Fast Analysis of Cefepime and Related Impurities on Poroshell 120 EC-C18

Application Note
Pharmaceuticals

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Introduction
Cephalosporins are the most frequently prescribed class of antibiotics. They are structurally and pharmacologically related to the penicillins. Like the penicillins, cephalosporins have a beta-lactam ring structure that interferes with synthesis of the bacterial cell wall which means that they kill bacteria. Cephalosporin compounds were first isolated from cultures of Cephalosporium acremonium in 1948 by Italian scientist Giuseppe Brotzu. The first commercial product, Cephalothin was launched by Eli Lily in 1964.

Cephalosporins are bactericidal agents and have the same mode of action as other beta-lactam antibiotics (such as penicillins). All bacterial cells have a cell wall that protects them. Cephalosporins disrupt the synthesis of the peptidoglycan layer of bacterial cell walls, which causes the walls to break down and eventually the bacteria die. Cephalosporins are beta-lactam compounds in which the beta-lactam ring is fused to a 6-membered dihydrothiazine ring, thus forming the cephem nucleus. Modifications to the side chain modifications of the ring structure can improve antibacterial and pharmacokinetic activity. Based on their spectrum of activity, cephalosporins can be broadly categorized into four generations.

First generation cephalosporins are predominantly active against gram-positive bacteria, and successive generations have increased activity against gram-negative bacteria (often with reduced activity against gram-positive organisms). Gram-negative bacteria have a unique outer membrane that prevents many drugs from penetrating them, making gram-negative bacteria generally more resistant to antibiotics than are gram-positive bacteria [1].
In addition, two 4.6 × 250 mm, 5 µm C18 competitive columns were also examined, and are designated C1 and C2.

Acetonitrile used was Burdick and Jackson ACS/HPLC Certified solvent, purchased from Honeywell. Monobasic Potassium Phosphate ACS/USP Grade purchased from VWR. Water used was produced on site using a Millipore Milli-Q system, 18 M filtered to 0.2 µm. 0.45 µm Regenerated Cellulose Filter media (Agilent Technologies) was used for buffer filtration. USP Cefepime Hydrochloride and USP Cefepime Hydrochloride System Suitability RS was purchased from United State Pharmacopeia. Sample and mobile phase preparation are made following directions from the USP and EP [3,4].

**Mobile Phase Preparation**

This method uses a gradient composed of a monobasic potassium phosphate buffer mixed with an amount of acetonitrile. The buffer is prepared by dissolving 0.68 g of monobasic potassium phosphate in 1000 mL of water. This buffer is adjusted with potassium hydroxide or phosphoric acid to a pH of 5.0, filtered through 0.45 µm filter media (regenerated cellulose was used in our lab) and degassed ultrasonically. The initial USP method uses a 9:1 ratio of buffer to acetonitrile for Mobile Phase A. All samples are subsequently prepared from this mobile phase. In the course of method adjustments, varied ratio’s of “Mobile Phase A” are prepared. Mobile Phase B is prepared from the monobasic potassium phosphate buffer mixed with a 1:1 ratio v/v acetonitrile. The European Pharmacopeia (EP) method is similar but specifies the concentration of Potassium Hydroxide or Phosphoric acid used to adjust the solution pH, (0.05 M) and specifies that the mobile phase pH is adjusted before the addition of acetonitrile.

**Assay Preparation**

About 70 mg of Cefepime Hydrochloride, should be accurately weighed and transferred, to a 50-mL volumetric flask, dissolved in and diluted with Mobile Phase A to volume. This solution should be sonicated for approximately 30 minutes. The system suitability sample is prepared at 7 mg/5 mL in Mobile Phase A. Sonication is important as impurity B is reluctantly soluble. NOTE: These solutions should be used immediately, or stored in a refrigerator and injected within 12 hours.

