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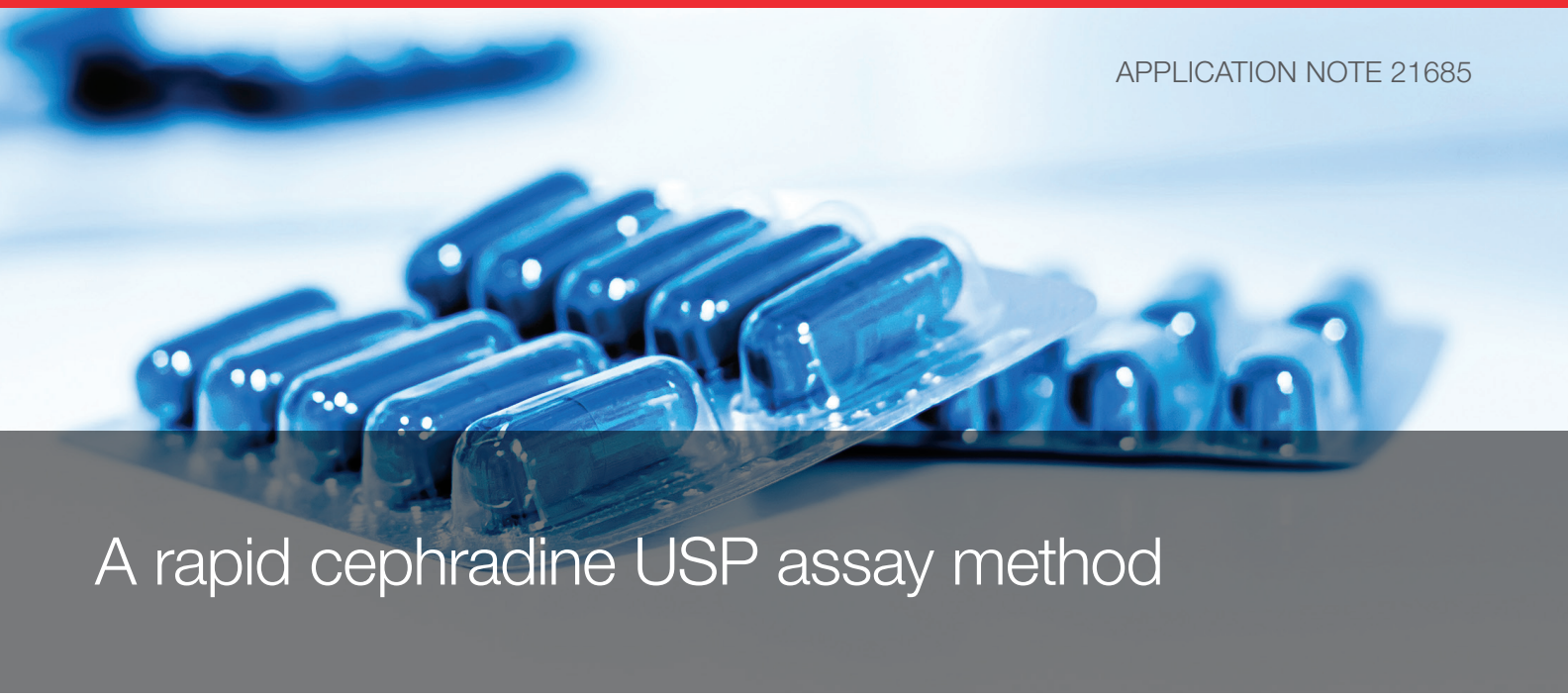
APPLICATION NOTE 21685



A rapid cephradine USP assay method

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Keywords

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Application benefits

- Seventeen-fold increase in method throughput compared to original method (fifty samples/hour)
- Associated 30-fold reduction in cost per sample through reduced mobile phase consumption and waste generation
- Additional reduced method complexity from easy to prepare mobile phase

Goal

To demonstrate practical approaches that can be used to significantly improve throughput of the cephradine USP assay monograph keeping to the spirit of USP-NF Chapter <621> guidelines while maintaining USP quality acceptance criteria

Introduction

Most existing pharmacopeial methods were established prior to the turn of the century and are configured for large particle size (≥5 µm) and long columns (>200 mm). As a consequence, the method times are long and the mobile phase often contains additives that are not compatible with modern aerosol-based HPLC detectors such as MS, ELS, and CAD. The volume of mobile phase consumption and subsequent waste disposal is high compared to modern equivalents, contributing to a significant cost when using these types of assay.

