Chemical properties of bile acids. IV. Acidity constants of glycine-conjugated bile acids

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Abstract The dissociation constants for the carboxyl group of a series of glycine (N-acyl)-conjugated and unconjugated bile acids were determined by potentiometric titration using dimethylsulfoxide-water and methanol-water mixtures of varying proportions. The pKₐ values in water were calculated by extrapolating the experimental values determined in different mole fractions of the organic solvent mixtures. The following values were obtained: 3.9 ± 0.1 for glycine-conjugated bile acids and 5.0 ± 0.1 for unconjugated bile acids, as general pKₐ values for the two classes of bile acids, respectively. The amidation of bile acids with glycine lowers the pKₐ value because of the proximity of the amide bond to the terminal carboxyl group. Bile acid dissociation constants are independent of the substituents in the steroid nucleus, since inductive effects of the hydroxyl groups on the steroid nucleus are too distant from the acidic group at the end of the side chain to influence its ionization.

Furthermore, the micelles formed by A⁻ can solubilize the sparingly soluble HA and a simple acid-base equilibrium is not achieved (5). The pKa values determined in these experimental conditions have no thermodynamic meaning, although they have empirical value since they are related to the pH value at which precipitation of the protonated form occurs (6).

EXPERIMENTAL

Materials
Bile acids were gifts or commercial samples of extremely high purity; GCBA in particular were purchased from Calbiochem (La Jolla, CA). Bile acids were further purified by preparative thin-layer chromatography and used as sodium salts. The final purity was assessed by thin-layer chromatography (9) or high-pressure liquid chromatography (10). DMSO and MeOH were of analytical grade and were used without further purification. Solu-

Abbreviations: BA, bile acid; UCBA, unconjugated bile acid; GCBA, glycine-conjugated bile acid; DMSO, dimethylsulfoxide; MeOH, methanol; LR, linear relationship.

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tions were prepared by weight, diluting the solvents with twice-distilled water as appropriate.

METHODS

For potentiometric titration in aqueous DMSO, a Beckman pH 140 potentiometer, equipped with a combined electrode was used. For measurements in aqueous MeOH, a potentiometer Radiometer pHM26 with a saturated calomel and a glass electrode was used. The titration vessel was thermostated at 25.0 ± 0.1°C. Perchloric acid was used as titrant since it is known to be completely ionized and dissociated in these solvent systems (11).

For determination of the ionization constants in the mixed solvent, thermodynamic ionization constant $K_a^*$ for the equilibrium:

$$HA = H^+ + A^- \quad Eq. 1)$$

of a general uncharged acid $HA$ in pure organic solvent or in water organic solvent mixture can be represented by equation 2:

$$K_a^* = \frac{(CH^+ Y^*H^+)(CA^- Y^*A^-)}{C_{HA} Y^*HA} \quad Eq. 2)$$

where the asterisk means that the activity coefficients $y$ refer to infinite dilution in the solvent selected for the determinations and $C$ is molar concentration (12). The activity coefficient of the ionic species can be estimated (13) from the equation 3 (or from similar expressions):

$$\log yH^+ = \frac{Az^{1/2}}{1 + I^{1/2}} + 0.3AzI \quad Eq. 3)$$

where $z$ is the charge of the ion, $I$ is the ionic strength; the

Debye-Hückel function $A$, appropriate to the solvent system, can be calculated from the equation 4:

$$A = \frac{1.825 \times (10^6 \cdot d^{1/2})}{(DT)^{3/2}} \quad Eq. 4)$$

where $T$ is the absolute temperature, $D$ and $d$ are the dielectric constant and the density of the solvent system, respectively. The activity coefficient of the neutral species can be assumed to be unity.

$C_H^+$ value to be inserted in equation 2 can be obtained by the following equation:

$$pa^*H^+ = -\log a^*H^+ = -\log C_H^+ - \log y^*H^+ \quad Eq. 5)$$

where $-\log a^*H^+$, a term related to the hydrogen ion activity, corresponds to the reading of a pH meter, $pH^*$, provided that the apparatus was standardized against buffer of known $pa^*H^+$ in the solvent used for measurements. In water system $pa^*H^+$ equals the pH meter reading, $pH$, in dilute solutions, when the electrodes are adjusted with appropriate aqueous buffer, e. g., phosphate or phthalate buffers, according to Robinson and Stokes (14).

