# 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 $pK_a$ 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: $3.9 \pm 0.1$ for glycine-conjugated bile acids and $5.0 \pm 0.1$ for unconjugated bile acids, as general $pK_a$ values for the two classes of bile acids, respectively. The amidation of bile acids with glycine lowers the $pK_a$ 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.—**Fini, A., and A. Roda.** Chemical properties of bile acids. IV. Acidity constants of glycine-conjugated bile acids. *J. Lipid Res.* 1987. **28:** 755–759.

**Supplementary key words** thermodynamic acidity constant • unconjugated bile acids • $pK_a$ values

The ionization constant of acidic endo- and xenobiotics is a physicochemical constant of recognized utility in clinical, analytical, and pharmaceutical research. This value, which is usually converted to a $pK_a$ value, can be used to predict the relationship between solubility and pH in aqueous solution and also influences the bioavailability of a drug (1, 2).

Because of their physiological importance, bile acids have been studied extensively, but to date only a few data are reported in the literature on their physicochemical properties in water (3). This scarcity of physicochemical characterization of bile acids is in part related to their properties in aqueous solution. Thus, the protonated forms of bile acids (HA) are generally rather insoluble, whereas the fully ionized forms ($A^-$) self-associate over a narrow concentration range to form multimers or micelles. As a consequence of the formation of this self-association, the $pK_a$ values of bile acids have been found to vary markedly as a function of the concentration of the ionized species (4).

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).

To circumvent the solubility problems and micelle-forming properties of bile acids, a potentiometric method in aqueous methanol has been used (7) to provide thermodynamic $pK_a^*$ values in the mixed solvent and reliable $pK_a$ in water for a series of unconjugated bile acids (UCBA) (8).

The determination of $pK_a^*$ values of a series of glycine-conjugated bile acids (GCBA) is presented in this report by means of the above mentioned method, both in aqueous DMSO and MeOH. The $pK_a$ values in water, which were determined by extrapolation, were compared with those obtained by a simpler method based on potentiometric measurement in mixed solvent of a single composition.

## 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:

$$HA \rightleftharpoons H^+ + A^- \qquad \textit{Eq. 1)}$$

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

$$Ka^* = \frac{(C_{H+}\, y^*_{H+}) \bullet (C_{A-}\, y^*_{A-})}{(C_{HA}\, y^*_{HA})} \qquad \textit{Eq. 2)}$$

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):

$$\log y_{H^+} = \frac{AzI^{1/2}}{1 + I^{1/2}} + 0.3AzI \qquad \textit{Eq. 3)}$$

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:

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

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:

$$pa^*_{H^+} = -\log a^*_{H^+} = -\log C_{H^+} - \log y^*_{H^+} \qquad \textit{Eq. 5)}$$

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 $HClO_4$ 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 1. $pK_a^*$ Values in aqueous MeOH and aqueous DMSO at different compositions for two unconjugated (UC) and two glycine-conjugated (GC) bile acids at 25°C

| Steroid Substituent | Solvent System | Weight Percent[a] 10 | 20 | 30 | 40 | 50 | 70 | 80 |
|---|---|---|---|---|---|---|---|---|
| 3α,7β-Dihydroxy UC | MeOH[b] | 5.24 | 5.56 | 5.68 | 6.00 | 6.28 | | |
| 3α,7β-Dihydroxy UC | DMSO | | | 5.70 | | 6.51 | 7.92 | 8.68 |
| 3α,7α-Dihydroxy UC | MeOH[b] | 5.25 | 5.52 | 5.63 | 6.04 | 6.34 | | |
| 3α,7α-Dihydroxy UC | DMSO | | | 5.69 | | 6.53 | 7.92 | 8.62 |
| 3α,12α-Dihydroxy GC | MeOH | | 4.28 | 4.63 | 4.08 | 5.08 | | |
| 3α,12α-Dihydroxy GC | DMSO | | | 4.26 | | 5.31 | 6.28 | 6.98 |
| 3α,7α,12α-Trihydroxy GC | MeOH | 4.11 | 4.30 | 4.60 | 4.78 | 5.08 | | |
| 3α,7α,12α-Trihydroxy GC | DMSO | | | 4.25 | | 5.25 | 6.38 | 6.98 |

[a]Mole fraction values (χ) were obtained from the weight percent values (%) by the following equation (MW, molecular weight):

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

[b]From ref. 8.

