Structural Analysis of an Unknown Compound and Determination of its $pK_a$
by NMR Spectroscopy

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1. Theoretical Development

Acid-Base Equilibria

Molecules in aqueous solution can take multiple forms. For example, a weak acid is either protonated (HA) or deprotonated ($A^-$), depending on the pH of the solution, and the two forms exist in equilibrium Eq. (1-1). This equilibrium is characterized by the acid dissociation constant $K_a$ or $pK_a$, which is defined as Eq. (1-2) and (1-3) using molar concentrations.

$$\text{HA} + \text{H}_2\text{O} \rightleftharpoons \text{A}^- + \text{H}_3\text{O}^+ \quad (1-1)$$

$$K_a = \frac{[A^-][H_3O^+]}{[HA]} \quad (1-2)$$

$$\text{pH} = pK_a + \log \frac{[A^-]}{[HA]} \quad (1-3)$$

Eq. (1-3) can also be written in terms of relative populations of protonated and deprotonated states using mole fractions as Eq. (1-4).

$$\text{pH} = pK_a + \log \frac{\chi_{A^-}}{\chi_{HA}} \quad (1-4)$$

The $pK_a$ value is of fundamental importance in science with implications in drug design as well as the normal function of enzymes and stability of protein folds. Site specific measurement of an amino acids $pK_a$ in biological systems is often a difficult yet active pursuit seen in the literature and NMR provides one of the few, and perhaps best method of such measurements to date.

Populations of Protonated and Deprotonated States and $^1$H Chemical Shifts

The composition of protonated and deprotonated species of a compound in a solution varies depending on the pH of the solution. For example, pyridine cation, the conjugate acid of a heterocyclic base, dissociates in water as follows Eq. (1-5)

$$\text{C}_5\text{H}_5\text{NH}^+ + \text{H}_2\text{O} \rightleftharpoons \text{C}_5\text{H}_5\text{N} + \text{H}_3\text{O}^+ \quad (1-5)$$

or in general,
\[ BH^+ + H_2O \rightleftharpoons B + H_3O^+ \] (1-6)

At an extremely low pH, almost 100% of the molecules are protonated (BH\(^+\)). On the other hand, almost 100% of the molecule is deprotonated (B) at an extremely high pH. If the pH of the solution is intermediate, both species exist in solution, and populations of each species are expressed with mole fractions \( \chi_{BH^+} \) and \( \chi_B \). The sum of the two mole fractions is 100% or 1 as stated in Eq. (1-7).

\[ \chi_{BH^+} + \chi_B = 1 \] (1-7)

As well as the populations, the chemical shift of \(^1\)H is also affected by the pH of the solution. Protonation and deprotonation change the charge distribution in the molecule, and thus the chemical environment of \(^1\)H would change. Positively charged atom withdraws electrons from other atoms, making those nuclei more deshielded, and therefore, the chemical shifts of those nuclei shift downfield (higher ppm value).

If the chemical shift of a completely protonated species (100% BH\(^+\), i.e., at extremely low pH) is denoted as \( \nu_{BH^+} \) in ppm and completely deprotonated species (100% B, i.e., at extremely high pH) as \( \nu_B \) in ppm, the chemical shift at any pH is given by Eq. (1-8)

\[ \nu = \nu_{BH^+} \chi_{BH^+} + \nu_B \chi_B \] (1-8)

where \( \nu \) is the chemical shift in ppm at a specific pH, and \( \chi_{BH^+} \) and \( \chi_B \) are the mole fractions of protonated and deprotonated species, respectively, at the same specified pH.

Populations of each species at a pH can be calculated by solving Eq. (1-7) and (1-8) and using experimentally measured chemical shifts at two extreme pHs. Once the two mole fractions are calculated, the \( pK_a \) of the molecule can be determined using the Henderson-Hasselbalch equation Eq. (1-4).

Note the observed chemical shift in Eq. (1-8) is a function of a weighted average of protonated and deprotonated species. A proton bound to an electron withdrawing group, such as an amine, has its electron density depleted somewhat and shifts downfield (higher ppm values). The degree of electronegativity of the amine in turn will depend on neighboring functional groups as well. For example, electron pushing aliphatic groups bound \textit{meta} on an aromatic ring will change the acidity of the amine group and hence its \( pK_a \) likewise goes up.

