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

Application Note

Pharmaceuticals

Author

William J. Long

Agilent Technologies, Inc.

2850 Centerville Road

Wilmington, DE 19808

USA

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].
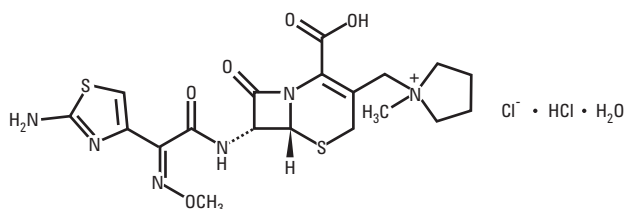
Agilent Technologies

Figure 1. Structure of Cefepime.

Cefepime, fourth generation cephalosporin, is a broad spectrum antibiotic with improved activity against Gram-negative bacteria over other commercially available cephalosporin drugs. The structure of Cefepime is shown in Figure 1.

Despite extensive research on this class of drugs, quantitative analysis and purity assays remain problematic [2]. The EP and USP have published methods determine cefepime and the related compounds [3,4]. These methods use a phosphate/acetonitrile eluent at 1.0 mL/min on a 4.6 × 250 mm, 5 µm column. In this work, an Agilent Poroshell 120 EC-C18 column, 4.6 × 75 mm, 5 µm will be used to quickly analyze the Cefepime related compounds while meeting the requirements for both USP and EP methods. The shorter column rate used with this column saves time solvent due to less frequent eluent preparation, and produces less waste compared to the original EP and USP methods. Moreover, using a shorter column allows quick evaluation of allowable adjustments to methods resulting in better performance in less time.

Experimental

An Agilent 1200 Series Rapid Resolution LC (RRLC) system with an Agilent:

- G1312B Binary Pump SL Gradient times vary depending on column dimensions and flow rate, (Table 1).

- G1367C Automatic Liquid Sampler (ALS) SL, injection volumes are dependent upon specific method parameters, see Table 1.

- G1316B Thermostated Column Compartment (TCC) SL with temperature controlled at 25 °C.

- G1367C Diode Array Detector, set to 254 nm as described in the method.

Two Agilent columns were used in this work:

- Agilent ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 µm, p/n 959990-902

- Agilent Poroshell 120 EC-C18, 4.6 × 75 mm, 2.7 µm, p/n 697975-902

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 suitablility 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

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

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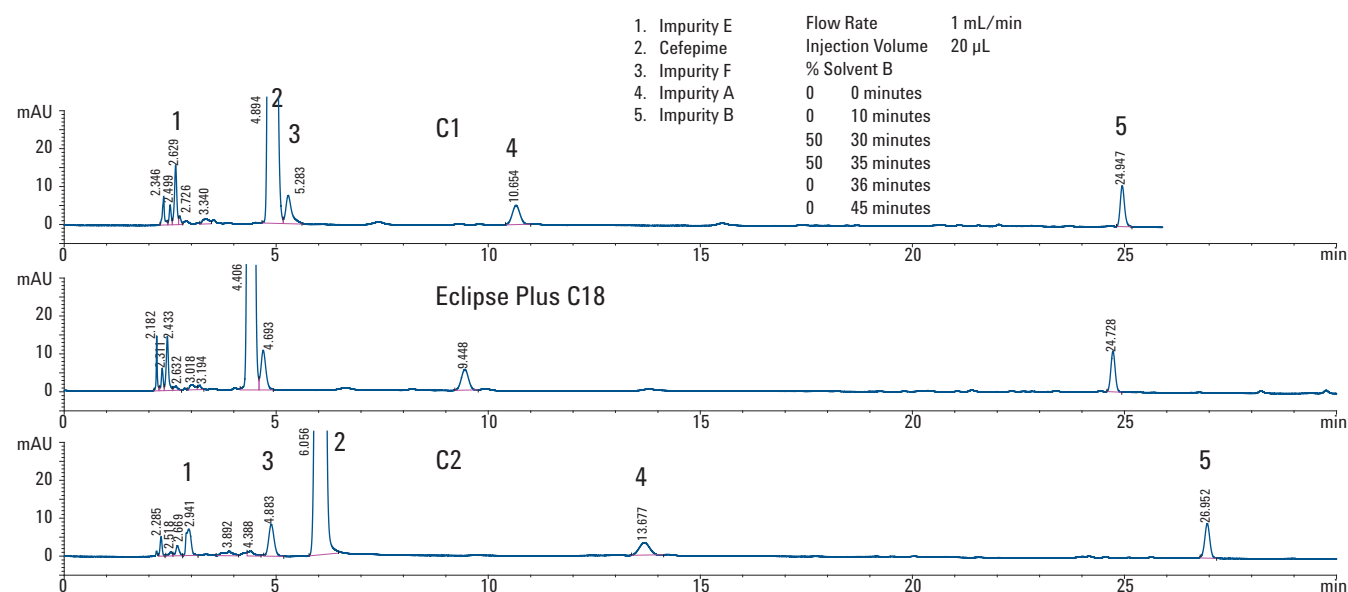