**Results and Discussion**

Chromatographic conditions as described in the USP and EP
were followed. In both cases an L1 (C18 column) is 4.6 × 250 mm, 5 µm is specified. The gradient program shown in Table 2. The liquid chromatograph uses an initial isocratic hold for 10 min of 100% mobile phase A increasing to 50% over the next 20 min. An isocratic hold at 50% A is maintained for 5 min, after which the solvent re-equilibrates to the initial 100% composition A. The total run time is 36 min.

In the original USP and EP methods a 4.6 × 250 mm, 5 µm L1 column is specified. Three different columns are shown in Figure 2. The method specifies a gradient and with recovery time approximately 45 min are required for each sample. Speeding up this method through adjustments presents a good opportunity for improving the method.

The chromatographic and performance requirements of the method are listed in the USP method. These are summarized below [1].

- 4.6 mm × 250 mm column, L1 column (C18)
- N of the analyte not less than 4000 plates.
- The resolution, R, between cefepime and cefepime related compound A is not less than five.
- The resolution, R between cefepime and cefepime related compound B is not less than 10.
- The capacity factor, k’, of cefepime, is more than 0.6.
- Column efficiency is not less than 4000 theoretical plates.
- The tailing factor is not more than 1.5.

For the purpose of identification, the relative retention times are about 1.0 for cefepime, 2.7 for cefepime related compound A, and about 4.3 for cefepime related compound B.

The USP updated chapter <621> presents recommendations on how much a method can be modified such that the changes are considered an adjustment. [5] Table 1 summarizes these modifications. In previous work, modification of particle size and column dimensions have been demonstrated. [6,7]. In addition, new changes to these compendial methods are proposed that could allow linear velocity to remain constant as particle size decreases, thus increasing the flow rate beyond the present ± 50% [8]. In this case, we also look at the advantages of modifying the mobile phase composition on a substantially smaller column.

Table 1. Allowable Method Modifications under USP Chapter 621

<table>
<thead>
<tr>
<th>Allowable Method Modifications</th>
<th>± 70%</th>
<th>± 25%</th>
<th>Reduction of up to 50%, no increase</th>
<th>± 50%</th>
<th>Changes are allowed as long as system suitability testing (SST) criteria are met</th>
<th>± 10%</th>
<th>± 0.2</th>
<th>No change outside manufacturer specifications</th>
<th>± 10%</th>
<th>Composition of mobile phase (adjustment of the minor component is allowed ± 30% or ± 10% absolute whichever is smaller (as discussed in the USP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column length</td>
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<td>Column internal diameter</td>
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<td>Column material particle size</td>
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<td>Flow rate</td>
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<td>Injection volume</td>
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<td>pH of mobile phase</td>
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<td>Concentration of salts in buffer</td>
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</table>

The USP/EP Cefepime impurity method can be run on many columns but requires 45 minutes per sample with equilibration.

For the purpose of identification, the relative retention times are about 1.0 for cefepime, 2.7 for cefepime related compound A, and about 4.3 for cefepime related compound B.
Gradient Conditions were scaled according to the formula:

When a constant $k^*$ value is to be maintained, the equation above can be reduced into the following equation to calculate new gradient times:

$$t_{g2} = \frac{(t_{g1}d_2^2L_2F_1)}{(d_1^2L_1F_2)}$$

$t_{g1}$ and $t_{g2}$ are the original and new gradient times

d$_1$ and d$_2$ are the original and new column id’s

L$_1$ and L$_2$ are the original and new column lengths

F$_1$ and F$_2$ are the original and new flow rates

For this work several allowed modifications were made. First the particle size is changed from 5 µm to 2.7 µm. This change yields an increase in efficiency as well as an increase in pressure. Because the column is 70% shorter the increase in pressure is minimized. An advantage of an Agilent Poroshell 120 is the narrow particle size distribution because of this, a 2 µm frit can be used, the same size used on 5 and 3.5 µm columns. This means that no additional care must be made in preparing samples than was used in the original method. Columns with particle sizes of 3, 2.5, and of course sub 2 µm use smaller size frit to retain the packing material in the column and as such are more apt to clogging [9].

