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Determination of Cefepime and Cefepime-Related Substances Using HPLC with UV Detection

INTRODUCTION

Cephalosporins are currently among the most widely prescribed antibiotics in hospitals.¹ Development of these antibiotics has led to compounds with a broad spectrum of activity against both Gram-positive and Gram-negative bacteria and with low toxicity profiles. Derivatives of penicillins, these drugs universally contain a β -lactam ring (Figure 1). This four member ring is inherently strained and prone to hydrolysis and photolysis, limiting its stability.² In addition to degradation products, isomers and dimers of the synthesis reagents are produced during manufacture of the compound. These impurities can persist in the drug product and many are of unknown toxicity.

Despite extensive research on this class of drugs, quantitative analysis and purity assays remain problematic.³ The chemical instability of the strained β -lactam ring system and the variable stability of different substituted groups (R1 and R2 in Figure 1) require that analysis of these compounds be rapid. In addition to the need for fast analysis times, superior resolution is necessary to separate synthetic byproducts. Both the chemical instability and the structural similarities of the impurities to the desired product make analysis of these antibiotics difficult.

Cefepime, a fourth generation cephalosporin, is a broad spectrum antibiotic with improved activity against Gram-negative bacteria over other commercially available cephalosporin drugs.⁴ Analysis of cefepime purity is particularly challenging due to isomeric and other impurities with similar structures (Figure 2). Additionally, cefepime is particularly labile and its stability is highly

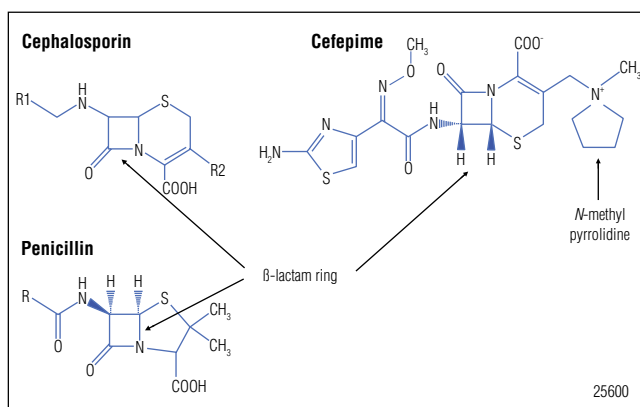


Figure 1. Penicillin, cephalosporin, and cefepime.

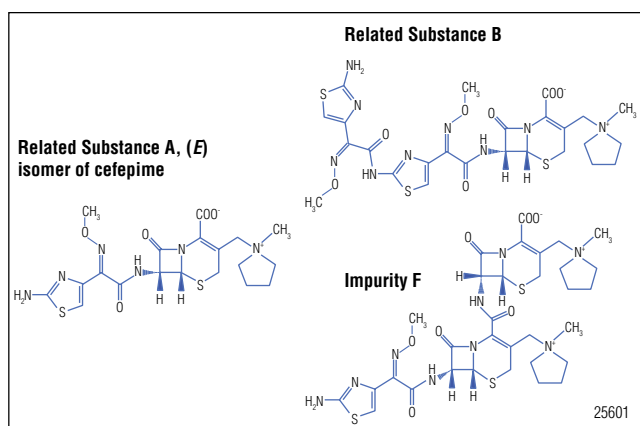


Figure 2. Cefepime-related substances.

pH dependent, in part due to rapid N-methylpyrrolidine (NMP) cleavage at room temperature.⁵ An IC method for the determination of NMP in cefepime preparations is described in Dionex Application Note 199.⁶

Both the United States Pharmacopeia (USP) and the European Pharmacopeia (EP) publish monographs for determining the concentration and purity of cefepime.⁷⁻⁸ The related substances methods for purity analysis that are provided by the EP and USP are chromatographically similar, requiring a type L1 column and chromatographic conditions consisting of a short isocratic elution using 10/90 acetonitrile/5 mM potassium phosphate followed by a linear gradient of acetonitrile in 5 mM aqueous monobasic potassium phosphate. Mobile phase preparation details and subsequent calculations to evaluate the purity of the cefepime differ, but the analytical methods are the same. In addition to the purity methods published by these two organizations, faster concentration assays are also available. The EP uses a modified version of the purity method to determine the cefepime concentration. This is convenient in that it does not require additional mobile phase preparation, column equilibration, or system set up. Rather than use the isocratic portion of the purity method, the USP method uses a pentane sulfonate/acetonitrile based mobile phase for the assay method.

