Chromatographic Determination of Thermodynamic Acid Dissociation Constants of Tetracycline Antibiotics and Their Epimers

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Abstract

The presented study describes the development of reversed-phase liquid chromatography method using a core-shell particle column with a pentafluorophenyl stationary phase for the dissociation constant (pKₐ) determination of the tetracycline group antibiotics (tetracycline, oxytetracycline, chlortetracycline) and their epimers (4-epitetracycline, 4-epioxytetracycline, 4-epichlortetracycline). The pH values were measured in the acetonitrile (ACN)–water binary mixtures, used as mobile phases, instead of in water and take into account the effect of the activity coefficients. Thermodynamic acid dissociation constant (pKₐ1) values of studied antibiotics and their epimers were calculated using retention factor (k) at different mobile phase pH values in studied binary mixtures with ACN percentages of 20, 25, 30 and 35% (v/v). Experimental data were analyzed by using an Origin 7.0 program to fit experimental data to the nonlinear expression derived. From calculated pKₐ1 values, the aqueous pKₐ values of studied compounds were calculated by different approaches and these values were compared.

Introduction

Tetracyclines (TCs) are among essential antibiotic families characterized by their wide range of antibacterial effect (1) that have anti-inflammatory properties (2) and have been used for >50 years for the treatment of bacterial infections in both humans and animal therapy (3).

TCs are derived from a hydronaphthacene skeleton consisting of four linearly linked six-membered rings (4). These drugs belong chemically to the polyketides. The structures of TCs (tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC)) and their epimers (4-epitetracycline (ETC), 4-epoxytetracycline (EOTC), 4-epichlortetracycline (ECTC)) are presented in Figure 1. Substitutions may occur in position C-1 through C-12. TCs are generally fairly stable to acids but quite sensitive to alkali (5).

TCs undergo rearrangements and degradations under mild conditions. In aqueous solutions at pH 2–6, reversible epimerization at C-4 takes place and the reaction is influenced by citrate, phosphate and neutral molecules (4). Generation of TCs degradation products depends on different pH, redox and light conditions and high temperature and humidity so it must be realized that degradation products of TCs can also occur in the stomach (6) because their molecular structure contains four connected benzene rings with multiple ionizable functional groups as shown in Figure 2.

TCs are difficult to analyze due to their instability. The stability of TC is poor under strong acidic and alkaline conditions. Degradation products can be formed via the epimerization, dehydration and proton transfer pathways (5); ETC from TC, EOTC from OTC and ECTC from CTC can be formed in aqueous conditions that are mildly acidic (pH 2–6), and can be reversed back to their active form under specific alkaline conditions in the presence of a complexing metal. The products can also epimerize and form epi-analogs (7).

The knowledge of acid-base equilibria of TCs and their epimers has great pharmacological importance. Moreover, knowing the...
structure and the different acid-base species, the best way of drug administration, the absorption rate, the distribution profile and the excretion percentage can be established. The lipophilicity, solubility and permeability of drug compounds are dissociation constant (pK) dependent, so it is important to obtain reliable information about pKa in the drug development process (8). Several experimental approaches have been employed for the determination of pKa values of TCs including potentiometric titration (9), spectrophotometry (10) and reversed-phase liquid chromatography (RPLC) (11). RPLC for acid dissociation constant (pKa) determination is one of the popular methods. The reported determination of pKa values with RPLC techniques have provided accurate and reliable measurements of TC antibiotics, most of these previous studies have been focused on parent TC agents, but no research has been published on the determination of epimers of TCs.