In case of measurements in the water-MeOH system, the potentiometer was standardized with the buffer oxalic acid-ammoniumhydrogen oxalate 0.01 M of known $pa^*H^+$ (15). As a similar buffer for water-DMSO mixtures is not available, the electrodes were calibrated with solutions of $\text{HClO}_4$ of known molarity, $C_H^+$, in each of the mixtures used for measurements and the pH meter readings, $pH^*$, were plotted against calculated $pa^*H^+$ values (see equation 5). Under these experimental conditions the glass electrode is known to be responsive (16).

During each titration the pH meter readings could be related to thermodynamic $pa^*H^+$ values by means of the calibration plot obtained for each composition. The buf-

<table>
<thead>
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<th>Steroid Substituent</th>
<th>MeOH</th>
<th>DMSO</th>
<th>MeOH</th>
<th>DMSO</th>
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<tr>
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<td>4.60</td>
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<td>3α,7α,12α-Trihydroxy GC</td>
<td>4.11</td>
<td>4.30</td>
<td>4.60</td>
<td>4.78</td>
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Mole fraction values ($x$) were obtained from the weight percent values (%) by the following equation (MW, molecular weight):

$x = \frac{\% \times MW \text{ of organic solvent}}{(100 - \% \times MW \text{ of water})}$

*From ref. 8.
Fig. 1. Plot of pKₘ* values in aqueous DMSO (—) and aqueous MeOH (—) for ursodeoxycholic (3α,7β-dihydroxy) (□), chenodeoxycholic (3α,7α-dihydroxy) (●), glycodeoxycholic (3α,12α-dihydroxy) (■), and glycocholic (3α,7α,12α-trihydroxy) (○) acids versus mole fraction (x) of the organic solvent in the mixture.

The initial concentration of the bile salt and the concentration of titrant were 2 • 10⁻³ M in each case. These values are below the critical micellar concentration of the bile salt in pure water (17). Ionic strength of the solution was calculated at any point during the titration.

In these experimental conditions thermodynamic Kₘ* values can be obtained for both mixed solvent systems. Kₘ* are converted to pKₘ* values

\[ pKₘ* = pH* - \frac{Cₘ}{Cₘₐ} \log yₘ*ₘ⁻ \quad \text{Eq. 6} \]

and pKₘ* values are listed in Tables 1 and 2 with an observed mean standard deviation of ±0.02 for experimental determinations and ±0.05 pKₘ units for the extrapolated or estimated values.

RESULTS

Table 1 reports the pKₘ* values determined in aqueous MeOH and DMSO at different concentrations of aqueous solvent. These values fitted in a linear relationship against the mole fraction (x) of the organic solvent in the mixture (Fig. 1). Regression analysis of the pKₘ* values gives the following common equations.

For UCBA:

\[ pKₘ* = 5.07 + 7.70x \quad (r = 0.996; n = 8) \quad \text{DMSO} \quad \text{Eq. 7} \]

\[ pKₘ* = 5.05 + 3.47x \quad (r = 0.992; n = 10) \quad \text{MeOH} \quad \text{Eq. 8} \]

For GCBA:

\[ pKₘ* = 3.81 + 6.85x \quad (r = 0.989; n = 9) \quad \text{DMSO} \quad \text{Eq. 9} \]

\[ pKₘ* = 3.93 + 3.23x \quad (r = 0.994; n = 9) \quad \text{MeOH} \quad \text{Eq. 10} \]

These equations were obtained by fitting together all the pKₘ* values available in Table 1 for each single class of bile acid. A similar equation was previously reported (8), using pKₘ* values in aqueous MeOH of six UCBA. This was possible since it has been shown that pKₘ* values are the same for all C₂₆ bile acids bearing substituents in the steroid nucleus (7). Table 1 and Table 2 confirm that this is true when pKₘ* values of different UCBA and GCBA are compared in the two different solvent mixtures used in this work.

Alternatively, it has been recently reported (18) by our group that a reliable value for the pKₘ in water can also be determined by a potentiometric determination of pKₘ in a mixed solvent at a single composition. Thus, a systematic study carried out on over 100 acids has allowed assessment of a linear relationship between pKₘ* values obtained in DMSO–water (80% w/w) and the experimentally determined pKₘ in water for the same acids. Using such a linear relationship it has been possible to obtain reasonably accurate pKₘ* values in water for a series of sparingly soluble acids (19). Standard deviations were within ±0.05 pKₘ units. The following linear relationship was found:

\[ \text{Fini and Roda Acidity constants of glycine-conjugated bile acids} \]
\[ \text{pK}_a = -0.80 + 0.67 \times \text{pK}_a \text{ (DMSO, 80% w/w)} \quad \text{Eq. 11} \]

By applying this equation to present systems, the pK\(_a\) values reported in Table 2 were obtained (LR column).