This application note describes modifications to the related substances method to maximize either the speed or the resolution. The Acclaim® 120 C18, 3 µm column is used, an L1 column as defined by the USP. It is manufactured using high purity silica with a 120 Å pore diameter, with very high surface coverage and extremely low metal content. This C18 phase exhibits low polarity, high hydrophobicity, and good steric selectivity, which results in a high-capacity column with unique selectivity. This steric selectivity makes it an excellent choice for resolving structurally similar compounds. The performance of this column is compared to data from the same method using the Acclaim PolarAdvantage (PA), a sulfonamide-embedded polar stationary phase column. Finally, a modified version of this method for use as a concentration assay for cefepime is discussed. Linearity, precision, the limit of detection (LOD), and the limit of quantification (LOQ) are demonstrated for the concentration assay.

The Acclaim 120 C18, 3 µm can be used to meet and exceed the criteria set by the USP for determining related substances and assaying the purity of cefepime. The improved efficiency of the column allows for shorter run times without sacrificing resolution, leading to fast, high-resolution methods. The lower flow rate used with this column saves resources and produces less waste than the original assay.

EQUIPMENT

Dionex UltiMate® 3000 Intelligent LC system:

SRD-3600 Solvent Rack (Dionex P/N 5035.9230)

DGP-3600M pump (Dionex P/N 5035.0050)

WPS-3000T autosampler (Dionex P/N 5820.0020)

FLM-3100 flow manager (Dionex P/N 5720.0010)*
or TCC-3200 column compartment (Dionex
P/N 7522.0025)

VWD-3400 detector (Dionex P/N 5074.0010)

Semi-Micro Peek flow cell, 2.5 µL (Dionex
P/N 6074.0300)

Chromeleon® 6.8 Chromatography Workstation

Glass injection vials with caps and septa, 1.5 mL (Dionex
P/N 055427)

Nalgene® Filter Unit, 0.2 µm nylon membrane, 1 L
capacity (Nalgene P/N 164-0020)

**An FLM-3100 flow manager was used as a temperature controlled column compartment with the flow controller disabled. The FLM is not necessary and a thermostatted column compartment (TCC) can be used for this application.*

REAGENTS AND STANDARDS

Deionized water, Type 1 reagent grade, 18 MΩ-cm
resistivity.

Acetonitrile, HPLC Grade or better (B&J P/N 015-4)

Potassium phosphate, monobasic, HPLC grade (Fisher
P/N P286-1)

Potassium hydroxide concentrate, 45% (JT Baker
P/N 314301)

ortho-Phosphoric acid, HPLC grade, (Fisher
P/N A260-500)

pH buffers, 4.00 (VWR P/N 34170-127) and 7.00 (VWR
P/N BDH5046-500mL)

SAMPLES

Cefepime Hydrochloride (USP, Catalog # 1097636),
Lot G0D116

Cefepime Hydrochloride System Suitability RS (USP,
Catalog # 1097647), Lot F0C095