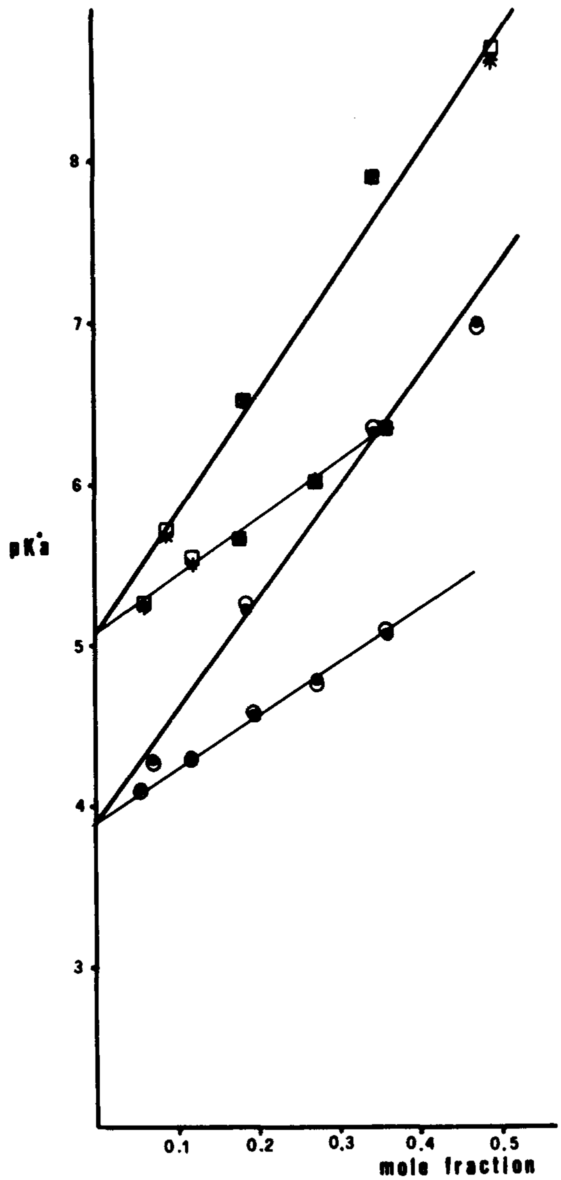


**Fig. 1.** Plot of $pK_a^*$ values in aqueous DMSO (——) and aqueous MeOH (——) for ursodeoxycholic (3α, 7β-dihydroxy) (□), chenodeoxycholic (3α,7α-dihydroxy)(*), glycodeoxycholic (3α,12α-dihydroxy) (●), and glycocholic (3α,7α,12α-trihydroxy) (○) acids versus mole fraction (χ) of the organic solvent in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

fer ratio $C_{A^-}/C_{HA}$ was calculated from the stoichiometric composition of the solutions during the titration with standard $HClO_4$ solution in the mixed solvent under investigation.

The initial concentration of the bile salt and the concentration of titrant were $2 \cdot 10^{-3}$ 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 $K_a^*$ values can be obtained for both mixed solvent systems. $K_a^*$ are converted to $pK_a^*$ values

$$pK_a^* = pH^* - \frac{C_{A^-}}{C_{HA}} - \log y^*_{A^-} \qquad \textit{Eq. 6)}$$

and $pK_a^*$ values are listed in Tables 1 and 2 with an observed mean standard deviation of $\pm 0.02$ for experimental determinations and $\pm 0.05$ $pK_a$ units for the extrapolated or estimated values.

