

# Application News

## No. L444

### High Performance Liquid Chromatography

## Effective Method Development Using Online Blending of Buffer Solutions for Mobile Phase Preparation

When investigating analytical conditions during HPLC method development, selection of the mobile phase greatly influences the separation. In particular, when the analyte is an ionic compound, it is especially important that the mobile phase contains a buffer solution of appropriate pH because the analyte retention varies greatly depending on its ionic dissociation equilibrium. From an analyst's perspective, however, the preparation of several mobile phases at various pH values for each analysis in the investigation of the analytical conditions represents a tedious and time-consuming effort.

Here, we introduce a technique in which multiple solutions are blended online to control and adjust the pH of mobile phases for use in analysis.

### ■ Online Mobile Phase Blending System

The mobile phase pH was adjusted by mixing multiple buffer solutions online using a pump with a low-pressure gradient valve installed. Fig. 1 shows an example of the flow line diagram.

This is a high-pressure gradient elution system in which each pump contains a low-pressure gradient valve that permits mixing of up to four different solutions in any desired ratios.

This example illustrates the process in which aqueous mobile phase delivered by pump A and organic mobile phase\*<sup>1</sup> delivered by pump B are mixed entirely online.

\*<sup>1</sup> For online preparation of organic mobile phase and aqueous mobile phase, refer to Application News No. L443 and L445.

### ■ Online Blending of Phosphate Buffer Solution

In general, a buffer solution is prepared by mixing a weak acid with its conjugate base (such as sodium salt) or a weak base with its conjugate acid. The pH of the final solution is determined by the concentration ratio of the acid and salt.

Here, buffer solutions with different pH values were prepared online by mixing 20 mmol/L phosphoric acid aqueous solution ( I ) with 20 mmol/L disodium hydrogen phosphate aqueous solution ( II )<sup>\*2</sup>. Table 1 shows a comparison of the measured pH values of the pump-blended solutions versus those of corresponding buffer solutions prepared manually offline using a graduated cylinder and volumetric flask.

Even in the vicinity of pH 5.7 at which there is no buffering capacity (mixing ratio of ( I )/( II ) = 50/50), approximately the same pH values were obtained for both online and offline preparation.

Table 1 Measured pH of Buffer Solutions

Mixing ratio (I)/(II)	Measured pH value	
	Online blending	Offline blending
80/20	2.38	2.37
60/40	3.01	3.00
50/50	5.67	5.72
40/60	6.50	6.50
20/80	7.19	7.19

\*<sup>2</sup> Shown here are the results of tests carried out under conditions in which pH differences were likely to occur due to the mixing ratio of aqueous phosphoric acid and the disodium hydrogen phosphate salt. For greater pH accuracy under acidic conditions, a combination of aqueous phosphoric acid and aqueous sodium dihydrogen phosphate is recommended. For slightly acidic or neutral conditions, use a blend of sodium dihydrogen phosphate and disodium hydrogen phosphate solutions.

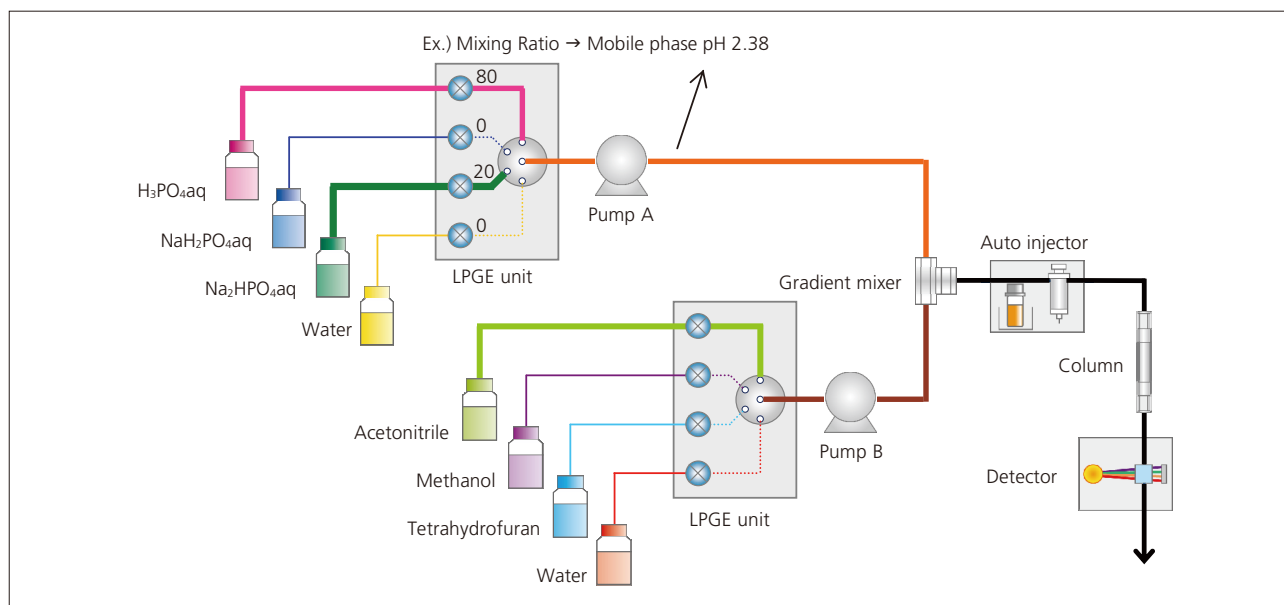


Fig. 1 Flow Diagram of Online Mobile Phase Blending System

### ■ Analysis by Online Blending of Phosphoric Acid Buffer Solution

Fig. 2 shows the chromatograms of a standard mixture of benzoic acid (pKa 4.0), sorbic acid (pKa 4.8), and methylparaben (neutral substance) that were analyzed using phosphate buffer solutions prepared by pump A online blending of 20 mmol/L aqueous phosphoric acid (I) with 20 mmol/L aqueous disodium hydrogen phosphate (II) at various mixing ratios. The analytical conditions are shown in Tables 2 and 3.

