The Combined Effects of pH and Percent Methanol on the HPLC Separation of Benzoic Acid and Phenol

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Reversed-phase high-performance liquid chromatographic separation studies have been extensively reported in the literature (*1–7*). One of the primary reasons for this is HPLC's widespread applicability to substances that are of interest to industry and to the public (*8*).

Background

Many mobile-phase variables can affect an HPLC separation (*7–9*). Among these are pH and the percent and type of organic modifier. The pK_a of a weak acid is the pH at which the acid is equally distributed between its protonated (uncharged) and unprotonated (charged) forms. This is illustrated by the Henderson–Hasselbalch equation

$$\text{pH} = \text{p}K_a + \log([A^-]/[HA])$$

where $[A^-]$ is the concentration of the weak acid in its unprotonated form and [HA] is the concentration of the weak acid in its protonated form. If the weak acid is equally distributed between its two forms, $([A^-]/[HA]) = 1$, $\log([A^-]/[HA]) = 0$, and pH = pK_a.

If the weak acid is *not* equally distributed between its two forms, then the pH will be either less or greater than the pK_a of the weak acid. For example, if $[A^-] < [HA]$, $([A^-]/[HA]) < 1$, $\log([A^-]/[HA]) < 0$, and pH < pK_a. Thus, a weak acid exists primarily in its protonated form at a pH below the pK_a and therefore has a greater affinity for the nonpolar stationary phase. If $[A^-] > [HA]$, $([A^-]/[HA]) > 1$, $\log([A^-]/[HA]) > 0$, and pH > pK_a. Thus, a weak acid exists primarily in its unprotonated form at a pH above the pK_a and therefore has a greater affinity for the polar mobile phase.

Organic modifiers also have an affect on the retention of solutes in HPLC. In the reversed-phase mode (polar mobile phase, nonpolar stationary phase), the most polar solute component will elute first. This is because the most polar component interacts least with the nonpolar stationary phase. As the polarity of the mobile phase is increased, those solute components that were previously highly retained (nonpolar components) will be retained even more (*8*).

Two species that are of public interest because of their classification as moderate environmental and health hazards are benzoic acid (pK_a = 4.202) and phenol (pK_a = 9.98). The purpose of this study is to investigate the combined effects of pH and percent methanol on the reversed-phase HPLC separation of these compounds. A three-level, two-factor full-factorial experimental design (*10, 11*) will be used to specify nine mobile phases for consideration in this study. The levels of pH were chosen to bracket the pK_a value of benzoic acid (below, near, and above 4.202). It was not possible to study a mobile phase with a pH > 7.5 owing to the pH range limit of the column. A methanol/water mobile phase was selected for this study because methanol is readily available in most undergraduate labs and relatively inexpensive. In addition, both solutes elute in a relatively short time, making completion of this lab during one or two lab periods possible.

Experimental

Instrumentation

The liquid chromatographic system consisted of a model 2350 solvent delivery system (ISCO), a C6W injector (Valco Instruments), a V^4 variable-wavelength absorbance detector (ISCO) set at 254 nm, and a 250 mm × 4.6 mm Spherisorb ODS-2 analytical column (ISCO). The detector time constant was set at 0.36 s and 10-µL sample volumes were injected. The column was allowed to equilibrate at 1.00 mL/min for 20 min prior to initial sample injection. The mobile phases and column were at ambient temperature and the mobile phase flow rate was 1.00 mL/min.

The analog output from the detector was recorded by an OmniScribe recorder (Industrial Scientific) and simultaneously digitized by a laboratory computer (Gateway) running Lab Works II-100 (SCI Technologies). All pH measurements were done using an Accumet model 425 digital pH/ion meter (Fischer) and a glass electrode (Leeds and Northrup).

Mobile Phase and Sample Preparation

A three-level, two-factor full-factorial experimental design (Fig. 1) was used to specify nine mobile phases (Table 1) corresponding to combinations of pH (3.0, 4.5, and 6.0) and percent methanol (25, 50, and 75%). The order of mobile phases

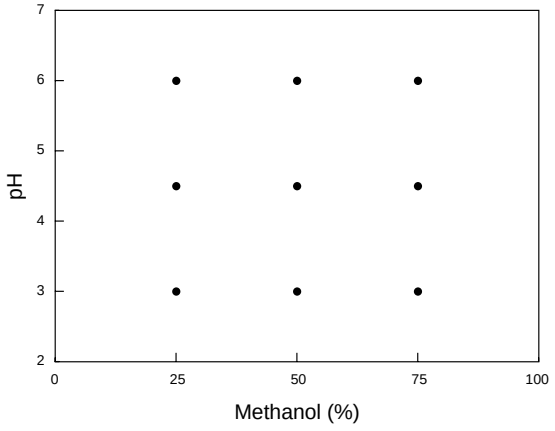

Figure 1. A three-level, two-factor full-factorial experimental design.

