Separation and Quantitative Analysis of An Unknown Pharmaceutical mixture using RP-HPLC
CHEM 4303
Analytical Separations

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Abstract

High-performance liquid chromatography utilizing the reverse-phase…is a separation technique blah blah blah …

1 Introduction

High performance liquid chromatography (HPLC) is a type of chromatographic technique in which this instrumentation uses high pressure in order to push the solvent through the column [1]. In an HPLC, the stationary phase is usually inorganic particles that are made up of silica that are porous, and the type of chromatographic techniques that utilizes this type of stationary phase are normal phase (NP), ion pair, and reversed phase (RP) [2]. While NP chromatography is usually utilized in the purification of organic molecules, RP is used to purify proteins that are dissolved in organic solvents and aqueous buffers and the adsorption between the stationary phase and the solute becomes unhinged with the increase in concentration of the aforementioned solvent [3]. In RP, the solvent is less polar than the stationary phase but has the higher eluent strength [1].

Acetaminophen (APAP), an antipyretic and a famous analgesic for humans, is known to cause poisons in cats and dogs [4]. And para-Aminophenol (PAP), an APAP metabolite, is a known nephrotoxicant for rats [5]. Thus, there is a need to separate as well as analyze these pharmaceutical components, and that is the main purpose of this experiment. And this is done with an RP-HPLC.

There were three main objectives in this experiment; in the first week, the purpose was to test the efficiency of separations in RP-HPLC by varying the composition of the mobile phase (methanol). In the qualitative part of the experiment, the purpose was to identify the types of compounds present in an unknown pharmaceutical mixture. Finally, in the quantitative part of the experiment, the purpose was to find out the mass of one of the drugs present in the aforementioned unknown pharmaceutical mixture.

2 Chemicals, Methods and Instrumentation

2.1 Chemicals

Benzaldehyde (Sigma Aldrich, Lot: 41696 PKV), nitrobenzene (Alfa Aesar, Lot: A06V040, 99%, CAS: 98-95-3), methanol (HPLC grade, Caledon, Lot: 103267, CAS: 108-90-7), salicylamide (Sigma Aldrich, Lot: S51885-279, CAS: 65-45-2), phenacetin (Sigma Aldrich, Lot 78C-0014), acetominophen (Sigma Aldrich, Lot: 32F-0073), caffeine (Sigma Aldrich, Lot: 0316B4), and an unknown pharmaceutical mixture was used in this experiment.

2.2 Instrumentation

The separation and analysis of the unknown pharmaceutical mixtures, and the standards, were performed on an Agilent 1100 Series, with a 1260 Infinity degasser (both by Agilent Technologies), fitted with a diode array detector (DAD). The type of column used was a Symmetry® C_{18} (particle size of $5\,\mu\mathrm{m}$, diameter of $4.6\,\mathrm{mm}$, and a length of $150\,\mathrm{mm}$). The flow rate for each analysis was kept at a constant value of $1\,\mathrm{mL/min}$, and the injection volume was $5\,\mu\mathrm{L}$ for each

analysis.

2.3 Methods

In "testing the retention behavior in RP-HPLC" part of the experiment, benzaldehyde, nitrobenzene and chlorobenzene, with volumes of $30\,\mu\mathrm{L}$, $30\,\mu\mathrm{L}$ and $4\,\mathrm{mL}$, respectively, were mixed together. $20\,\mu\mathrm{L}$ of this mixture was diluted with $5\,\mathrm{mL}$ of HPLC-grade methanol. This diluted mixture was analysed in the HPLC utilizing the RP technique, and the mobile phase was varied until the separations were deemed satisfied. Standards were also prepared for benzaldehyde, nitrobenzene and chlorobenzene. For nitrobenzene, $30\,\mu\mathrm{L}$ of it were diluted with $4.3\,\mathrm{mL}$ of methanol. And from this mixture, $20\,\mu\mathrm{L}$ were diluted with $5\,\mathrm{mL}$ of methanol. The same procedure was done for making the benzaldehyde standard. However, for the chlorobenzene standard, $4\,\mathrm{mL}$ of it were added to $60\,\mu\mathrm{L}$ of methanol, and then $20\,\mu\mathrm{L}$ of this mixture was transferred to $5\,\mathrm{mL}$ of methanol. The UV detection was set at $254\,\mathrm{nm}$.

In the qualitative part of the experiment, $0.0615\,\mathrm{g}$ of an unknown pharmaceutical mixture was grinded with a mortar and pestle, and dissolved in $25\,\mathrm{mL}$ of methanol. $500\,\mu\mathrm{L}$ of this mixture was added to $5\,\mu\mathrm{L}$ of water and $5\,\mu\mathrm{L}$ of methanol. This diluted mixture underwent RP-HPLC chromatography with an initial condition of $70:30$ methanol:water. This composition would be varied if necessary in order to get an optimal separation of the mixture. Standards of concentration $2\,\mathrm{mg/mL}$ were made for salicylamide, phenacetin, caffeine and acetominophen so that the compounds in the mixture can be recognized. Note that these standards were diluted 10-fold before being analyzed. The UV detection was set to 245 and $275\,\mathrm{nm}$.