The mean pK\(_a\) values obtained by means of this linear relationship were 5.01 for UCBA and 3.83 for GCBA, respectively. The agreement between the values obtained by the two methods can be summarized thus: differences among single values are below 0.1 pK\(_a\) unit, and because of uncertainties present in any type of extrapolation or estimation, this value can be taken as very satisfactory. Therefore the following values, 5.0 ± 0.1 and 3.0 ± 0.1, can be confidently accepted as general pK\(_a\) values in water for UCBA and GCBA, respectively. These values agree satisfactorily in some cases with those obtained from precipitation curves of some UCBA and GCBA, respectively (6).

The bile acids studied show the same susceptibility of the acidity to the change in the composition of the mixed solvent; the two lines in Fig. 1 relative to UCBA and GCBA have about the same slope when the solvent is the same. On the other hand, a different behavior was observed toward the two mixed solvents by both acid systems, as shown by the different values of the slopes. Table 2 reports pK\(_a\) values determined in aqueous DMSO (80% w/w) and in aqueous MeOH (50% w/w) for six UCBA and GCBA; the pK\(_a\) (w) (LR) values estimated in water were calculated by means of equation 11. pK\(_a\) (w) Values quoted as extrapolated (extr.) were obtained by means of the linear relationship between pK\(_a^*\) and the mole fraction of the organic solvent in the mixture (see equations 7–10).

The estimation of the pK\(_a\) values in water from potentiometric measurements in mixed solvent has been the object of many studies (20). The mixed solvent offers a suitable tool to obtain pK\(_a\) values of acids sparingly soluble in water and, under clearly assessed experimental conditions, they may have a thermodynamic meaning.

In the case of bile acids, use of a mixed solvent removes not only the problems due to solubility but also those deriving from micelle formation. From Tables 1 and 2 it is clear that hydroxy substituents do not affect the ionization constant for either of the mixed solvents or any of the bile acids which were studied. Small differences observed are less than the experimental error and even less in some cases.

The pK\(_a\) values extrapolated to \(x = 0\), i.e., pure water, for both solvent systems are given in Table 2 as pK\(_a\)(w) extr. together with pK\(_a\) values obtained by the linear relationship (equation 11). The agreement between the two sets of mixed solvents obtained by the two methods was satisfactory.

It is well known that steroidal hydroxyls influence many chemical and physical properties of bile acids, such as UCBA solubility (21–23), critical micellar concentration values, (17), and interaction with human serum protein (24) or calcium ions (25–27). In general, steroidal hydroxyls affect the hydrophilic-hydrophobic balance on the bile

<table>
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<th>Steroid Substituent</th>
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<th>pK(_a^*)</th>
<th>pK(_a)(w)</th>
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<tr>
<td></td>
<td>DMSO</td>
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<td>LR(^a)</td>
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<td>Acetylglicine</td>
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<td>3.79</td>
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\(^{a}\)Values obtained by equation 11.

\(^{b}\)Values obtained by means of the pK\(_a^*\) versus DMSO mole fraction linear relationship (see equations 7 and 9).

\(^{c}\)Values obtained by means of the pK\(_a^*\) versus MeOH mole fraction linear relationship (see equations 8 and 10).
acid molecules and ions and therefore all the related properties. It has already been observed that steroidal substituents do not affect the ionization constants of bile acids (7, 8). In fact, inductive effects operating along saturated alkyl chains decline rapidly at a distance from the reaction center; therefore only functional groups close to the carboxyl group can significantly affect acidity. Thus, the UCBA behavior resembles that of pentanoic acid, bearing an alkyl sterol group in the γ position. Hence, a pKₐ for UCBA that differs by only about 0.1 unit from the pKₐ of pentanoic acid is not astonishing.

For the bile acid with glycine (GCBA), the insertion of an acid-strengthening peptide group in the side chain of a bile acid increases the ionization constant, as would be expected. Thus, amidation of a GCBA lowers the pKₐ value by about 1.1 units, because of the inductive effect of the acid-strengthening peptide group in the side chain of pentanoic acid is not astonishing.

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References