Determination of the polyacid dissociation constants of glycyrrhizic acid

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Determination of the three stepwise dissociation constants of glycyrrhizic acid in water by potentiometric titration method is described here. The experimental data have been processed with a stepwise linear regression and simultaneous equations algorithm. The three dissociation constants of glycyrrhizic acid assayed by the proposed method are 3.98 ($pK_{a1}$), 4.62 ($pK_{a2}$) and 5.17 ($pK_{a3}$), respectively. The distribution of the different charged ions of glycyrrhizic acid under different pH values has been simulated with Microsoft Excel software. The results are accurate and useful for further pharmacological and pharmacal studies of glycyrrhizic acid.

Licorice is well known and widely used in traditional Chinese medicine. Glycyrrhizic acid (GA) is the main active component of licorice root, and has a beneficial effect on inflammation and hepatitis. GA (1) belongs to polyprotic organic weak acid. (1) has three carboxyl groups in its molecular structure. When the pH value of the solution increases to a high value from low, the degree of ionization of (1) is enhanced and three stepwise ionization equilibrium of the solution occur. To our knowledge, so far there has been no report about the determination of dissociation constants of (1).

The determination of dissociation constants of basic and acidic drugs or active compounds is critical for predicting their ionization states at physiological pH value. The physical chemical parameters can be important for structure-activity relationship. Its determination is therefore essential in understanding the biological activity of drugs, allowing a more defined picture of the macromolecule-ligand binding interaction. Moreover, ionization equilibrium may influence the total concentration of drug available for the interaction with the enzyme active site. The important role of the degree of ionization in the biological behaviour of chemical substances, as well as in their ability to passive transcellular diffusion and/or in their suitability as substrates for active transport is well established.

Several experimental approaches have been employed for the determination of dissociation constants, including potentiometry, HPLC, UV spectrophotometry, capillary zone electrophoresis techniques and feedback-based flow ratiometry. Polyprotic weak acids or bases often profile with several overlapping dissociation constants, which may render difficulty in their correct attribution to the ionization centers. Thus, their dissociation constants cannot be exactly calculated. Among these methods, potentiometry is quite precise classical method, and suitable to determine dissociation constants of polyprotic weak acid or base. The operation is usually simple and the sensitivity of electrodes allows sub-ppm concentrations of drugs to be measured.

The three stepwise dissociation constants of glycyrrhizic acid in water determined by potentiometric titration method with a stepwise linear regression and simultaneous equations algorithm are reported here.

Experimental

The titration was performed with an automatic potentiometric titrator (Shanghai Precision & Scientific Instrument Co. Ltd) fitted with a glass-calomel conjugated electrode and a magnetic stirrer. (1) (>99%) was procured from Jiangsu ChiaTai Tianqing Pharmaceutical Co. Ltd. Sodium hydroxide...
of analytical grade was obtained from commercial sources. Oleanolic acid (99.5%) and cephalosporin C (99.0%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). Deionized water was used during the experiments, which were performed at room temperature (20 ± 2°C).

Solution of (1) (10 g\cdot L^{-1}) was freshly prepared using hot deionized water (70-80°C) by shaking it thoroughly, cooling and then diluting to volume. For preparing NaOH standard solution (0.01 mol\cdot L^{-1}), 10.162 g sodium hydroxide was dissolved in 150 mL of carbon dioxide-free water. After cooling it to room temperature, the solution was filtered through hardened filter paper. Then, 54.5 mL of the clear filtrate was transferred to a tight, polyolefin container, and diluted with carbon dioxide-free water to 1000 mL (USP NF, 2004). Then, the concentrated standard solution was diluted 100-fold with carbon dioxide-free water to yield NaOH standard solution (0.01 mol\cdot L^{-1}).

Before the titration, the calibration of the electrode system was achieved with two standard buffers of pH 4.00 (potassium acid phthalate) and 6.86 (KH2PO4 0.025 mol\cdot L^{-1}−Na2HPO4 0.025 mol\cdot L^{-1} mixture) in order to obtain the standard electromagnetic force of the cell and the slope of the glass electrode at 20°C.