### 2. Sample Preparation

In this experiment you are going to use deuterated solvents: deuterated chloroform and deuterated water (heavy water). These are expensive reagents, so limit the amount you use. \textbf{Please be very careful not to contaminate the source bottle!}
Sample Preparation for Structural Analysis

1. An unknown sample is prepared by TAs. The sample consists of 150 μL of the unknown and 450 μL of deuterated chloroform (CDCl₃) to give 600 μL of 25% (v/v) solution.

Sample Preparation for Titration Experiment (pH Dependence of ¹H Chemical Shift)

Preparation of Stock HCl and KOH Solutions:

1. **Work in the fume hood!** To prepare 4 mL of 1.0 M stock HCl solution, dilute 37% (w/w) HCl (MW = 36.46 g/mol, d = 1.18 g/mL at 25°C) with 10% D₂O (i.e., 90% H₂O) stock solution. Remember to add acid to water as the reaction is highly exothermic.

2. To prepare 4 mL of 1.0 M KOH stock solution, measure the mass of two KOH pellets (MW = 56.11 g/mol). Calculate the volume of 10% D₂O required and dissolve the pellets with 10% D₂O stock solution. Make sure the pellet is completely dissolved. Properly label each solution.

Calibration of pH Meter:

1. Select pH mode by pressing the **mode** button. Press the **setup** and select 4 7 10 buffer set. Press **enter** to clear previous standardization results.

2. Wash the pH probe with distilled water and blot dry. Immerse the probe in pH 4 standardization buffer and when the reading is stabilized, press **standardize**.

3. Repeat Step 2 with pH 7 and 10 buffers. Wash the probe whenever you change solutions and keep it in the storage solution when not in use.

Preparation of Unknown Solutions:

1. To prepare the sample solution, mix 500 μL of unknown sample with 9.5 mL of 10% D₂O. Continuously mix well with a small magnetic stir bar and magnetic stirring plate. This is important as the pH will be off if the solution is not mixing while you add HCl or KOH.

2. Record pH of the solution and transfer 500 µL of this sample into an NMR tube.

3. To prepare the rest of the samples, split the unknown sample in two. To one portion, add the 1.0 M HCl stock solution dropwise to adjust for the lower pH. To the other portion, add 1.0 M KOH stock solution dropwise to adjust for the higher pH. Draw 500 μL of each pH adjusted sample every ~0.4 pH unit between the pH range of 4.7 – 7.7 and collect every 2.0 pH unit outside of this range. Prepare 12 – 15 samples total to cover pH range of approximately 1 – 14.

Note chemical shifts change rapidly when \( |\text{pH} - \text{pK}_a| < 1.0 \), where the pKₐ range of the unknown is approximately 5.7 – 6.7. It is best to have a number of points where the derivative of
the curve is greatest (i.e., within a couple units of the $pK_a$). It is also good to have a few points each at the pH extremes for the baseline.

Setup for pH Adjustment of Unknown Samples:

Note there is a small stir bar inside the vial and the pH probe is about 1 cm above it. Adjust the pH and draw from this solution to prepare/fill the NMR tubes. Keep careful labeling and notes on pH of each sample.

Clean Up:

Clean NMR tubes with distilled water, methanol, and acetone, using an NMR tube cleaner (Figure on the right). Dispose of all waste in appropriate waste container.

1. Connect the cleaner to the aspirator and turn on the aspirator.
2. Place the NMR tube upside-down on Port A.
3. Pour a few squirts of distilled water from Port B.
4. Pour a few squirts of methanol from Port B.
5. Pour a few squirts of acetone from Port B.
6. Repeat Steps 2 – 5 for all used NMR tubes.
7. Turn off the aspirator when finished.
8. Wash the caps with distilled water and acetone in a small beaker.
9. Discard the waste in appropriate waste container.
3. Data Analysis for the Titration Experiment (pH Dependence of $^1$H Chemical Shift)

$^1$H Chemical Shift vs. pH Plot:

1. Prepare tables of experimentally measured pH values and obtained chemical shifts for the most acidic proton.
2. Plot the chemical shift of $^1$H vs. pH.

The table below is the sample data for pyridine. A sample plot for this set of data with fitting curve is also shown below. These figures were generated by Origin 9. Use Origin or any other professional piece of software for the analysis.