Figure 2.   Original Method on 3 different columns, approximately 45 minutes per analysis.

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:

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

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 2.   Table of Gradients

Inj. Volume % Solvent B	4.6 × 250 mm 5 μm, 1 mL/min, 20 μL	4.6 × 75 mm 2.7 μm, 1 mL/min, 6 μL	4.6 × 75 mm 2.7 μm, 1.5 mL/min, 6 μL	4.6 × 75 mm 2.7 μm, 2 mL/min, 6 μL
0	0 min	0.0 min	0.0 min	0.0 min
0	10 min	3.0 min	2.0 min	1.5 min
50	30 min	10.0 min	6.67 min	5.0 min
50	35 min	10.5 min	7.0 min	5.25 min
0	36 min	10.8 min	7.2 min	5.4 min
0	45 min	13.5 min	10 min	6.75 min

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
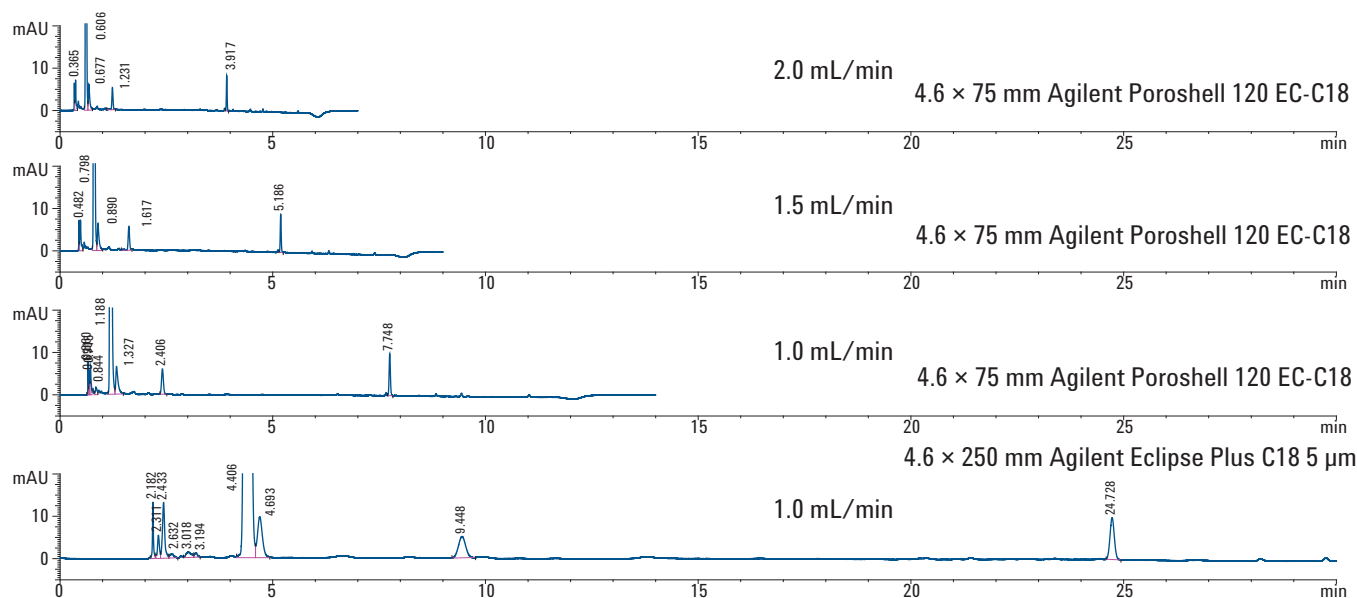

Figure 3.   Totally Porous Agilent Eclipse Plus C18 column and Agilent Poroshell 120 EC-C18 column.