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Since 2014 the USP-NF Chapter <621> has allowed adjustments to these methods, within certain criteria, to benefit from the increased performance of smaller particle size products. However, there are still restrictions on changing the mobile phase composition and chemistry and this can limit the benefit compared to establishing a new method without the legacy restrictions.

One of the key goals for the chromatographer is to achieve a consistent, reproducible separation. The selection of a highly reproducible HPLC column is essential if this goal is to be attained. Based on solid core technology, Accucore HPLC columns allow users of conventional HPLC methods to enjoy performance beyond that of columns packed with 5 µm or even 3 µm fully porous particles. High separation efficiencies provide increased peak resolution. An ultra-stable packed bed results in exceptionally robust columns that demonstrate excellent retention and response reproducibility. Accucore columns are available in a wide range of chemistries and particle sizes making them an ideal choice for this type of work.[1]

The Vanquish Flex Quaternary UHPLC system has the benefit of SmartInject technology, and improvements in injection system hardware synchronization. This results in excellent retention time precision providing the user with greater data confidence during method development. The Vanquish Flex Quaternary system also utilizes Thermo Scientific™ LightPipe™ flow cell technology designed for the diode array detector (DAD), which provides the user with low peak dispersion due to small internal volume.

The cephalexin method was selected as a good example of a legacy method with a complex mobile phase using a large column dimension (250 × 4.6 mm) and potential for method improvement. This will be demonstrated by developing a new method with simple mobile phase, compatible with MS and CAD, and a short 50 mm column providing a method time capable of fifty samples per hour and excellent retention time reproducibility. This process can be applied to other legacy methods to improve the productivity of the laboratory and reduce operating costs through mobile phase and waste reduction.

Experimental

Consumables and apparatus

- Accucore aQ, 50 × 2.1 mm, 2.6 µm column (P/N 17326-052130)
- LC-MS grade 18 MΩ water from Thermo Scientific™ Smart2Pure™ system (P/N 50129845)
- Fisher Scientific™ LC-MS grade acetonitrile (P/N A955-212)
- Fisher Scientific™ Optima™ LC-MS grade ammonium acetate (P/N A114-50)
- Fisher Scientific Optima LC-MS grade acetic acid (P/N A113-50)
- Thermo Scientific™ Virtuoso™ 9 mm wide opening, 2 mL screw thread vial and cap kit (P/N 60180-VT400)

Standards

The two compounds specified in the USP chromatographic assay method were cephalexin (1) and cephradine (2). These were purchased from a reputable supplier. The numbers relate to their elution order and peak labelling in the subsequent chromatograms.

Instrumentation

Analyses were performed using a Vanquish Flex Quaternary UHPLC System consisting of:

- Quaternary Pump F (P/N VF-P20-A)
- System Base Vanquish Flex (P/N VF-S01-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active Pre-heater (P/N 6732.0110)
- Diode Array Detector HL (P/N VH-D10-A)
- LightPipe Flow Cell, 10 mm (P/N 6083.0100)
- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT-100)

Software

Thermo Scientific™ Chromeleon™ 7.2 SR4

Sample preparation

Solutions of the compounds were prepared by dissolving a known amount in water/acetonitrile (80:20, v/v) to produce 1 mg/mL primary solutions. A mixed working standard solution and individual working standards were used to assess method development and were prepared in water/acetonitrile (80:20, v/v) at a concentration of 0.1 mg/mL.

Preparation of mobile phase

The 25 mM ammonium acetate solution was prepared by dissolving 1.575 g of ammonium acetate in 1 L of 18 MΩ water and adjusting the pH to 5.0 with Optima grade acetic acid.

Vial labeling was supported by the Virtuoso Vial Identification System.

USP criteria

Relative retention times are approximately 0.8 for cephalexin and 1.0 for cephradine, with resolution between the peaks not less than 2.0 and RSD not more than 2%.