Retention in RPLC is primarily controlled by the chromatographic strength of the mobile phase, with the strength frequently denoted as the percent of the organic modifier in the binary aqueous solution (12). The predicted effect of solute dissociation on the retention factors (k) was confirmed by experimental data. The dependence of the retention factors on the ionic strength of the eluent and pH of the mobile phase gives an indirect estimate of the dissociation constant (13). The solute retention in RPLC represents the molecular fraction weighted average of the retention factors of the undissociated (kHA) and dissociated (kA−) forms (Eq. 1).

\[
k = \frac{k_{HA} + k_{A^{-}} \cdot \frac{K_{a}}{K_{m} \cdot \alpha_{m}}}{1 + \frac{K_{a}}{K_{m} \cdot \alpha_{m}}}
\]

where \(a_{H^{+}}\) is the hydrogen ion activity in the mobile phase. The dissociation constant in the acetonitrile (ACN)–water mixture used as the mobile phase is represented by \(K_{a}\). \(\gamma_{m}\) is the activity coefficient of the dissociated acid in the mobile phase that can be calculated by the classical Debye–Hückel equation (Eq. (2)) (14). The Debye–Hückel equation is required to correct for binary mixtures ionic strength in order to obtain thermodynamic pK_a value.

\[
\log y = -A \sqrt{I} + a_0 \sqrt{I}
\]

where the values of the Debye–Hückel A and B constants and the ion size parameter, \(a_0\), in the ACN-water mixtures have been reported by Barbosa et al. (14). The ionic strength, I, of the mobile phases used, can be calculated for each pH value from charge and mass balances, taking into account the pK_a1 and pK_a2 values of phosphoric acid at each mobile phase composition, the analytical concentration of this acid in the mobile phase, the pH values and the activity coefficients, using iterative calculation (15).

Thermodynamic aqueous acid dissociation constant is a relevant technological property to know stability and solubility of drug. Many drugs are poorly soluble in water and therefore literature proposes several different approaches for their aqueous pKa estimation. This constant from the pKa values of hydro-organic mixtures is calculated using different approaches. In the first one, the value of the intercept of plots experimental dissociation constant amount fraction (X) of the organic solvent content in the mobile phase gives aqueous pKa value. This approach is performed by using Eq. (3).

\[
pK_a = aX + b
\]

where \([H_2O]\) denotes the molar water concentration and \(\varepsilon^{-1}\) denotes the reverse of the dielectric permittivity of the binary mixtures. \(b\) and \(a\) symbolize the intercept and slope of the plot, respectively (18). This extrapolation is crucial for accurate pKa determination in organic solvent–water mixtures.

In the second approach, aqueous pK_a values are calculated by using Yasuda–Shedlovsky equation (Eq. (4)). Yasuda (16) and Shedlovsky (17) independently derived a correlation by means of a plot of pK_a + log [H_2O] versus \(\varepsilon^{-1}\) producing a straight line.

\[
pK_a + \log [H_2O] = a\varepsilon^{-1} + b
\]

where \([H_2O]\) denotes the molar water concentration and \(\varepsilon^{-1}\) denotes the reverse of the dielectric permittivity of the binary mixtures. \(b\) and \(a\) symbolize the intercept and slope of the plot, respectively (18). This extrapolation is crucial for accurate pKa determination in organic solvent–water mixtures.

This paper primarily describes the retention factors of TC, OTC and CTC, and their epimers (ETC, EOTC, ECTC) were determined.
using RPLC method in different percentages of ACN–water binary mixtures at 25°C. In all cases, thermodynamic pK_{a1} values of studied compounds were calculated through the Debye–Hückel equation despite the fact that experimental values were obtained at constant ionic strength. The effect of changing ACN composition on the pK_{a1} was calculated. On the other hand, this study investigates on the use of different percentages of ACN–water binary mixtures and extrapolation to 100% water. The thermodynamic aqueous pK_{a1} values of studied antibiotics were calculated with Yasuda–Shedlovsky equation and amount fraction (X_{ACN}) of the ACN content in the mobile phase.

