Performance Evaluation of the Agilent 1290 Infinity II High Speed Pump using Phosphate Buffer as Mobile Phase

Analysis of Antibacterial Drugs

Technical Overview

Authors

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Abstract

Many analytical methods require mobile phases with a modified pH. The use of buffers in the aqueous mobile phase can drastically improve selectivity and resolution of a separation. The separation system, however, has to be flushed properly to avoid buffer salts impairing the column or moving parts of the pump. This Technical Overview demonstrates the performance of the Agilent 1290 Infinity II High Speed Pump when a phosphate buffer is used in the mobile phase. A sequence of 90 consecutive samples was analyzed and evaluated regarding retention time (RT) precision, resolution, and peak symmetry. The performance of the pump was found to be excellent even over a sequence of 90 injections, yielding a RT precision better than 0.07 %.

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Introduction

The use of pH modifiers in the mobile phase has become common practice in high-performance liquid chromatography (HPLC) when samples containing ionizable compounds are analyzed. For a classic reversed-phase (RP) HPLC separation, the nonionized form of acidic and basic structures is preferred, since this form is less polar, and therefore strongly retained on the RP stationary phase. To prevent ionization of an acidic or basic compound by proton exchange with the aqueous mobile phase, the pH of the mobile phase has to be adjusted depending on the sample. To determine a suitable pH of the mobile phase, the p*K*a value of a substance can be used as a starting point. When the pH equals the p*K*a of an acid, the protonated and deprotonated forms are in equilibrium, meaning half of the amount of sample is nonionized and therefore strongly retained. Shifting the pH by two units off the p*K*a changes this ratio from 1:1 to 99:1, enabling the analysis of only the nonionized form. Depending on the variety of different compounds present in the sample, an optimum mobile phase pH has to be determined during method development.

A variety of different small molecule buffers are available for pH adjustment in RP HPLC. Buffers are most effective when used within a range of ±1 pH unit of their p*K*a value, and at a concentration of 5 to 50 mM. When the percentage of the organic phase is increased, which is typical for an RP gradient, the solubility of the buffer in the mixed mobile phase is reduced. In extreme cases, this can lead to precipitation of buffer salts within the HPLC system, causing severe problems such as plugged columns and frits or damaged moving parts, especially in the solvent pump. Phosphoric acid, a highly polar compound, is likely to cause solubility problems in organic solvents. Depending on the phosphate concentration, the percentage of organic mobile phase should not exceed 70 to 80 %. Immediately after the analysis, the whole system should be purged thoroughly with a mobile phase composition as used in the analysis, but without buffer salts present. With these precautions, the performance of the column and the system can be maintained even over a long sequence of analyses.

This Technical Overview describes the application of a 25 mM phosphate buffer at pH 3.0 to separate a sample of four antibacterial drugs (Amoxicillin, Ampicillin, Penicillin G, and Penicillin V). The performance of the system was evaluated by retention time (RT) precision (expressed as relative standard deviation, RSD), resolution, and peak symmetry. The solvent pump has a major influence on these parameters, especially under the burden of a 25 mM phosphate buffer. To demonstrate the long-term stability of the Agilent 1290 Infinity II High Speed Pump, a series of 90 consecutive analyses with phosphate buffer in the mobile phase was evaluated.

Experimental

Instrumentation

The Agilent 1290 Infinity II LC consisted of the following modules:

- Agilent 1290 Infinity II High Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) with sample cooler (Option #100)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 1290 Infinity II Diode Array Detector (G7117B), equipped with a 10 mm Max-Light cartridge cell

Column

Agilent ZORBAX Eclipse Plus C18, 3.0 × 100 mm, 1.8 µm (p/n 959758-302)

Software

Agilent OpenLAB CDS ChemStation Edition for LC and LC/MS Systems, version C.01.07 SR1 [110]

Chromatographic conditions

Parameter	Value
Mobile phase	A) 25 mM KH_2PO_4 in water, pH 3.0 B) Acetonitrile
Flow Rate	1.6 mL/min
Gradient	0.0 minutes – 5 %B 1.5 minutes – 5 %B 11 minutes – 80 %B 15 minutes – 5 %B
Stop time	15 minutes
Post time	4 minutes
Injection volume	3 µL
Sample temperature	8 °C
Column temperature	40 °C
Detection	204/10 nm, reference wavelength 360/100 nm Peak width > 0.013 minutes (0.25 seconds response time, 20 Hz)