CONDITIONS

Column:	Acclaim 120 C18, 3 μ m Analytical, 2.1 x 150 mm, (Dionex P/N 059130)
Mobile Phases:	USP Method A: 90/10 5 mM potassium phosphate/ acetonitrile B: 50/50 5 mM potassium phosphate/ acetonitrile -or- Increased Resolution Method A: 94/6 5 mM potassium phosphate/ acetonitrile B: 50/50 5 mM potassium phosphate/ acetonitrile
Gradient:	USP Method 100% A for 10 min, 0–50% B in 20 min, 50% B for 5 min, 9 min of equilibration prior to injection -or- Shortened Runtime Method 100% A for 8 min, 0–50% B in 10 min, 50% B for 3 min, 5 min of equilibration prior to injection
Flow Rate:	0.20 mL/min
Temperature:	30 °C (column compartment)
Inj. Volume:	1 μ L
Detection:	Variable Wavelength UV-Vis detector, 254 nm
Noise:	~12-18 μ AU
System Backpressure:	~110 bar (~1600 psi)

PREPARATION OF SOLUTIONS AND REAGENTS

Mobile Phases

Mobile Phase A:

Dissolve 0.68 g of HPLC grade monobasic potassium phosphate in 1000 mL of DI water. Remove 100 mL of the solution and add 100 mL of HPLC grade acetonitrile. Adjust the pH to 5.00 ± 0.05 with 100 fold diluted 45% KOH. Filter the mobile phase through a 0.2 μ m nylon filter unit and degas. Transfer the solution to a glass eluent bottle.

Optional: To prepare 94/6 5 mM monobasic potassium phosphate/acetonitrile, dissolve 0.68 g of HPLC grade monobasic potassium phosphate in 1000 mL of DI water. Remove 60 mL of potassium phosphate solution and add 60 mL of HPLC grade acetonitrile. Continue the preparation as described above.

Mobile Phase B:

Dissolve 0.34 g of monobasic potassium phosphate in 500 mL of DI water. Add 500 mL of acetonitrile. Adjust the pH to 5.0 ± 0.05 with 100-fold diluted 45% KOH or 100-fold diluted HPLC grade phosphoric acid. Filter the mobile phase through a 0.2 μ m nylon filter unit and degas. Transfer the solution to a glass eluent bottle.

Consistency in the amount of acetonitrile in the mobile phase is critical to reproducible chromatography between mobile phase preparations. Care should be taken to ensure that the amounts of acetonitrile added are reproducible and that degassing does not remove the solvent from the aqueous phosphate solution. It is recommended that the mobile phase be used as soon as practical after filtration. Additionally, cefepime acts as a zwitterion over a broad pH range.⁹ With zwitterionic compounds, the mobile phase pH can strongly affect both retention time and peak shape. Care must be taken during preparation of the mobile phase to adjust the pH properly in order to avoid peak shifting and broadening.¹⁰

Autosampler Syringe Wash Solution

In order to prevent carryover from the autosampler, a wash solution of 10% acetonitrile in DI water was used. Carryover from injections of 1.4 mg/mL cefepime solutions was not observed when this wash solution was used.

Sample Solutions

Prepare stock solutions of cefepime hydrochloride gravimetrically by accurately weighing 10.0 mg of powder in a 1.5 mL vial, dissolving the powder in 1 mL (1 g) of deionized water, and mixing thoroughly. Prepare the cefepime system suitability standard (SSS) similarly by weighing 10 mg of the sample in a 1.5 mL glass AS vial, adding 1 mL of deionized water, and mixing. Store these stock solutions at -19 °C or below. Prepare samples volumetrically by diluting an aliquot of stock solution in Mobile Phase A to produce a final concentration of 1.4 mg/mL prior to analysis. Prepare standards for testing linearity of the assay method by volumetric dilution of the cefepime hydrochloride stock standard with Mobile Phase A to produce the desired concentration. Note: Samples should be analyzed within 24 h if stored in the dark at 4 °C. We strongly recommend that the WPS autosampler sample compartment temperature control be set to 4 °C for the duration of this method.