Experimental

Instrumentation and apparatus
Shimadzu HPLC system (Shimadzu Technologies, Kyoto, Japan) was equipped with a model series LC-20 AD pump, SPD-20A detector, DGU-20A3 degasser and CTO-20A column oven. The analyses were performed on Kinetex F5 (Phenomenex, 150 × 4.6 mm ID, 2.6 μm) column.

pH measurements were taken with an In Lab 412 combination glass electrode, equipped with automatic temperature compensation probe, using a Mettler Toledo MA 235 pH/ion analyzer (Schwerzenbach, Switzerland).

Chemicals and reagents
Tetracycline hydrochloride, oxytetracycline dihydrate, chlortetracycline hydrochloride, 4-epoxytetracycline and 4-epichlortetracycline were obtained from Acros (Acros Organics, USA) and 4-epitetracycline hydrochloride and uracil (for dead time) were acquired from Sigma–Aldrich (St. Louis, MO). ACN (HPLC grade), sodium hydroxide and orthophosphoric acid were of analytical quality and supplied by Aldrich (St. Louis, MO). ACN (HPLC grade), sodium hydroxide and orthophosphoric acid were prepared daily from stock solutions at a final concentration of 200 µg/mL for each compound.

Chromatographic procedure
In the present work, the effect of solvent composition in mobile phases was analyzed at three solvent levels. At each composition, different pH values were studied, ranging from 2.5 to 6.0. In this study, mobile phases used were different proportions of ACN ranging from 20–35% (v/v). Orthophosphoric acid was used as a buffer component because of their appropriate buffer capacity and appropriate peak shape of studied compounds in this buffer solution. The pH of the mobile phase containing 30 mM orthophosphoric acid was adjusted between 2.5 and 6.0 by adding 1 M sodium hydroxide. Chromatographic measurements were done at 25°C with an eluent flow rate of 0.5 mL/min. The volume of solution injected into the column was 20 µL for each run. TCs group compounds were monitored at 270 nm and their epimers were monitored at 255 nm.

pK_{a} measurements
The pK_{a} values of TCs and their epimers in different percentages were measured at 25°C. These values were determined by performing fit to Eq. (1) using the (non-linear regression software) Origin 7.0 (19).

Results

The TCs and epimers contain different ionizable functional groups. These group antibiotics show amphoteric behavior because of acidic substituents and the basic dimethylamino group. The first dissociation constant (pK_{a1}) is associated with tricarbonyl group (Figure 1). The retention factor of these compounds and the pK_{a1} value were determined in a stationary phase containing the pentafluorophenyl phase with a high selectivity. Kinetex F5 (150 × 4.6 mm ID, 2.6 μm) column selected for the assay was superior to conventional C18 columns. This column is both a polar and apolar compound as well as a highly reproducible column that is preferable in the separation between the epimer and the isomer. The second dissociation constant (pK_{a2}) value of the studied compounds is not determined in this study. It could not be determined by this LC method due to column pH range (1.5–8.5).

In this study, the retention factors were determined for each mobile phase composition and over a pH range of 2.5–6.0 in order to determine the pK_{a1} of these compounds. The examples of dependences of the retention factors on the pH value of the mobile phase (20%, v/v) were given in Figures 3 and 4. These graphs were plotted using Origin 7.0 (19).

Data acquisition and handling were performed by Origin 7.0 (19). The pK_{a1} values of the compounds studied were determined from the calculated k values, the pH measurements and calculated activity

Figure 3. Plots of the retention factors versus the pH of the mobile phase for 20% v/v ACN: (A) TC, (B) OTC, (C) CTC. The solid lines indicate the retention factors predicted by Eq. (1).
Figure 4. Plots of the retention factors versus the pH of the mobile phase for 20% v/v ACN: (A) ETC, (B) EOTC, (C) ECTC. The solid lines indicate the retention factors predicted by Eq. (1).