10.00 mL (1) solution (10 g\cdot L^{-1}) spiked with 10 mL deionized water was added into a 100 mL beaker, the combination electrode was plunged into the sample solution and the magnetic stirrer was operated. Then, the sample solution was titrated with NaOH solution (CNaOH=0.01 mol\cdot L^{-1}). Each three drops of the NaOH solution were added into the sample solution, its volume and the pH value corresponding to the volume of the titrant were accurately recorded after stabilization of the electrode response (15 sec). The procedure was repeated and NaOH was gradually added until the pH value approached 10.40.

**Results and discussion**

**Determination of the three dissociation constants of (1)**

Using the first step dissociation of (1), V ml of NaOH at concentration C_T is added into V_0 mL of (1) at concentration C_0 to form a buffer system. K_{a1} is the first dissociation constant of (1).

\[
\text{R(COOH)}_3 + \text{H}_2\text{O} \leftrightarrow \text{R(COOH)}_2\text{COO}^- + \text{H}_3\text{O}^+
\]

where, R(COOH)_3 represented (1). When the above equilibrium is achieved,

\[
K_{a1} = \frac{[\text{R(COOH)}_2\text{COO}^-][\text{H}^+]}{[\text{R(COOH)}_3]} \quad \cdots (1)
\]

So:

\[
[\text{R(COOH)}_2\text{COO}^-] = \frac{V \cdot C_T}{V + V_0} \quad \cdots (2)
\]

\[
[\text{R(COOH)}_3] = \frac{V_0 \cdot C_0 - V \cdot C_T}{V + V_0} \quad \cdots (3)
\]

From Eq. (1), we get:

\[
[H^+] = \frac{K_{a1} \times [\text{R(COOH)}_3]}{[\text{R(COOH)}_2\text{COO}^-]} \quad \cdots (4)
\]

When \[
\frac{[\text{R(COOH)}_3]}{[\text{R(COOH)}_2\text{COO}^-]} = 1 \]

\[\text{[H}^+] = K_{a1}, \text{pH} = pK_{a1}.
\]

If the volume of NaOH added is \(V^*\) mL, then 2 \(V^*\). \(C_T = V_0 \cdot C_0\). Thus,

\[
[H^+] = \frac{K_{a1} \times (2V^* - V)}{V} \quad \cdots (5)
\]

\[
\log V = p\text{H} - pK_{a1} + \log 2V^* + \log \left(1 - \frac{V}{2V^*}\right) \quad \cdots (6)
\]

From Eq. (6), a turning point is found in the log \(V -\) pH curve when \[
\frac{V}{2V^*}\]

is 0.5. \(pK_{a1}\) is equal to the \(\text{pH}\) at that point. For weak acids and bases, the two log \(V -\) pH curves in both sides of the turning point are almost linear, hardly affected by \[
\log \left(1 - \frac{V}{2V^*}\right)
\]
in the range of 0.2-0.8. So, the \(pK_{a1}\) value can be obtained by calculating the \(\text{pH}\) at the intersection of the two log \(V -\) pH curves. The rest two dissociation constants can be obtained analogically.

The potentiometric titration data were processed to get four regression equations for log \(V -\) pH curves of (1) (Table 1) by a subsection-linearity regression with Microsoft Excel software. The three stepwise dissociation constants of (1) were estimated by simultaneous equations of two log \(V -\) pH curves each. It was found that the relative standard deviation
(RSD) of $pK_a$ value was less when $\frac{V}{2V'}$ was in the range of 0.4-0.6 and regression coefficient of log $V$ vs $pH$ curve was more than 0.99. The results of the stepwise dissociation constants of (1) are shown in Table 2.

According to Henderson-Hasselbalch equation, the degree of dissociation and absorption of (1) in biological fluids of different $pH$ can be predicted:

$$pH = pK_{a1} + \log \frac{[A^-]}{[HA]}$$  \hspace{1cm} \text{(7)}

where HA and $A^-$ represent (1) and the deprotonated (1), respectively. $[HA]:[A^-]$ is about 1000:1 in gastric juice, and 1:1000 in small intestine. So, it indicates that (1) is absorbed better in stomach than in small intestine.