RESULTS AND DISCUSSION

Separation

The SSS was used to test the separation on the Acclaim 120 C18 column. Figure 3 shows the separation of both the SSS and cefepime using the USP conditions. The separation meets the USP requirements for the method. The asymmetry of the cefepime peak in the SSS sample is 1.4, meeting the requirement of ≤ 1.5 . The resolution between cefepime and cefepime-related substance A (RSA) is 23, and the resolution between cefepime and cefepime-related substance B (RSB) is 120, exceeding the requirements of 5 and 10 for RSA and RSB respectively. The capacity factor, k' , is 1.0, greater than the requirement of 0.6, and 9600 theoretical plates are calculated for cefepime, more than double the 4000 specified. The relative retention times are 2.5 for RSA and 6.6 for RSB. In addition to the related substances specified by the USP, another peak is visible just past cefepime. This peak has been assigned as impurity F as described in EP method 2126.

Improved Resolution

In order to improve the resolution between cefepime and impurity F, Mobile Phase A was modified by reducing the amount of acetonitrile from 10% to 6%. Figure 4 shows the separation of the SSS sample on an Acclaim 120 C18 3 μm under these conditions. In this case, the impurity F peak is baseline resolved from cefepime. Another option for improving the resolution that does not require changing the mobile phases, is to use an Acclaim PA 3 μm , 2.1 $\times$ 150 mm column. This column contains a phase with an embedded polar group that is compatible with 100% aqueous mobile phases, is well suited for samples containing polar and nonpolar analytes, and delivers excellent peak shapes for acidic and basic compounds. Using this column and the same mobile phase conditions shown in Figure 3, the resolution between cefepime and impurity F is significantly improved compared to the Acclaim 120 C18, increasing from 2.5 to 4.9 (Figure 5). The relative retention times for RSA and RSB are 2.1 and 6.2, respectively. In addition to the enhanced resolution, the asymmetry of the cefepime peak is also improved on this column (1.1 vs 1.4) and the overall peak shapes are excellent.

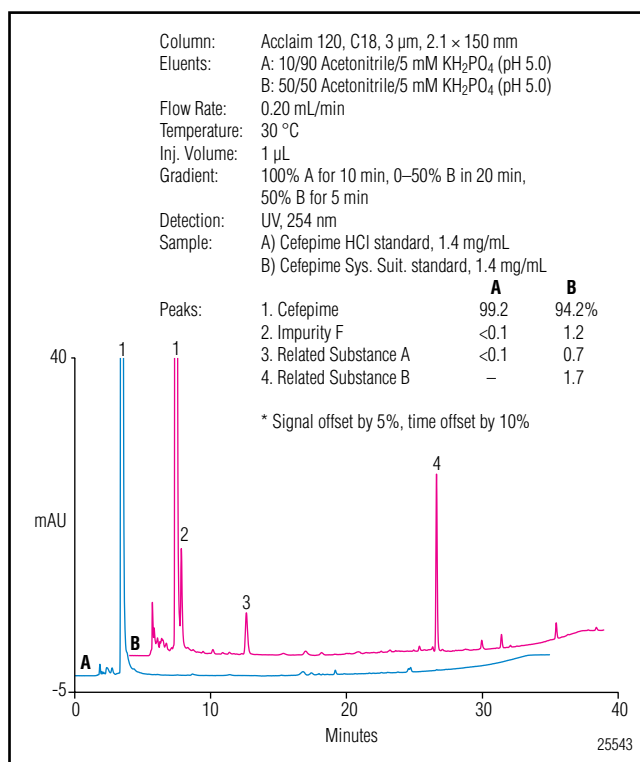


Figure 3. Separation of cefepime and Cefepime System Suitability Standard.