Solvents and samples

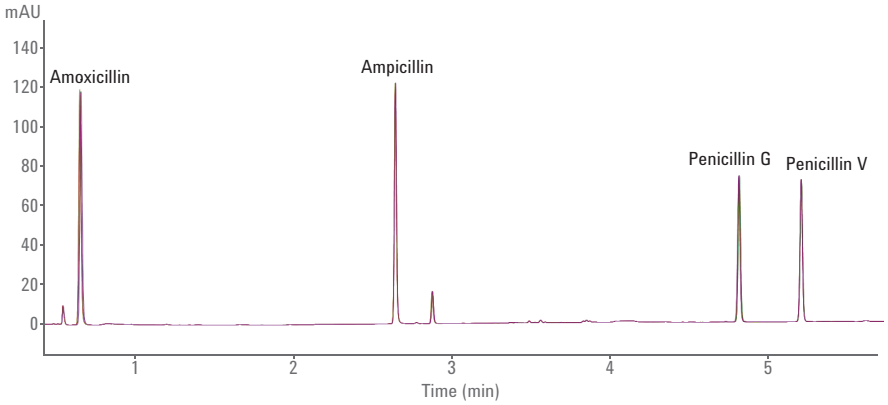
All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22-µm membrane point-of-use cartridge (Millipak). Potassium dihydrogen phosphate (KH_2PO_4), phosphoric acid for pH adjustments, and standards of the four antibacterial compounds were purchased from Sigma-Aldrich, St. Louis, Missouri, USA.

Results and Discussion

A mixture of four antibacterial drugs was separated on an Agilent 1290 Infinity II LC using a solvent gradient of an aqueous phosphate buffer and acetonitrile. The separation of the four compounds was evaluated regarding RT RSDs, resolution, and peak symmetry. All compounds were separated with high resolution and reproducibility. In a series of six consecutive runs, excellent RT RSDs of less than 0.05 % were achieved for all but the first analyte. Figure 1 shows a chromatogram overlay of six consecutive runs along with peak properties.

To test the long-term performance of the Agilent 1290 Infinity II High Speed Pump under the burden of a 25 mM phosphate buffer, the sample was injected and analyzed 90 times in sequence. The separation was evaluated using the same parameters as described above. Peak symmetry changed only marginally from 0.96 to 0.95 for the third compound. Resolution and RT RSDs did not change significantly, and were excellent even over 90 consecutive injections (< 0.07 % for all but the first analyte). Table 1 provides the details.

As a marker of column plugging, the backpressure of the system was observed and evaluated over all injections. The pressure difference between the first and ninetieth run was 18 bar, equaling a total backpressure increase of 0.02 % per run, which is negligible in practice.



Compound	RT (min)	RT RSD (%)	Resolution	Symmetry
Amoxicillin	0.653	0.476	—	0.85
Ampicillin	2.639	0.048	77.32	0.85
Penicillin G	4.807	0.030	74.71	0.96
Penicillin V	5.200	0.023	13.64	0.95

Figure 1. Separation of an antibacterial drug mixture using a 25 mM phosphate buffer in the mobile phase. Chromatogram overlay and peak properties of six consecutive runs.

Table 1. Peak properties of 90 consecutive analyses of an antibacterial drugs mixture using a 25 mM phosphate buffer in the mobile phase.

Compound	RT (min)	RT RSD (%)	Resolution	Symmetry
Amoxicillin	0.654	0.472	—	0.85
Ampicillin	2.640	0.068	81.34	0.85
Penicillin G	4.808	0.037	81.12	0.95
Penicillin V	5.201	0.038	13.47	0.95

Conclusion

The performance of the Agilent 1290 Infinity II High Speed Pump was demonstrated by the analysis of an antibacterial drug mix using a 25 mM phosphate buffer (pH 3.0) as aqueous mobile phase. RT precision, resolution, and peak symmetry were evaluated over six runs. Resolution, peak symmetry, and reproducibility were excellent, yielding RT RSDs below 0.05 % for all but one analyte. When a sequence of 90 consecutive runs was evaluated, the performance did not change significantly: RT RSDs were below 0.07 % for all but one analyte. These data underline the superior performance of the 1290 Infinity II High Speed Pump even under challenging conditions such as a phosphate buffer in the mobile phase.

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