Table 1. Mobile Phases Specified by the Experimental Design

Phase No.	Methanol (%)	pH	Phase No.	Methanol (%)	pH
1	25	3.0	6	50	6.0
2	25	4.5	7	75	3.0
3	25	6.0	8	75	4.5
4	50	3.0	9	75	6.0
5	50	4.5			

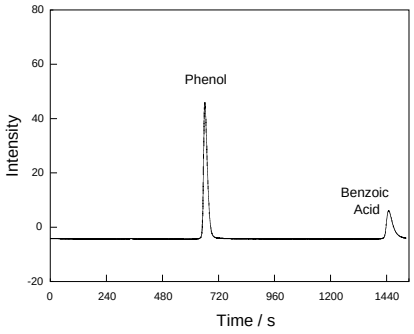

Figure 2. Liquid chromatogram using a 25% methanol/75% water mobile phase at pH 3.0.

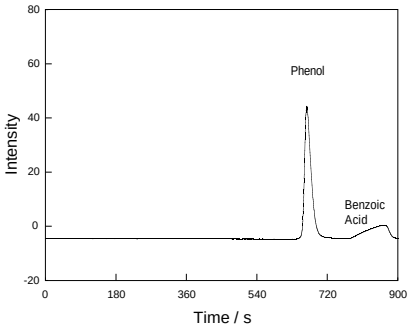

Figure 3. Liquid chromatogram using a 25% methanol/75% water mobile phase at pH 4.5.

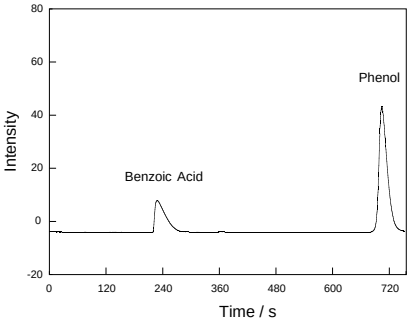

Figure 4. Liquid chromatogram using a 25% methanol/75% water mobile phase at pH 6.0.

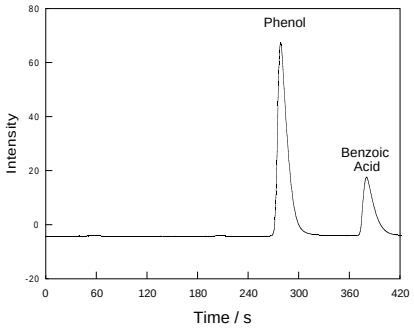

Figure 5. Liquid chromatogram using a 50% methanol/50% water mobile phase at pH 3.0.

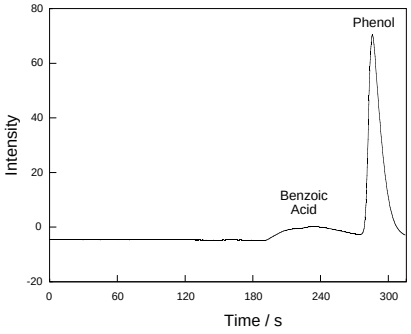

Figure 6. Liquid chromatogram using a 50% methanol/50% water mobile phase at pH 4.5.

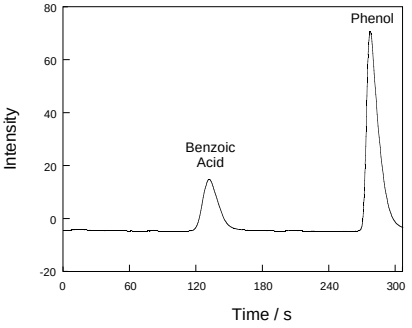

Figure 7. Liquid chromatogram using a 50% methanol/50% water mobile phase at pH 6.0.

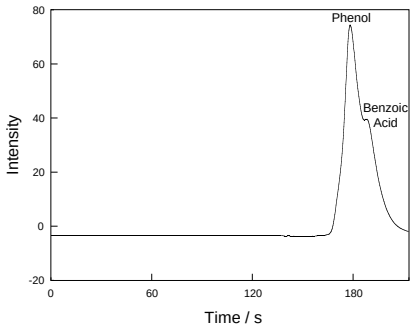

Figure 8. Liquid chromatogram using a 75% methanol/25% water mobile phase at pH 3.0. The phenol and benzoic acid peaks overlap.