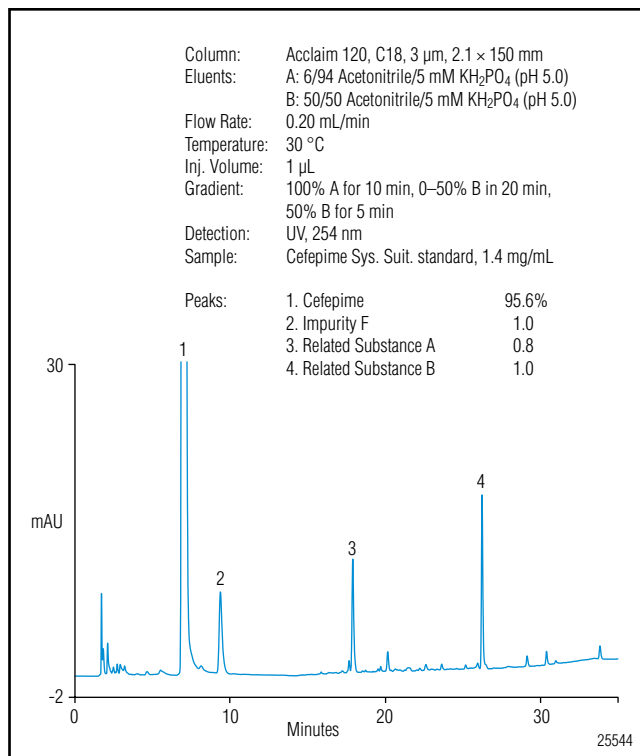


Figure 4. Resolution improvement with 6% acetonitrile in mobile phase A.

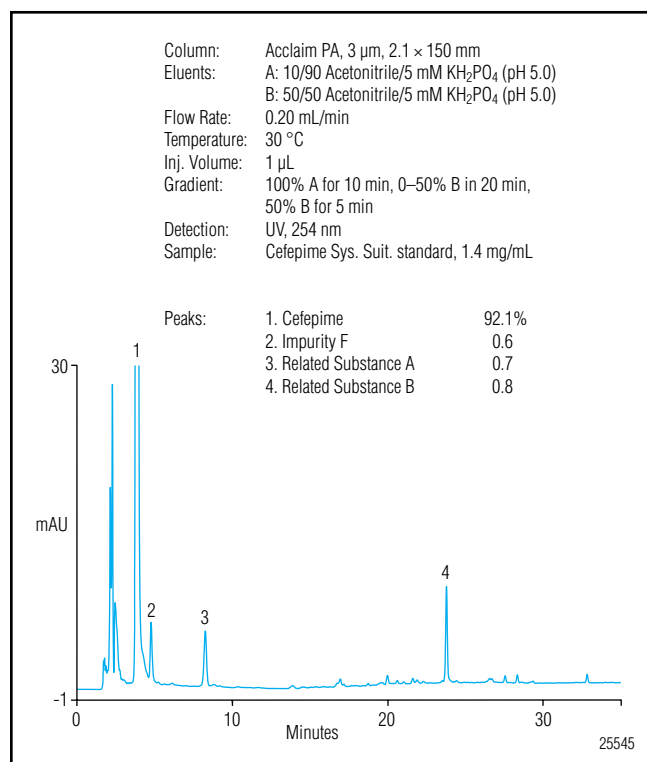


Figure 5. Improved resolution using the Acclaim PA column.

Faster Analysis

Due to the smaller column size and the 3 μ m particle size of the Acclaim 120, C18 column, the gradient can be considerably shortened and still meet the USP conditions. Figure 6 illustrates the separation possible with a gradient method that removes 20 min from the run time for each injection. This shortened gradient meets the USP criteria. The only differences are slight changes in the resolution between cefepime and RSA (25) and RSB (110). The precision of this shortened gradient was tested, and retention times and peak areas were reproducible (Table 1).