Finally, in the qualitative part of the experiment, a series of calibration solutions was prepared with caffeine acting as the internal standard (IS). $500\,\mu\mathrm{L}$ was the volume of caffeine that was added to each calibration solutions. Salicylamide and phenacetin was found out to be the two compounds in the unknown mixture, and phenacetin was chosen to be quantitatively analyzed. Volumes of $100\,\mu\mathrm{L}$, $300\,\mu\mathrm{L}$, $500\,\mu\mathrm{L}$, $1500\,\mu\mathrm{L}$ and $2500\,\mu\mathrm{L}$ of phenacetin was added to each $10\,\mathrm{mL}$ of volumetric flask. A constant amount of IS was also added each flask ($500\,\mu\mathrm{L}$), and it was filled with 50% methanol till the mark. These solutions was analyzed in the RP-HPLC.

3 Results and Discussion

3.1 Results

As stated earlier, in the first week of the experiment, the compositions of the mobile phase, methanol, was being varied with HPLC-grade water in order to notice the differences in separations in RP-HPLC. The corresponding chromatograms are given in the appendix (figures 1 to 4), and table 1 summarizes the aforementioned chromatograms.

In addition, figure **??** illustrates the log of the capacity factors as a function of the polarity of methanol.

Methanol/%	k'	log k'
100		
90		
80		
70		

Table 1: Data that shows how the capacity factors (k) differ with the change in composition in methanol.

Insert figure here...

Table 2 now shows how the selectivity factor (α) varies with the

Methanol/%	k'	log k'
100		
90		
80		
70		

Table 2: Data that shows how the selectivity factors (α) change with the polarity of methanol.

Figures 5, 6 and 7 show the chromatograms of a chlorobenzene, nitrobenzene and benzaldehyde standards, respectively.

In addition, figure 8 illustrates the chromatogram of the unknown pharmaceutical mixture. While figures 9, 10, 11 and 12 shows the chromatograms of the caffeine, phenacetin, salicylamide and the acetominophen standard, respectively. Note that the optimal composition of the mobile phase was found out to be $50:50$ methanol:water.

Finally, in the quantitative analysis of the experiment, figures 13, 14, 15, 16 and 17 represent the chromatograms .... Figures 18 and 19 show the chromatograms of the analysis of the aforementioned pharmaceutical unknown. Finally, figures 20, 21 and 22 represents the chromatograms of the blank that was run (50% methanol).

The pressure of the column(?) for each analysis is shown in table 3. Note that the figure headings correspond to the analysis of the chromatograms in the appendix.

Now draw a table for the data of the calibration curve, and include a figure.

3.2 Discussion

Give a reason as to why reverse-phase is used as a separation technique and not normal phase.

Maybe state why the pressure increases as the polarity of the mobile phase increases. Hint: its because of the hydrogen bonding of the water molecules, hence a larger pressure is needed to push it through the column.

In the quantitative identification of the pharmaceutical unknown mixture, the initial concentration of the mobile phase was chosen as 50% methanol, and based on the chromatogram

Figure number	Pressure/bar
Figure 1	72
Figure 2	98
Figure 3	122
Figure 4	143
Figure 5	143
Figure 6	143
Figure 7	143
Figure 8	178
Figure 9	178
Figure 10	178
Figure 11	178
Figure 12	178
Figure 13	
Figure 14	
Figure 15	
Figure 16	
Figure 17	
Figure 18	
Figure 19	
Figure 20	
Figure 21	
Figure 22	

Table 3: Table that tabulates how the pressure changes with each analysis. The figure number references the chromatogram in the appendix.

received, this composition was considered to be desirable for the analysis of the aforementioned unknown mixture. This was based on the resolution of the peaks and the retention time for each peaks.

The reason for using internal standards and not standard addition for calculating the concentration of the unknown analyte is that internal standards are preferred when give an accurate answer when

In conclusion, reversed-phase HPLC gives a ...

4 References

References

(1) Harris, D. C., *Quantitative chemical analysis*, 8th ed; W.H. Freeman and Co: New York, 2010.

(2) Moldoveanu, Ș.; David, V., *Essentials in modern HPLC separations*; Elsevier: Waltham, MA, 2013.

(3) Wellings, D. A., *A practical handbook of preparative HPLC*; Elsevier: Amsterdam ; Boston, 2006.

(4) McConkey, S. E.; Grant, D. M.; Cribb, A. E. *Journal of Veterinary Pharmacology and Therapeutics*, *32*, 585–595.

(5) Shao, R.; Tarloff, J. B. *Toxicological Sciences* **1996**, *31*, 268–278.

5 Appendix

5.1 Calculations

Calculating the response factor

$$\frac{\textit{Area of Analyte Signal}}{\textit{Concentration of Analyte}} = F\left(\frac{\textit{Area of Standard Signal}}{\textit{Concentration of Standard}}\right)$$
$$\frac{A_X}{[X]} = F\left(\frac{A_S}{[S]}\right) \tag{1}$$

Equation 1 was taken from [1], where $[X]$ and $[S]$ represent the concentrations of analyte and of the standard.

%RSD

5.2 Chromatograms

There are 22 sheets of HPLC chromatograms.