The second modification made is a change from 250 mm length to 75 mm. This 70% reduction in length is allowed and can easily lead to higher throughput of samples if the performance of the column allows such a change.

<table>
<thead>
<tr>
<th>Table 2. Table of Gradients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inj. Volume</td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>0</td>
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<tr>
<td>0</td>
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<tr>
<td>50</td>
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<tr>
<td>50</td>
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<tr>
<td>0</td>
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</tbody>
</table>

The minor component in the mobile phase is the Acetonitrile at 10%. Under USP modification rules an allowed change of ±30% or ±10% absolute whichever is smaller means that the acetonitrile can be reduced to 7% or increased to 13%. Under EP rules this change is still 7 to 13%. In this work, several concentrations of acetonitrile can be quickly calculated using this dramatically shorter column. It is important to note that the chromatographic solvents are varied in this method but

![Figure 3. Totally Porous Agilent Eclipse Plus C18 column and Agilent Poroshell 120 EC-C18 column.](image-url)
the sample preparation is left intact. It was noticed during the course of this work that Impurity B is less soluble than other components, and without sufficient ultra-sonication as specified in the method, this compound is not dissolved.

In Figure 4, the flow rate is increased to 2 mL/min to allow even faster method evaluation.

As can be seen, the initial acetonitrile is varied from 11% to 8%, within compendial guidelines for adjustment. At 10% the resolution of the cefepime and impurity B is 2, but by decreasing the initial organic modifier content to 8% the resolution between these peaks is increased to 4.9. It becomes evident that decreasing the initial concentration beyond 7% could potentially lead to additional peak resolution, but modifications outside these ranges are considered changes and require re-validation.
Table 3.
Requirement: Agilent Poroshell 120 EC-C18 4.6×75 mm, 2.7 µm

<table>
<thead>
<tr>
<th>Flow Rate</th>
<th>156 bar</th>
<th>221 bar</th>
<th>300 bar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mL/min</td>
<td>1.28</td>
<td>1.28</td>
<td>1.30</td>
</tr>
<tr>
<td>1.5 mL/min</td>
<td>18288</td>
<td>11993</td>
<td>7250</td>
</tr>
<tr>
<td>2 mL/min</td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>k’ &gt; 0.6</td>
<td>5.9</td>
<td>5.9</td>
<td>5.9</td>
</tr>
<tr>
<td>Rs C/a</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
</tr>
<tr>
<td>Rs C/b</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
</tr>
</tbody>
</table>

Modifications outside these ranges are considered changes and require re-validation. If the analyst chooses to use a shorter column, such as a 4.6 mm × 50 mm, the same analysis could be accomplished in potentially 20% of the time. Further only 20% of the solvent would be used. However, this would require a complete revalidation. In cases such as assay methods, it might be easier to justify revalidation of a method but impurity methods, are run less frequently. Tables 2 and 3 indicate that this analysis could easily be reduced in time from 36 minutes to 7.2 minutes without any need for new equipment, with an 80% reduction in analysis time. This would allow a lab to assay an incoming raw material within two hours of receipt instead of within 24 hours of receipt.

Conclusion

Laboratories performing compendia analysis with 250 mm fully-porous LC columns can benefit from the increased speed, resolution, and sensitivity that superficially porous, Poroshell 120 columns provide without having to replace existing instrumentation. The 75 mm column length is within the allowed modification range of USP and EP guidelines. Faster analysis times resulting in higher throughput and greater productivity can be achieved with Poroshell 120 columns. Method adjustments to these compendia methods with shorter length columns and the smaller 2.7 µm particle size provide these improved results.

References

6. William Long, Anne Mack and Yun Zou, “Improved Simvastatin Analysis Using ZORBAX Eclipse Plus C18 5 µm, 3.5 µm, and 1.8 µm Columns”, 5990-3883EN.