HPLC conditions (USP method)

HPLC column:	L1, C18, 10 µm, 250 mm × 4.6 mm
Mobile phase:	Water/methanol/0.5M sodium acetate/0.7 N acetic acid
Off-pump mixing:	782:200:15:3
Flow rate:	Approximately 1 mL/min
Column temperature:	Not specified
Injection volume:	Approximately 10 µL
UV detection:	254 nm

UHPLC conditions (final method)

UHPLC column:	Accucore aQ, 2.6 µm, 50 mm × 2.1 mm
Mobile phase A:	25 mM Ammonium acetate pH 5.0
Mobile phase B:	Acetonitrile
On-pump mixing:	95% A: 5% B
Flow rate:	0.6 mL/min
Column temperature:	50 °C, still air with eluent pre-heating
Injection volume:	1 µL
Mixer:	50 µL capillary + 350 µL static in combination
UV detection:	254 nm

Results and discussion

The Accucore aQ UHPLC column was configured on the Vanquish Flex Quaternary system and an initial flow of 0.4 mL/min established with a column temperature of 40 °C. The mobile phase proportioning was adjusted to provide a retention time correlating to a capacity factor of at least two and resolution between the cephradine and cefalexin peaks of at least two. Figure 1 shows the UV chromatogram with a mobile phase proportion of 95:5 (buffer/acetonitrile).

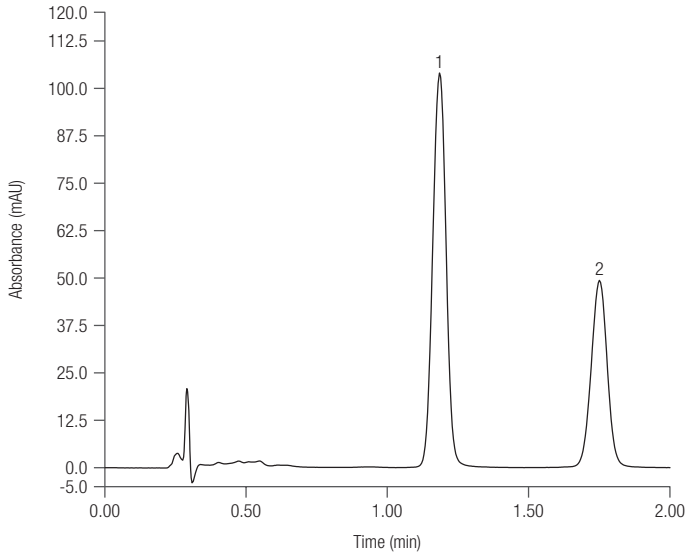

Figure 1. UV chromatogram of standard mixture with a flow rate of 0.4 mL/min of buffer: acetonitrile (95:5) at a column temperature of 40 °C.

The typical retention time for cephradine using the standard USP method[2] is nearer 20 minutes, so already a 10-fold reduction in method time is feasible.

Modern UHPLC systems are equipped with accurate column ovens, and for robust method transfer between different laboratories it is essential to maintain the column at a consistent temperature.

The effect of temperature on this separation was investigated by running the same standard mixture at column temperatures of 40, 50, and 60 °C (Figure 2). The mobile phase proportion and flow rate remain unchanged. The results are shown in Figure 2 and Table 1.

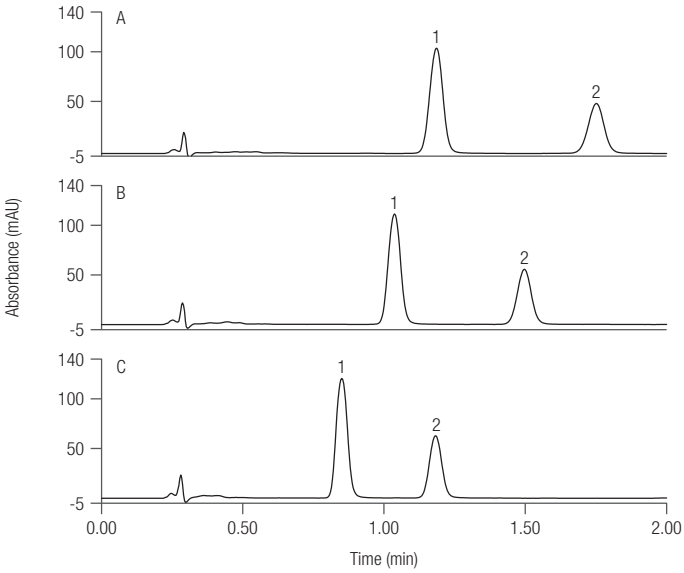

Figure 2. Chromatogram showing standards mixture analyzed at three different temperatures a) 30 °C, b) 40 °C, and c) 50 °C.