Table 1. Thermodynamic acid dissociation constant values of investigated compounds in various ACN–water ratios

<table>
<thead>
<tr>
<th>Compounds</th>
<th>20% (ενv)</th>
<th>25% (ενv)</th>
<th>30% (ενv)</th>
<th>35% (ενv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline</td>
<td>4.278 (0.016)</td>
<td>4.436 (0.030)</td>
<td>4.684 (0.009)</td>
<td>4.933 (0.075)</td>
</tr>
<tr>
<td>4-epitetracycline</td>
<td>4.805 (0.032)</td>
<td>5.003 (0.025)</td>
<td>5.264 (0.022)</td>
<td>5.499 (0.011)</td>
</tr>
<tr>
<td>Chlortetracycline</td>
<td>4.309 (0.052)</td>
<td>4.523 (0.077)</td>
<td>4.782 (0.067)</td>
<td>4.988 (0.020)</td>
</tr>
<tr>
<td>4-epichlortetracycline</td>
<td>4.799 (0.014)</td>
<td>5.023 (0.011)</td>
<td>5.254 (0.052)</td>
<td>5.519 (0.004)</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>3.960 (0.071)</td>
<td>4.094 (0.028)</td>
<td>4.298 (0.048)</td>
<td>4.502 (0.087)</td>
</tr>
<tr>
<td>4-epioxytetracycline</td>
<td>4.706 (0.003)</td>
<td>4.906 (0.051)</td>
<td>5.122 (0.019)</td>
<td>5.305 (0.033)</td>
</tr>
</tbody>
</table>

*The values between parentheses are the standard deviations.

Table 2. Linear equations between experimental pK_a values and the X_{ACN}

<table>
<thead>
<tr>
<th>Compound</th>
<th>Equation</th>
<th>r^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline</td>
<td>pK_{a1} = 8.980(0.373) X_{ACN} + 3.561(0.044)</td>
<td>0.997</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>pK_{a1} = 7.423(0.276) X_{ACN} + 3.369(0.032)</td>
<td>0.997</td>
</tr>
<tr>
<td>Chlortetracycline</td>
<td>pK_{a1} = 9.275(0.431) X_{ACN} + 3.596(0.050)</td>
<td>0.996</td>
</tr>
<tr>
<td>4-epichlortetracycline</td>
<td>pK_{a1} = 9.677(0.115) X_{ACN} + 4.048(0.013)</td>
<td>0.999</td>
</tr>
<tr>
<td>4-epitetracycline</td>
<td>pK_{a1} = 9.483(0.237) X_{ACN} + 4.064(0.028)</td>
<td>0.999</td>
</tr>
<tr>
<td>4-epioxytetracycline</td>
<td>pK_{a1} = 8.127(0.387) X_{ACN} + 4.085(0.045)</td>
<td>0.995</td>
</tr>
</tbody>
</table>

Coefficient values. The calculated pK_{a1} values are listed in Table I and were calculated by using a non-linear least-squares fit of the data to Eq (1).

Thermodynamic aqueous acid dissociation constant is a relevant technological property to know the stability and solubility of the drug. Many drugs are poorly soluble in water and therefore literature proposes several different approaches for their aqueous pK_a estimation. Simple approaches for the calculation of accurate pK_a values in water were used in this work. In the first described approach, Equation 3 was used to calculate the aqueous pK_a values of studied compounds. There is actually a linear relationship between pK_{a1} values of the TC, CTC, OTC, ETC, ECTC, EOTC and amount fraction of ACN (X_{ACN}) in the binary mixtures. The results of linear equations for compounds used are provided in Table II. The intercept of linear equations obtained from this approach was the thermodynamic aqueous acid dissociation constant values of these compounds (Figure 5).

Then, the following expression, Equation 4 was used to estimate the aqueous pK_a of investigated substances. Yasuda–Shedlovsky equation was used to correlate pK_a values in studied ACN–water binary mixtures. Table III represents the data obtained by RPLC method. pK_a values of TCs and their epimers were determined using Yasuda–Shedlovsky equation (16, 17). The extrapolated values represent the pK_a value at 100% water concentration (0% ACN).