<table>
<thead>
<tr>
<th>pH</th>
<th>$\delta_{^1H}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.64</td>
<td>8.610</td>
</tr>
<tr>
<td>2.96</td>
<td>8.610</td>
</tr>
<tr>
<td>4.69</td>
<td>8.450</td>
</tr>
<tr>
<td>5.07</td>
<td>8.300</td>
</tr>
<tr>
<td>5.24</td>
<td>8.220</td>
</tr>
<tr>
<td>5.40</td>
<td>8.140</td>
</tr>
<tr>
<td>5.91</td>
<td>7.940</td>
</tr>
<tr>
<td>6.60</td>
<td>7.830</td>
</tr>
<tr>
<td>8.30</td>
<td>7.790</td>
</tr>
<tr>
<td>10.10</td>
<td>7.800</td>
</tr>
<tr>
<td>11.50</td>
<td>7.800</td>
</tr>
<tr>
<td>12.50</td>
<td>7.810</td>
</tr>
</tbody>
</table>

Nonlinear Curve Fitting – Henderson-Hasselbalch Equation Fitting:

1. Click on the plot window to make it active.
2. From the menu bar, go to Analysis → Fitting → Nonlinear Curve Fit… to open the NLFit dialog box.
3. Under the Settings tab, select Growth/Sigmoidal from the Category drop-down menu and select DoseResp from the Function drop-down menu.
4. Click on Fit until converged icon located at the middle of the dialog box. Nonlinear regression will be performed. Check whether the fit converges.
5. Click OK to close the NLFit dialog box. The report sheet will be created and parameters will be summarized in the report sheets.
Removal of Data Points:

1. Calculate the pKₐ of the unknown.
2. Activate the plot window.
3. From the tool bar, select Mask Points on Active Plot from Regional Mask Tool drop-down list.
4. Select the data points to be removed on the plot. In this case, remove data points outside the range of pKₐ – 1 ≤ pH ≤ pKₐ + 1. Changes will be automatically reflected on the fit curve as the data points are masked. Fit curve will be truncated outside of this range.
5. Once finished, press Esc key to quit the masking mode.

The figure below is the sample plot without and with the data point removal. The red line corresponds to the fitting which includes all the data points. The blue line corresponds to the fitting which only includes data points in blue. Note the fitting parameters change slightly before and after the data point removal.

Determination of pKₐ:

1. The equation of Dose Response (DoseResp) fitting function is

   \[ y = A1 + \frac{A2 - A1}{1 + 10^{(Logx0 - x) p}} \]  

   (3-1)

   where A1, A2, Logx0, and p are the parameters. To calculate pKₐ of the unknown based on the fitting parameters, you need to modify the Henderson-Hasselbalch equation.

2. Express the Henderson-Hasselbalch equation Eq. (1-4) in terms of chemical shifts rather than mole fractions, using Eq. (1-7) and (1-8).
3. Since the plot is chemical shift $\nu$ vs. pH, and the equation derived in Step 2 is pH as a function of $\nu$, arrange the equation in the form $\nu = f(pH)$.

4. Compare the function $\nu = f(pH)$ derived in the previous step with Eq. (3-1) and calculate $pK_a$ of the unknown.

5. Calculate the uncertainty based on the standard errors in fitting parameters. The reference value should be within the uncertainty of your experimental value.

6. Calculate the percent error with respect to the reference value.

4. Laboratory Report Requirements

The following information is required in your laboratory report for this experiment. Please note that both students of each group are required to have copies of all spectra, printouts, and any other data, notes, etc., recorded during the laboratory meetings. The overall guidelines for the report should follow Physical Chemistry Laboratory Reports on the course website.

I. Title Page

II. Abstract

III. Introduction

1. Introduce what NMR is. (How is NMR used in chemistry and other fields?)
2. Briefly summarize the principles of NMR spectroscopy. (What is detected in NMR? How is the NMR signal generated, acquired, observed, and analyzed? What are $^{13}$C NMR and $^1$H NMR?)
3. Briefly discuss how DEPT and 2D $^1$H/$^1$H and $^1$H/$^{13}$C correlation NMR can be used for determining the structure of an unknown compound.
4. Briefly discuss what the titration experiment (pH dependence of $^1$H chemical shifts) is and why it is used.