Table 1. Peak parameters for cephradine and resolution against cephalexin, under different column temperatures.

Temp. (°C)	Retention Time (min)	Peak Height (mAU)	Peak Width @50% (min)	Resolution
40	1.751	49	0.103	6.0
50	1.497	56	0.091	5.4
60	1.182	63	0.080	4.4

As expected, the retention time decreases with the increase in temperature. Peaks become narrower and taller, thus improving signal to noise, and there is a slight reduction in resolution though still well above the USP criteria of at least two.

Solid core HPLC columns are capable of equivalent efficiencies to much smaller fully porous particles. This allows high efficiency separations at a fraction of the cost in pressure. This, coupled with the solid-core columns ability to run at optimum (highest) efficiency over a much larger linear velocity range[1] allows the chromatographer to significantly speed up their methods.

The effect of flow rate was investigated at temperatures of 50 and 60 °C with flows of 0.4, 0.5, and 0.6 mL/min. Figure 3 and Table 2 show key data obtained from these experiments.

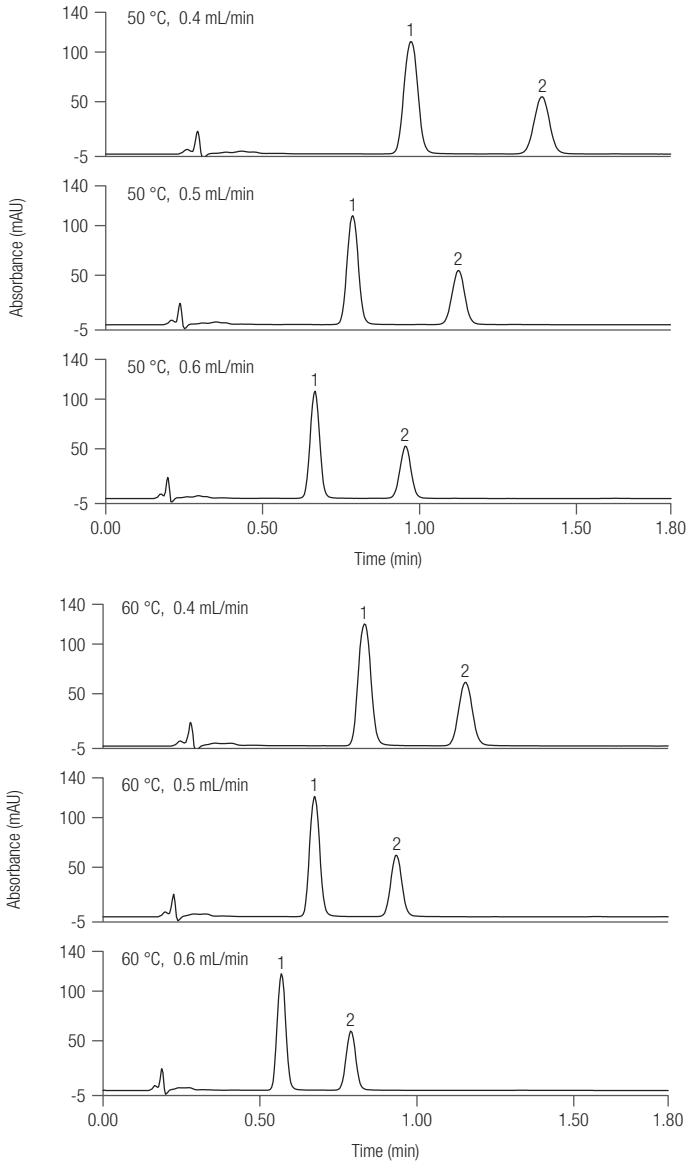

Figure 3. Chromatograms showing separation of cephalexin and cephradine under different temperature and flow rate.

Table 2. Retention time and resolution data from experiments at two temperatures and three flow rates.

