totally ionized in the operational pH interval of CE separations (pH 2-10). In order to keep the migration time of anions reasonably short without flow reversal, the pH was adjusted to 5.0, with acetic acid, and CE runs were performed with the use of short-end injection (effective capillary length 8.5 cm).

Initially, to evaluate the separation of the chloride, sulfate, nitrate, perchlorate and carbonate, a 5 mmol L\(^{-1}\) molybdate solution was used as electrolyte. Additionally, for sample introduction two injection modes, the hydrodynamic and electrokinetic sampling, were also tested. Figure 1 shows the electropherograms obtained. Under both injection conditions, all the compounds studied were separated in less than 3 min but electrokinetic injection was superior with respect to sensitivity and efficiency. However, both injection modes provided poor repeatability (> 2% RSD, n=10) for migration time and peak area. This behavior might be associated with the equipment design. The end part of the capillary is not inside the thermostatted cassette and in this set up, heat which dissipates through the capillary walls may not be removed so efficiently. As a consequence, the precision may be poor because of the lack of temperature control.

Separation of inorganic anions with flow reversal

Reversal of the EOF is achieved by the addition of a cationic surfactant, such as CTAB to the electrolyte. Initial conditions were based on the literature. In the work of Fung and Lau, the separation of organic and inorganic anions in rain water was conducted using a electrolyte containing 5 mmol L\(^{-1}\) molybdate, 0.15 mmol L\(^{-1}\) cetyltrimethylammonium hydroxide (CTAH), 0.01% (m/v) polyvinil alcohol (PVA) and 5 mmol L\(^{-1}\) Tris buffer, pH 7.9. Since the methodology parameters seemed to have been optimized according to the sample nature, it was decided to explore in better detail the buffer composition. The influence of the molybdate concentration was studied in the interval 5-20 mmol L\(^{-1}\) in electrolytes with a fixed amount of CTAB (0.15 mmol L\(^{-1}\)). When the molybdate concentration was increased, a substantial gain in resolution of chloride and sulfate was achieved (not shown). However, concentrations above 10 mmol L\(^{-1}\) produced loss of resolution between sulfate and nitrate peaks (not shown). Moreover, the Joule heating effect is also increased rapidly at higher molybdate concentration, leading to baseline noise. Therefore in this study, 10 mmol L\(^{-1}\) molybdate was chosen as the optimum concentration.
Buffered solutions tend to be used in CE to limit the possible changes in the composition of the electrolyte and to deliver results with better reproducibility. In the case of anion separations, buffering using counter ion, such as Tris, is a useful option. However, when 5 mmol L\(^{-1}\) Tris buffer was added to the molybdate electrolyte baseline oscillations were observed. In order to provide stabilization against pH change, when unbuffered molybdate electrolyte was used, electrolyte reservoirs were replenished each five runs.

In Figure 2, a typical electropherogram of a standard mixture is given, showing the separation of five anions at optimized conditions, i.e. unbuffered molybdate solution containing a flow modifier.

**Method validation**

The proposed CE methodology was validated by determining its performance characteristics regarding specificity, linearity, limit of detection, limit of quantification, precision and accuracy. The precision of the proposed method regarding peak area and migration time repeatability for ten consecutive injections of the sulfate standard solution at 10 \(\mu\)g mL\(^{-1}\) was estimated. Since much of the variance in precision is attributable to variable injection volume, voltage or EOF, the incorporation of an appropriate internal standard (IS) is required to minimize these source errors. It can be seen in Table 2 that the precision of injection for relative peak area (analyte/IS) was significantly improved from 8.0% RSD to 0.87%.

<table>
<thead>
<tr>
<th>Anion</th>
<th>MT (min)</th>
<th>MT(^{a}) RSD (%)</th>
<th>RMT(^{b}) RSD (%)</th>
<th>PA(^{c}) RSD (%)</th>
<th>RPA(^{d}) RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfate</td>
<td>3.2</td>
<td>0.27</td>
<td>0.13</td>
<td>8.0</td>
<td>0.87</td>
</tr>
</tbody>
</table>

RSD = relative standard deviation (10 consecutive injections); \(^{a}\) Migration time; \(^{b}\) Relative migration time (sulfate/IS); \(^{c}\) Peak area; \(^{d}\) Relative peak area (sulfate/IS).

The repeatability of sample preparation was also evaluated by preparing independently ten samples of indinavir sulfate raw material. Each solution was injected once and the average of relative peak area (sulfate/IS) was used for calculation. The intra-day precision expressed as RSD was 2.8%.

**Specificity**

Specificity is described as the ability of a method to discriminate the analyte from all potential interfering substances. Inorganic anionic impurities as chloride, nitrate, and carbonate can derive from the manufacturing process of indinavir sulfate. The non-interference of chloride, nitrate and carbonate on the sulfate peak was assessed by injection the corresponding solutions (Figure 2). As it can be observed, none of these inorganic anions interfered in the analysis of sulfate, establishing therefore the method specificity.

**Linearity, limit of detection (LOD) and limit of quantification (LOQ)**

Linearity is the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range. The specified range is normally derived from linearity studies and depend on the intended application of the procedure. Acceptable coefficients of correlation (0.99 or greater) and an intercept close to the origin should be achieved. For quantitative purposes, analytical curves based on relative peak area (sulfate/IS) versus concentration were built. The analytical curves consisted of five points and three replicate injections of standards at each concentration level were performed. As shown by the statistical data organized in Table 3 the method exhibited good linearity over the concentration range from 10.1 \(\mu\)g mL\(^{-1}\) to 79.8 \(\mu\)g mL\(^{-1}\) (\(r = 0.999\)).
Conclusions

A CE method has been validated for the counter ion determination in indinavir sulfate raw material. Good method performance was obtained for linearity, precision, accuracy, LOD, LOQ and specificity. Satisfactory precision data were obtained by using an internal standard. Based on the performance characteristics, the proposed methodology was shown to be eligible for the routine monitoring of sulfate in indinavir sulfate raw material.

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