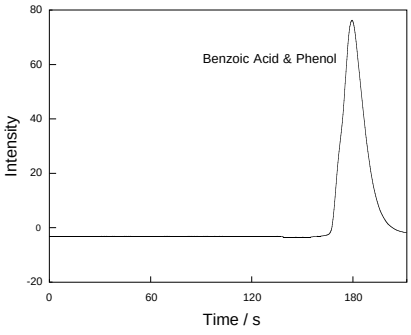

Figure 9. Liquid chromatogram using a 75% methanol/25% water mobile phase at pH 4.5. The phenol and benzoic acid peaks overlap.

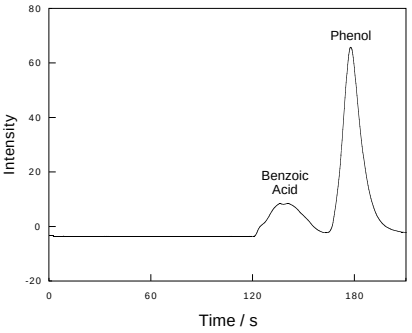

Figure 10. Liquid chromatogram using a 75% methanol/25% water mobile phase at pH 6.0.

investigated was randomized to avoid the confounding of time trends with factor effects (*10, 11*). Five replicate injections were done at the center point to obtain an estimate of the reproducibility of the system.

Mobile phases were prepared by adding HPLC-grade methanol (Burdick and Jackson) to a 500-mL volumetric flask using a graduated cylinder. This was diluted to volume with deionized water and adjusted to the appropriate pH with either 20% H_2SO_4 (Chempure) or 1.0 M NaOH (Chempure). A 0.01 M benzoic acid solution was prepared by placing 0.6105 g of benzoic acid (Eastman Organic Chemicals) in a 500-mL volumetric flask and diluting to volume with deionized water. A 0.05 M phenol solution was prepared by placing 2.3528 g of phenol (Eastman Kodak Company) in a 500-mL volumetric flask and diluting to volume with deionized water. A mixture of benzoic acid and phenol was prepared by mixing equal volumes of 0.01 M benzoic acid solution, 0.05 M phenol solution, and a 50% (v/v) methanol/50% (v/v) water solution.

Hazards

Wear appropriate safety goggles throughout the course of this experiment. Dispense all chemicals and prepare all solutions in a well-ventilated area. If any chemical is spilled on the body, wash the afflicted area with copious amounts of water for at least 15 minutes. Consult the MSDS for complete information regarding toxicity of all chemicals.

Results and Discussion

Figures 2–10 show the chromatograms obtained in this study. Pure benzoic acid and phenol were also injected (their chromatograms are not shown) to assist with peak identification. While a statistical treatment of the data (calculating peak-to-valley ratios, model-fitting, etc.) is possible, it is obvious from the chromatograms that five of the nine mobile phases give baseline separation of the two-component system. The interesting results are the effects of pH and percent methanol on the retention of the benzoic acid and phenol.

At low mobile-phase methanol concentration (25%), as pH increases (Figs. 2–4), the retention time of phenol appears to be unaffected, whereas the retention time of benzoic acid decreases significantly. Over the pH range investigated, the mobile-phase pH is below the pK_a of phenol. Thus, phenol will remain in its protonated form and should be unaffected by these mobile-phase changes. However, as pH increases, benzoic acid shifts from its protonated to its unprotonated form, decreasing its affinity for the nonpolar stationary phase and decreasing its retention time.

At intermediate (50%) and high (75%) mobile-phase methanol concentrations, as pH increases (Figs. 5–10), the retention time of phenol remains unaffected by increases in pH while the retention time of benzoic acid decreases. This is consistent with the behavior at low methanol concentration.

At pH 3.0, as percent methanol increases, the retention times of both phenol and benzoic acid decrease significantly. Because both solutes are polar, increasing mobile-phase polarity causes both to be retained less tightly.

At pH 4.5 (slightly above the pK_a of benzoic acid) and pH 6.0 (well above the pK_a of benzoic acid), as percent methanol increases, the retention times of phenol and benzoic acid decrease. This is consistent with the retention behavior at pH 3.0.

Summary

Baseline separation of benzoic acid and phenol is observed in five of the nine mobile phases studied. At low levels of methanol, as pH increases, the elution order of the benzoic acid and phenol peaks reverses. A similar trend is observed at intermediate levels of methanol. At high levels of methanol, as pH increases, the two-component sample begins to resolve, but complete resolution is never achieved.

In general, as percent methanol increases, the retention times of both analytes decrease. This trend is independent of mobile-phase pH.

Using a factorial experimental design allows one to rapidly see the effects of pH and percent methanol on this separation.

Acknowledgment

The Robert A. Welch Foundation is acknowledged for funding this project.

WSupplemental Material

An equipment list, a summary of the lab procedure, and the suggested format for the lab report are available in this issue of *JCE Online*.

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