The proposed method would be applicable to the determination of dissociation constants of mono- and polyary weak acids or bases except polyary acids or bases which could be titrated hierarchically like $H_3PO_4$. In order to confirm it, $pK_a$ values of oleanolic acid and cephalosporin C were determined by the proposed method and the results are shown in Table 3. Each titration was performed in triplicate. The experimental results show that $pK_a$ values assayed by the proposed method coincide with the data in Merck Index. The dissociation constants of $H_3PO_4$ were also determined. But only the first dissociation constant could be exactly calculated ($pK_{a1}=2.27$) and its $pK_{a1}$ value in Merck Index is 2.15. However, reliable $pK_{a2}$ and $pK_{a3}$ values of $H_3PO_4$ could not be obtained by the procedure. The reason is that a very small addition of titrant causes a large increase of $pH$ value when the first titration jump happened, the linearity of log $V$ vs $pH$ was affected very much by $\log \left(1 - \frac{V}{2V'}\right)$. So, the two log $V$ vs $pH$ curves for $pK_{a2}$ or $pK_{a3}$ were not linear.

The dissociation constants of (1) were also determined by UV spectrophotometry; but, the $pK_a$ values could not be exactly determined. Because the maximum absorption wavelength of chromophore (\(-COOH\)) is about 210 nm, near to the UV end absorption, and the absorbance of (1) in the various $pH$ buffers at this wavelength could not be accurately assayed. So, the $pK_a$ values based on the absorbance deviated from the true values.

### Table 1 — Regression equations for log $V$ vs $pH$ curves of (1)

<table>
<thead>
<tr>
<th>Titrant volume (mL)</th>
<th>Regression equation</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.50-3.80</td>
<td>$\log V = 0.7282 pH - 2.2423$</td>
<td>0.9986</td>
</tr>
<tr>
<td>3.98-12.60</td>
<td>$\log V = 0.8009 pH - 2.5296$</td>
<td>0.9997</td>
</tr>
<tr>
<td>15.50-20.88</td>
<td>$\log V = 0.2475 pH + 0.0339$</td>
<td>0.9961</td>
</tr>
<tr>
<td>22.14-24.55</td>
<td>$\log V = 0.13 pH + 0.646$</td>
<td>0.9959</td>
</tr>
</tbody>
</table>

### Table 2 — The stepwise dissociation constants of (1)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>$pK_{a1}$</th>
<th>$pK_{a2}$</th>
<th>$pK_{a3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.98</td>
<td>4.59</td>
<td>5.15</td>
</tr>
<tr>
<td>2</td>
<td>3.95</td>
<td>4.63</td>
<td>5.21</td>
</tr>
<tr>
<td>3</td>
<td>4.02</td>
<td>4.65</td>
<td>5.17</td>
</tr>
<tr>
<td>4</td>
<td>3.94</td>
<td>4.59</td>
<td>5.16</td>
</tr>
<tr>
<td>Average</td>
<td>3.97</td>
<td>4.62</td>
<td>5.17</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>0.90</td>
<td>0.65</td>
<td>0.51</td>
</tr>
</tbody>
</table>

### Table 3 — The $pK_a$ of some weak acids determined by the proposed method (n=3)

<table>
<thead>
<tr>
<th>Weak acid</th>
<th>$pK_a$</th>
<th>Values in Merck Index (2001, 13th edn)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleanolic acid</td>
<td>2.55</td>
<td>2.52</td>
</tr>
<tr>
<td>Cephalosporin C</td>
<td>2.64, 3.03</td>
<td>2.6, 3.1</td>
</tr>
</tbody>
</table>

The species distribution as a function of $pH$ in (1) system is shown in Fig. 1. From the chemical
structure and the distribution curves of (1), it is shown that the ionization of (1) does not happen at pH < 2. The carboxyl group marked with ⑥ would be ionized first in a pH range 2-3, the ionizations of the carboxyl groups marked ⑤ and ⑦ overlap in a pH range 3-4, and the ionization of the three carboxyl groups overlaps in a pH range 4-5. Also, the ionizations of the carboxyl groups marked with ⑥ and ⑦ overlap in a pH range 5-6. Only the fully deprotonated (1) exists at pH > 7.

Conclusions

The three stepwise dissociation constants of glycyrhrizic acid are found to be: $pK_{a1}=3.98$, $pK_{a2}=4.62$, $pK_{a3}=5.17$, respectively. The potentiometric titration determines the $pK_a$ values with high precision (RSD<1%). The results are accurate and helpful for further pharmacological and pharmacal study of glycyrrhizic acid. The species distribution as a function of pH in (1) has been simulated with Microsoft Excel software.

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References