## RESULTS

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

For UCBA:

$$pK_a^* = 5.07 + 7.70\chi \ (r = 0.996;\ n = 8)\ (DMSO) \qquad \textit{Eq. 7)}$$

$$pK_a^* = 5.05 + 3.47\chi \ (r = 0.992;\ n = 10)\ (MeOH) \qquad \textit{Eq. 8)}$$

For GCBA:

$$pK_a^* = 3.81 + 6.85\chi \ (r = 0.989;\ n = 9)\ (DMSO) \qquad \textit{Eq. 9)}$$

$$pK_a^* = 3.93 + 3.23\chi \ (r = 0.994;\ n = 9)\ (MeOH) \qquad \textit{Eq. 10)}$$

These equations were obtained by fitting together all the $pK_a^*$ values available in Table 1 for each single class of bile acid. A similar equation was previously reported (8), using $pK_a^*$ values in aqueous MeOH of six UCBA. This was possible since it has been shown that $pK_a^*$ values are the same for all $C_{24}$ bile acids bearing substituents in the steroid nucleus (7). Table 1 and **Table 2** confirm that this is true when $pK_a^*$ 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 $pK_a$ in water can also be determined by a potentiometric determination of $pK_a^*$ 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 $pK_a^*$ values obtained in DMSO-water (80% w/w) and the experimentally determined $pK_a$ in water for the same acids. Using such a linear relationship it has been possible to obtain reasonably accurate $pK_a$ values in water for a series of sparingly soluble acids (19). Standard deviations were within $\pm 0.05$ $pK_a$ units. The following linear relationship was found:

$$pK_a = -0.80 + 0.67\ pK_a\ (\text{DMSO, } 80\%\ \text{w/w}) \quad \textit{Eq. 11)}$$

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, $5.0 \pm 0.1$ and $3.0 \pm 0.1$, 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).

## DISCUSSION

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 $\chi = 0$, 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 2. $pK_a^*$ Values for six unconjugated (UC) and glycine-conjugated (GC) bile acids determined in 80% w/w aqueous DMSO and 50% w/w aqueous MeOH, and $pK_a$ values in water determined by two different means at 25°C

| | | $pK_a^*$ | | $pK_a$(w) | | |
|---|---|---|---|---|---|---|
| Steroid Substituent | Bile Acid | DMSO | MeOH | LR[a] | Extr.[b] | Extr.[c] |
| 3α,-Hydroxy | UC | 8.70 | 6.30 | 5.03 | | |
| 3α,-Hydroxy | GC | 6.92 | 4.99 | 3.84 | | |
| 3α,7β-Dihydroxy | UC | 8.68 | 6.28 | 5.02 | 5.05 | 5.08 |
| 3α,7β-Dihydroxy | GC | 6.95 | 4.99 | 3.86 | | |
| 3α,7α-Dihydroxy | UC | 8.62 | 6.34 | 4.98 | 5.08 | 5.03 |
| 3α,7α-Dihydroxy | GC | 6.97 | 5.10 | 3.87 | | |
| 3α,12α-Dihydroxy | UC | 8.68 | 6.26 | 5.02 | | |
| 3α,12α-Dihydroxy | GC | 6.98 | 5.08 | 3.88 | 3.81 | 3.93 |
| 3α-Hydroxy 7 keto | UC | 8.65 | 6.34 | 5.00 | | |
| 3α-Hydroxy 7 keto | GC | 7.00 | 5.06 | 3.89 | | |
| 3α,7α,12α-Trihydroxy | UC | 8.66 | 6.30 | 5.00 | | |
| 3α,7α,12α-Trihydroxy | GC | 6.98 | 5.08 | 3.88 | 3.81 | 3.93 |
| Pentanoic acid | | 8.44 | 6.02 | 4.85 | (experimental | 4.83) |
| Acetylglicine | | 6.85 | | 3.79 | (experimental | 3.79) |

[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 $\gamma$ position. Hence, a $pK_a$ for UCBA that differs by only about 0.1 unit from the $pK_a$ 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 carbonyl group of the amide bond. The effect of the substituent agrees well with the reported acid strengthening effect of the CONH group in terms of $\Delta pK_a$, i.e., 1.12(28).

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