Analyte	Retention Time (min)	Area (mAU*min)	Relative Area (%)	Retention Time Precision (RSD)	Peak Area Precision (RSD)
Cefepime	6.95	168.5	95.6	0.05	0.10
Related Substance A	9.39	1.73	1.0	0.06	0.22
Related Substance B	15.0	1.40	0.8	0.03	0.82
Impurity F	19.0	1.76	1.0	0.03	0.52

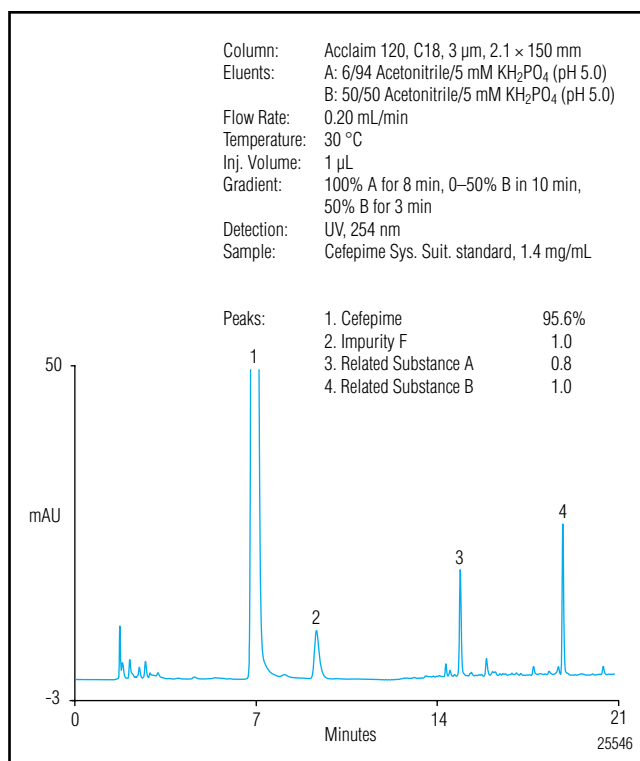


Figure 6. Separation with a rapid gradient using the Acclaim 120 C18 column

Table 2. Linearity, Precision, LOD, and LOQ for Isocratic Cefepime Assay Methods							
Mobile Phase Composition	Range (µg/mL)	Coor. Coeff. (r ²)	LOD (µg/mL)	LOQ (µg/mL)	Retention Time (min)	Retention Time Precision (RSD)	Peak Area Precision (RSD)
6/94 Acetonitrile/ 5mM KH ₂ PO ₄ (pH 5.0)	1.0–1400	0.99995	0.062	0.20	6.95	0.02 ^a	0.08 ^a
10/90 Acetonitrile/ 5mM KH ₂ PO ₄ (pH 5.0)	1.0–1400	0.99989	0.032	0.12	3.62	<0.01 ^b	0.04 ^b

^an=10

^bn=15

Quantification Assay Linearity, Limit of Quantitation, Limit of Detection, and Precision

Isocratic methods for assaying the concentration of cefepime using mobile phase A were tested for linearity, LOQ, LOD, and precision. Both 10% acetonitrile and 6% acetonitrile in 5 mM monobasic potassium phosphate mobile phases were tested. The 10% acetonitrile mobile phase A run time is very fast (6 min), but the 6% acetonitrile mobile phase provides better resolution with a 10 min run time. The EP recommends a run time of 1.4 times the retention time of cefepime. However, if the samples contain significant amounts of RSA, the time should be extended to 12 min for mobile phase A containing 10% acetonitrile and 25 min for mobile phase A containing 6% acetonitrile, in order to avoid quantification interference from RSA in subsequent injections. The linearity, LOQ, LOD, and precision data for both mobile phases using the isocratic assay method are listed in Table 2. In both cases, the linearity and precision are excellent. The LOD is improved for 10% acetonitrile mobile phase due to the shorter retention time leading to narrower peak widths. In this method, RSB and other less polar compounds are retained on the column. For this reason a periodic 30 min wash of 50:50 mobile phase A/mobile phase B is recommended to preserve column life.

Sample Stability

Cefepime solutions are sensitive to hydrolytic and photolytic decompositions. To determine the stability of the cefepime solutions in the mobile phase, samples were studied at ambient room temperature (average of 25 °C), 4 °C (WPS autosampler), and -19 °C (freezer).