The retention time of the neutral compound methylparaben showed little change regardless of the mixing ratio, but the retention times of benzoic acid and sorbic acid, ionic compounds whose dissociative/non-dissociative states change depending on the pH region used in the analysis, both showed large variations.

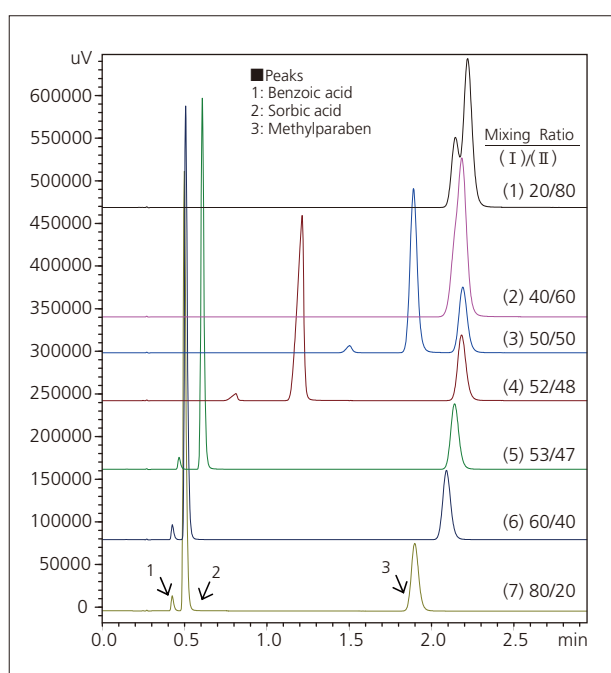


Fig. 2 Chromatograms of a Standard Mixture of Three Compounds (200 mg/L each, 1: Benzoic Acid, 2: Sorbic Acid, 3: Methylparaben)

Table 2 Analytical Conditions

Column	: Shim-pack XR-ODS (50 mm L. × 3.0 mm I.D., 2.2 μm)
Mobile Phase	: A: Online blended 20 mmol/L Phosphate (Sodium) Buffer (I): 20 mmol/L Phosphoric acid (H <sub>3</sub> PO <sub>4</sub> ) (II): 20 mmol/L Disodium hydrogenphosphate (Na <sub>2</sub> HPO <sub>4</sub> ) Mixing Ratios of (I)/(II) are shown in Table 3 B: Methanol A/B = 7/3 (v / v)
Flowrate	: 1.0 mL/min
Column Temp.	: 40 °C
Detection	: SPD-M20A at (260 nm)
Injection Vol.	: 1 μL

Table 3 Online Mixing Ratios of Solutions (I) and (II)

No.	Mixing ratio (I)/(II)
(1)	20/80
(2)	40/60
(3)	50/50
(4)	52/48
(5)	53/47
(6)	60/40
(7)	80/20

### ■ Comparison of Chromatograms and Retention Time Repeatability Obtained Using Online- and Offline-Blended Mobile Phases

Fig. 3 shows the chromatograms obtained from five repeat analyses of the above mixture of three compounds using a 60/40 mixing ratio of solutions I and II as the mobile phase prepared using online mixing by pump A, and the chromatogram of the same sample obtained using the same blend of mobile phase premixed offline.

The chromatograms obtained using online and offline mobile phase preparation show excellent correlation. In addition, the retention time repeatability obtained from five repeat analyses of the sample using online- and offline-blended mobile phases are shown in Table 4. The results obtained indicate excellent equivalence of retention time repeatability between the two methods.

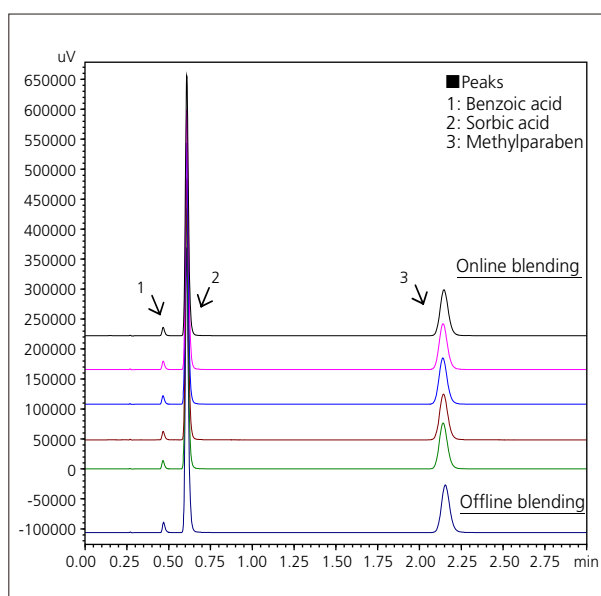


Fig. 3 Chromatograms Obtained Using Online Blending and Offline Blending (I)/(II) = 60/40

Table 4 Repeatability of Retention Time

Compound	Repeatability of retention time RSD (%) n5	
	Online blending	Offline blending
Benzoic acid	0.136	0.086
Sorbic acid	0.132	0.123
Methylparaben	0.124	0.091