Discussion

In this study, the pK_a values were found to increase slightly with increasing percentage of ACN in the medium. This observation is consistent with the pK_a values of compounds bearing similar tricarbonyl group and nearly between 3.0–3.5 in water (9–11, 21). In Figures 3 and 4, data pairs of k/pH for investigated compounds in different percentages of ACN–water binary mixtures are shown, together with the corresponding experimental and calculated retention factors. It can be concluded that plots of sigmoidal curves of retention factor (k) versus pH of the mobile phase are related to the influence of an organic modifier on the dissociation of acidic solute. For a few very acidic compounds whose ionization either does not change at all or very little, the change in retention with pH is very small. The retention is slightly higher at acidic pH, indicating that the factor that causes the small shift in retention is partial deionization.
in the acidic mobile phase (20). Furthermore, when the $k$ values calculated in 20% ACN (v/v) medium are examined, it shows that the $k$ values of the compound and its epimer are very different from each other. This shows that the selectivity of the selected HPLC column is high.

Aqueous $pK_a$ values calculated from these two approaches are compatible with each other. In the first described approach, all these straight lines show reasonably satisfactory correlation coefficients ($r^2$) and standard deviations. It can be seen that acidic functional groups have positive slopes. In this study, a plot of the Yasuda–Shedlovsky equation is a straight line. Results obtained by Yasuda–Shedlovsky extrapolation from ACN–water binary mixtures were found to show excellent agreement with those obtained under aqueous medium ($r^2 = 0.99$). Table III shows the Yasuda–Shedlovsky plots of acidic functional group of TCs and epimers, which are characterized by positive slopes. A positive slope is indicative of decreasing acidity with increasing organic solvent concentration in the mixture. Moreover, the slopes of the Yasuda–Shedlovsky plots for compounds of comparable size, possessing the same functional groups, are expected to be broadly similar. In this study, the slope values of studied compounds are close to each other. As a result, the extrapolated aqueous $pK_a$ (eqs 3 and 4) values were found to be in close agreement with the literature $pK_a$ values.

In literature reports, $pK_a$ values for CTC and OTC were calculated by Sanli et al. (11). In this study, $pK_a$ values in different percentages of ACN (10–20%, v/v) and water were determined with RPLC and potentiometric method. In the study conducted by Sanli et al., the HPLC column used was the classic C18 column. Calculated values are not thermodynamic values. Moreover, no good sigmoidal behavior was observed since the pH range studied (2.5–4.5) was limited. However, the results are consistent. In the study by Zrnčić et al. (21), thermodynamic $pK_a$ values for TC, CTC and OTC were determined in the buffer solution, with constant ionic strength of 0.05 M, by capillary electrophoresis method. Data obtained from this study (average value of $pK_a$ 3.59) are compatible with our work. In the study by Stephens et al. (9), TC, CTC and OTC were also determined in aqueous solutions with potentiometric method. Average value of $pK_a$ is 3.29.

The resulting data shows that there are many studies conducted with different methods for the determination of the acid dissociation constant values of studied TCs group antibiotics. The available literature was scanned thoroughly; there were no reported methods for determination of thermodynamic acid dissociation constant values of studied epimers.

**Conclusion**

The present work represents the first study dealing with the RPLC determination of thermodynamic $pK_a$ values of TCs and their epimers at different proportions of ACN–water binary mixtures with pentafluorphenyl phenyl phase containing HPLC column. The determination of the compounds with this column was carried out in a shorter time than the columns containing the conventional porous particles. Additionally, aqueous $pK_a$ values of these compounds were calculated by way of different approaches. The important data extracted from this work can be used for pharmacokinetic and pharmacological studies of these drugs. Moreover, the knowledge of the retention factor values of the drug compounds, in different water–organic solvent media, is a useful parameter to optimize analytical procedures for the separation of ionizable compounds by liquid chromatography.
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