All samples were stored in the dark, with the exception of the room temperature samples that were stored in a covered HDPE container. A single solution of 1.4 mg/mL cefepime was prepared in a glass 10 mL volumetric flask. The solution was transferred to individual vials for storage under the three conditions. Samples were injected for 5 consecutive days at 24, 48, 72, and 96 h from the initial injections. From these data, storage at both 4 °C and -19 °C conditions were nearly equivalent, with no change in the peak area of cefepime over 96 h (4 days). Storage at room temperature resulted in a 2.9% loss of peak area after 24 h. After 96 h of storage at room temperature there was a 12% loss of peak area (Figure 7A). Storage at room temperature prior to analysis for any extended length of time is not recommended. Comparison of the purity of the cefepime by relative peak area revealed more subtle effects of thermal instability (Figure 7B). Over 96 h there was no change in the absolute peak area or the relative peak area of cefepime in samples stored at -19 °C. However, storage at 4 °C did result in a slight decrease in the purity of cefepime from 99.4% to 99.0%. The absolute peak area data for samples stored at 4 °C does not show this change. Similar to the results from measuring the absolute peak area, the purity data for samples stored at room temperature showed dramatic loss of cefepime from the solution. During the 96 h of the study, the purity by relative peak area of the remaining cefepime dropped from 99.4% to 93.3%. It is strongly recommended that the samples are stored in the autosampler at 4 °C and that analysis for related substances be completed within 24 h of sample preparation.

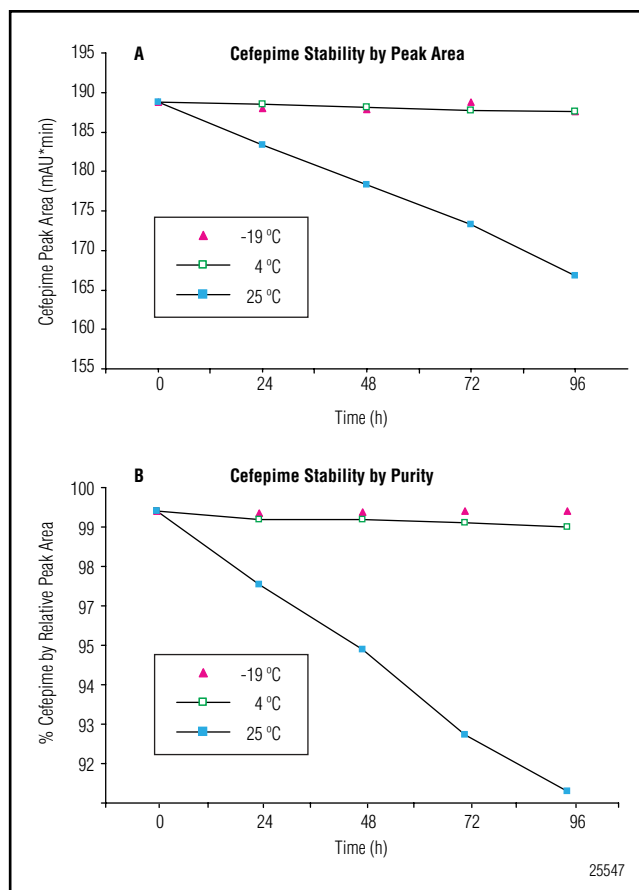


Figure 7. Stability of cefepime over 96 h. A) Absolute cefepime peak area. B) Relative cefepime peak area.

CONCLUSION

In this application note, the Acclaim 120 C18 column, an L1 column with good steric selectivity, combined with UV detection was successfully used for the determination of cefepime and cefepime-related substances, and for assaying cefepime hydrochloride. The methods were modified to decrease the time needed for analysis and improve the resolution as compared to the current methods described in USP monograph USP 30-NF 25-Supplement 1. In addition, the described method uses the same mobile phase preparation for both methods, rather than two separate types, adding convenience and time savings to the method. Finally, the low flow rates used in this method save time and resources spent on mobile phase preparation and reduce waste production.

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