IV. Data and Calculations

Structural Analysis:

1. Table of $^1$H chemical shifts, integration, multiplicity, and, if possible, $J$ values. Arrange peak information according to the chemical shift values, increasing from left to right.
2. Table of $^{13}$C chemical shifts. Arrange peak information in a similar manner to $^1$H NMR data.
3. Table of $^{13}$C DEPT 45, 90, and 135 chemical shifts. Indicate the presence or absence of peaks and their signs compared to $^{13}$C NMR data.
4. Separate tables for COSY and HETCOR correlations. Summarize which peak is correlated to which peak.
Determination of $pK_a$:

1. An overlaid spectrum of all $^1$H NMR spectra for titration experiment. Peaks should be labeled, and each spectrum should be labeled with pH.
2. Table of pH vs. $^1$H chemical shifts.
3. Plot of $^1$H chemical shifts vs. pH with the Henderson-Hasselbalch fitting curve.
4. Table of fitting parameters.
5. Calculations of $pK_a$ based on the fitting parameters and its error propagations.

V. Results

1. Name and give the chemical structure of the unknown. Each hydrogen and carbon should be clearly labeled (e.g. $H_A$, $H_B$, $H_C$, …, and $C_a$, $C_b$, $C_c$, …).
2. Peak table for $^1$H spectrum. List ppm positions, assignments, relative integral, multiplicity ($J$ coupling value, if possible), and $pK_a$ of corresponding hydrogen.
3. Peak table for $^{13}$C spectrum. List chemical shift positions and assignments.

Example of $^1$H and $^{13}$C labeling of an unknown:

```
O
\|
C_a-C_b-C_c-C_d-O
H_A
```

Unknown A: 4-hydroxybutanoic acid

Example of signal assignments for $^1$H NMR

<table>
<thead>
<tr>
<th>Chemical shift (ppm)</th>
<th>4.6</th>
<th>3.5</th>
<th>2.1</th>
<th>1.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative integral</td>
<td></td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Multiplicity</td>
<td></td>
<td>Doublet of triplet</td>
<td>Triplet of triplet</td>
<td>Triplet</td>
</tr>
<tr>
<td>$pK_a$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assignment</td>
<td>$H_2O$</td>
<td>$H_D$</td>
<td>$H_C$</td>
<td>$H_B$</td>
</tr>
</tbody>
</table>

Example of signal assignments for $^{13}$C NMR

<table>
<thead>
<tr>
<th>Chemical shift (ppm)</th>
<th>182</th>
<th>55</th>
<th>40</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assignment</td>
<td>$C_a$</td>
<td>$C_b$</td>
<td>$C_c$</td>
<td>$C_d$</td>
</tr>
</tbody>
</table>
VI. Discussion

1. Discuss the reasoning for your $^{13}$C NMR assignments of the unknown sample based on your 1D $^{13}$C NMR, 1D $^{13}$C DEPT, and 2D $^{13}$C/$^1$H HETCOR NMR spectra. What is the unknown?
2. Discuss your reasoning for your $^1$H NMR assignments of the unknown sample based on your 1D $^1$H NMR, 2D $^{13}$C/$^1$H HETCOR, and $^1$H/$^1$H COSY NMR.
3. What is the reference value of $pK_a$? How accurate is the experimental $pK_a$ value compared to the reference value? Discuss the source of error.

VII. Discussion Questions

1. Discuss potential problem of 1D NMR for large molecule.
2. Discuss how 2D NMR enhances resolution. How does 2D NMR aid signal assignments?
3. Discuss why titration experiment is useful. How does it help to identify unknowns?

VIII. Conclusions

IX. References

X. Appendix

1. Copy of NMR spectra used for structural analysis. Each spectrum should be properly labeled.
2. Copy of NMR spectra used for titration experiment. Include three spectra (high, intermediate, and low pH spectra). Properly label each spectrum.
3. Copy of inlab and postlab notebook pages.

List of Possible Unknowns:

- 2-methylpyridine
- 3-methylpyridine
- 4-methylpyridine
- 2-ethylpyridine
- 3-ethylpyridine
- 4-ethylpyridine
- 2,3-dimethylpyridine
- 2,4-dimethylpyridine
- 2,5-dimethylpyridine
- 2,6-dimethylpyridine
- 3,4-dimethylpyridine
- 3,5-dimethylpyridine
- 2-ethyl-3-methylpyridine
- 2-ethyl-4-methylpyridine
- 2-ethyl-5-methylpyridine
- 2-ethyl-6-methylpyridine
- 3-ethyl-2-methylpyridine
- 3-ethyl-4-methylpyridine
- 3-ethyl-5-methylpyridine
- 4-ethyl-2-methylpyridine
- 4-ethyl-3-methylpyridine
- 5-ethyl-2-methylpyridine