	Temp. (°C)	Flow Rate (mL/min)		
		0.4	0.5	0.6
RT cephradine (min)	50	1.390	1.127	0.966
Rs		4.9	4.9	4.8
RT cephradine (min)	60	1.155	0.935	0.790
Rs		4.3	4.3	4.2

Some customers prefer working with lower column temperatures so this was set to 50 °C to collect method reproducibility data using a flow rate of 0.6 mL/min. Twenty-four replicate injections were made under these conditions (Figure 4).

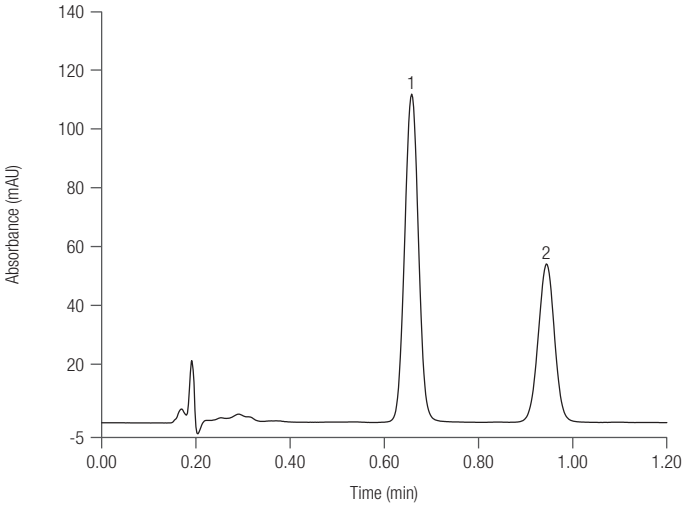

Figure 4. Overlay of 24 replicate injections of standards mixture at 50 °C and a flow rate of 0.6 mL/min. RT RSD for cephradine was 0.14%.

By applying the developed method, the method time has been decreased from ~20 minutes to 1.2 minutes. The consumption of mobile phase (and generation of waste) per assay has also been reduced, from 20 mL to 0.72 mL, thus contributing a saving in both assay cost and an increase in throughput (Figure 5).

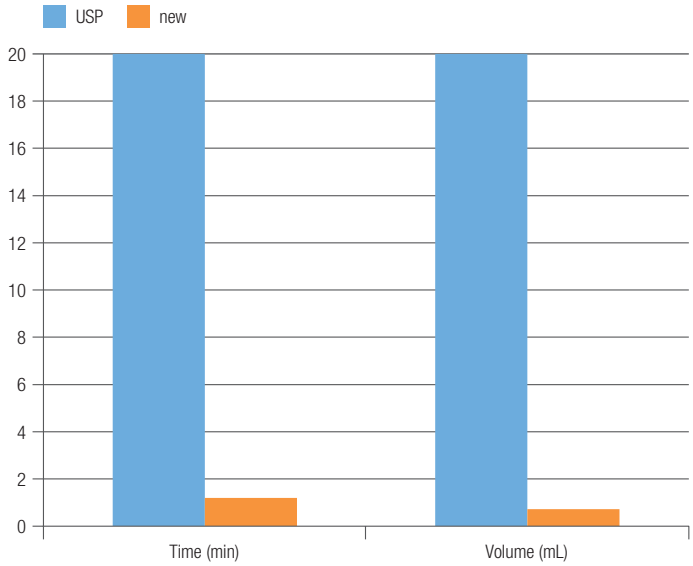

Figure 5. Indicative savings in time and mobile phase volume between the original USP and the improved method.

Conclusions

A high-throughput assay for cephradine was developed keeping to the spirit of USP-NF Chapter <621> guidelines for method modernization that significantly increased throughput and maintains USP quality acceptance criteria. When compared to the original USP method, the updated method demonstrates the following:

- Significant increase in assay throughput (seventeen fold) allowing fifty samples per hour to be assessed
- Substantial associated cost reduction, through reduced mobile phase consumption and waste generation
- Associated reduced method complexity from simplified mobile phase preparation

References

1. Accucore HPLC columns technical guide https://tools.thermofisher.com/content/sfs/brochures/TG-20666-Accucore-HPLC-Columns-TG20666-EN.pdf
2. Transfer the USP method for cephradine from a traditional 5 µm column to Poroshell™ 120, Agilent Application Note 5991-3356EN

Find out more at **thermofisher.com/LC-columns**

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