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.
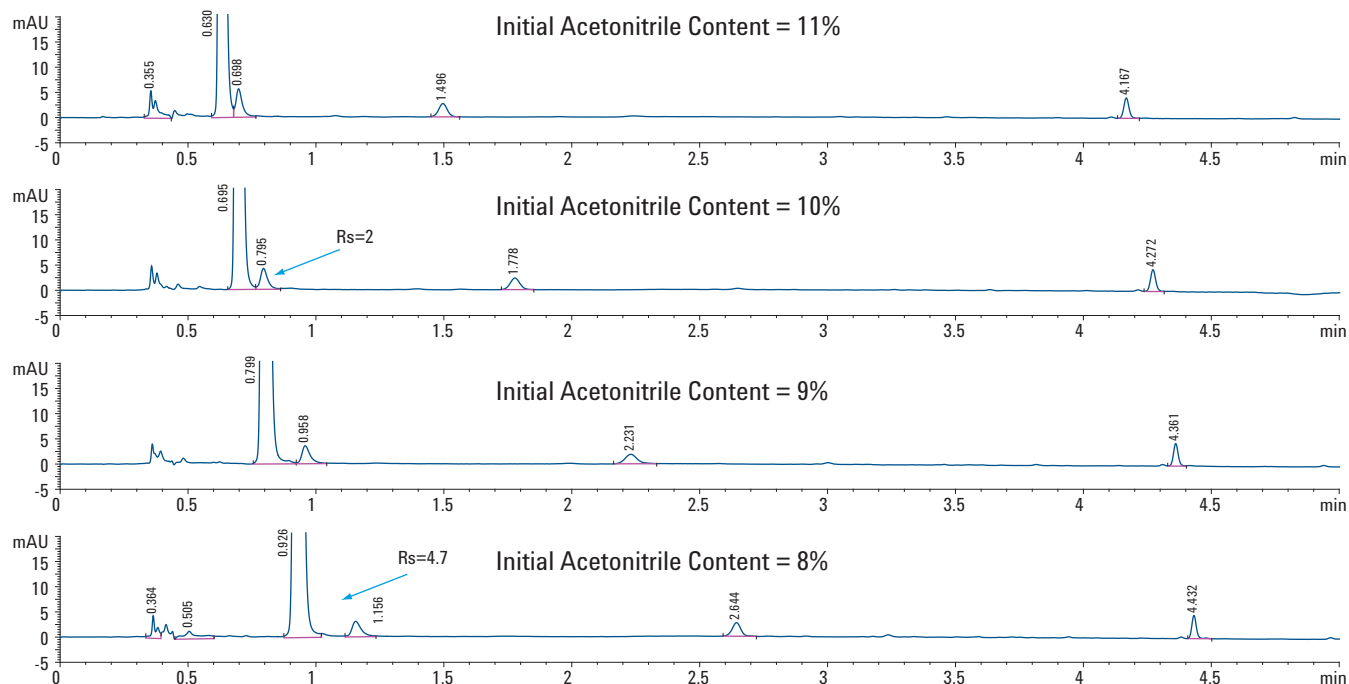

Figure 4. Modification of organic content to improve resolution. Fast method development with Agilent Poroshell 120 EC-C18.
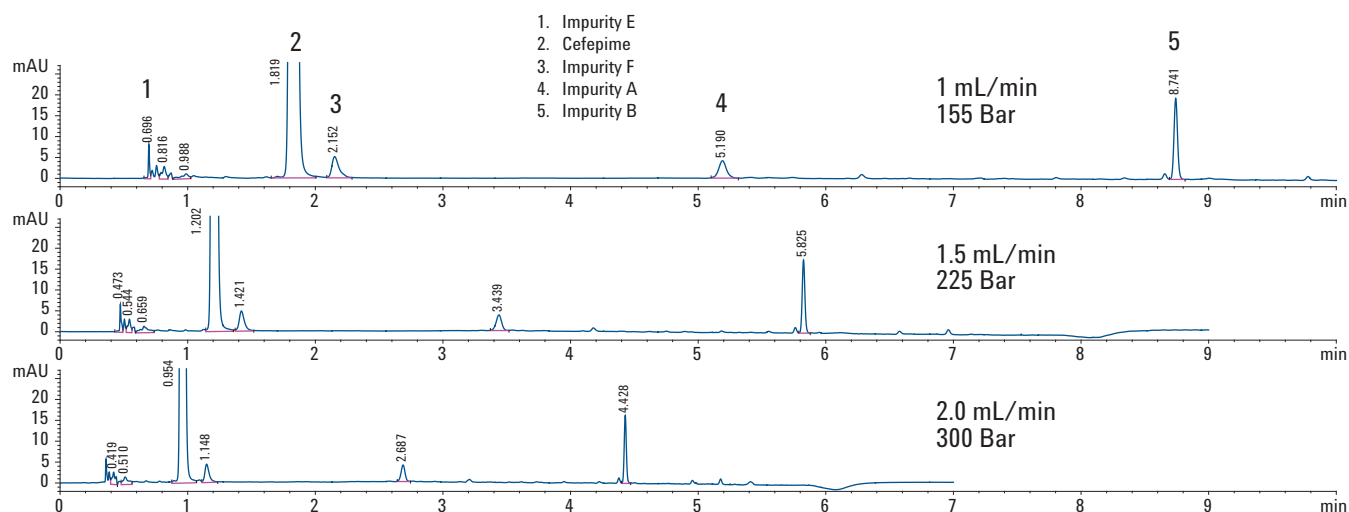

Figure 5. Final optimization of USP cefepime impurity Method, 8% initial acetonitrile at varied flow rates, using Agilent Poroshell 120 EC-C18 4.6 × 75 mm column.

5

Table 3.
Requirement: Agilent Poroshell 120 EC-C18 4.6×75 mm, 2.7 µm

	1 mL/min 156 bar	1.5 mL/min 221 bar	2 mL/min 300 bar
Tf<1.5	1.28	1.28	1.30
N>4000	18288	11993	7250
k'>0.6	1.4	1.4	1.4
Rs C/a	5.9	5.9	5.9
Rs C/b	10.7	10.7	10.7

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

1. D. Yahav, M. Paul, A. Fraser, N. Sarid, L. Leibovici, "Efficacy and Safety of Cefepime: A Systematic Review and Meta-Analysis". Lancet Infect Dis. 2007, 7, 338–348.

2. S. R. El-Shaboury, G. A. Saleh, F. A Mohamed, A. H. Rageh, "Analysis of Cephalosporin Antibiotics". J. Pharm. Biomed. Anal. 2007, 45, 1–19.

3. USP Cefepime Impurity Method, "United States Pharmacopeia 31 NF 26" Rockville, MD. 2008.

4. Cefepime Impurity Method "The European Pharmacopeia", seventh ed., Council of Europe, 2011.

5. USP Method Validation Guidance. "United States Pharmacopeia 30 Supplement 2: System Suitability Testing, Rockville, MD. 2007, Chapter <621>.

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.

7. William J. Long, "Faster analysis of Naproxen Tablets, Improvements on the USP Method", Agilent Technologies, LC GC Magazine, June 2010.

8. Uwe D. Neue, Doug McCabe, Vijaya Ramesh, Horacio Pappa, Jim DeMuthc, "Transfer of HPLC Procedures to Suitable Columns of Reduced Dimensions and Particle Sizes", Pharmacopeial Forum, Vol. 35(6) [Nov.–Dec. 2009].

9. Dorothy J. Phillips, Mark Capparella, Uwe D. Neue, Zoubair El Fallah, "A new small particle packing for faster analysis with high resolution", Journal of Pharmaceutical and Biomedical Analysis 15 (1997) 1389-1395.

For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.

www.agilent.com/chem

Agilent Technologies