Relative acidity scale of bile acids through ESI-MS measurements†

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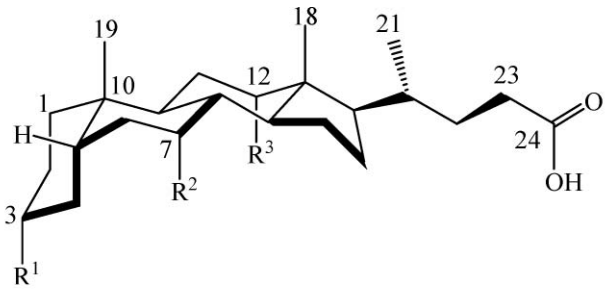
The anion proton affinity of the most important human bile acids and those of the corresponding keto bile acids have been examined in order to establish a true (intrinsic) relative acidity scale. The measurements have been carried out in the gas-phase using the Cooks' kinetic method. The remarkably high acidity of cholic acid with respect to the other bile acids was confirmed. Rationalization of the differences found for the various acids and comparisons with the available solution-phase data are discussed with the help of theoretical calculations.

Introduction

The bile acids are a group of steroid-based acidic compounds produced from cholesterol that possess important pharmaceutical applications related to their ability to dissolve cholesterol gallstones and for the treatment of bile acid deficiency and cholestatic liver diseases.[1,2] Bile acids, their conjugates and bile salts are natural products that represent 67% of the soluble components of bile. The most abundant mammalian bile acids are derivatives of cholanic acid (5β-cholan-24-oic acid) **1** (Scheme 1), and in humans these consist mainly of cholic acid **2** and chenodeoxycholic acid **3**, in the forms of glycine and taurine conjugates.[1,3] The bile acid group also includes conjugates of deoxycholic acid **4** and lithocholic acid **5**, commonly known as secondary bile acids, produced from cholic and chenodeoxycholic acids by intestinal bacteria dehydroxylation.[3]

Most of the physical chemical data available for these molecules have been obtained in water solutions, that is in conditions as close as possible to a physiological medium.[2] This aspect, however, represents the problem and the limit of the information, since bile acids are poorly soluble in water contrary to the corresponding salts that, in addition, self-aggregate to form micelles.[4] The consequence is that in non-conventional conditions extrapolation of the experimental data must be used. This is the reason why, for example, acidity measurements appear as a set of scattered values as for those of cholic acid, with pK_a values ranging from 4.98 to 5.6 and up to 6.3, depending on the different experimental conditions adopted.[4] These observations prompt us to bypass the use of the solvent, shifting the measurements to gas-phase conditions,[5] in order to establish an intrinsic relative acidity scale of the most important bile acids, not affected by the drawbacks discussed above. Once the various bile acids are ordered and the intrinsic acidity differences explained, using for example geometry optimization or other theoretical calculations, also the effect of the solvent and that of the self-aggregation could be understood. Gas-phase basicities (GB) and proton affinities (PA) of a molecule, in fact, are considered very useful in providing insights into molecular structure, stability and Brønsted acid–base reactivity.[6] Proton affinities, defined as the negative of the enthalpy change ($-\Delta H$) for the reaction:[7] $A^- + H^+ \rightarrow HA$, are available by measuring the equilibrium constant for a reverse proton-transfer reaction to a second base, as a function of the temperature.[6,8] A closely related procedure for ordering PA values is the bracketing method.[8,9] In this case the occurrence or non-occurrence of proton transfer from a series of compounds of known proton affinities to an unknown sample indicates whether the unknown compounds has a higher or lower PA than the reference derivatives.

Another alternative is the Cooks' kinetic method, based on the rates of competitive dissociation of mass-selected cluster ions.[10,11]



	R^1	R^2	R^3
cholanic acid **1**	H	H	H
cholic acid **2**	OH	OH	OH
chenodeoxycholic acid **3**	OH	OH	H
deoxycholic acid **4**	OH	H	OH
lithocholic acid **5**	OH	H	H
ursodeoxycholic acid **6**	OH	β-OH	H
3-ketocholanic acid **7**	=O	H	H
3,7-diketocholanic acid **8**	=O	=O	H
3,12-diketocholanic acid **9**	=O	H	=O
3,7,12-triketocholanic acid **10**	=O	=O	=O

Scheme 1 Structures of the most important bile acids and derivatives.

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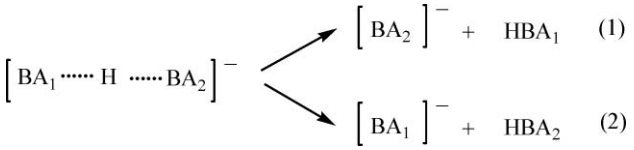
† Electronic supplementary information (ESI) available. Mass spectra for the fragmentation of the proton bound dimers investigated in this study.

For proton affinities the experiment involves the competitive fragmentation of proton-bound dimers $[BA_1 \cdots H \cdots BA_2]^-$ formed by a proton and the two compounds under examination, in our case two different bile acid anions BA_1^- and BA_2^-, according to Scheme 2.



Scheme 2 Relative proton affinities by Cooks' kinetic method.

If the relative abundance of the peak pertaining to BA_1^- is higher with respect to BA_2^-, this species possesses a lower proton affinity with respect to BA_2^-.

In this paper we report the determination of the anion proton affinity relative scale of the most abundant human bile acids, and a comparison of these data with carboxylic acid anions of known proton affinity, in order to bracket the different bile acids in a relatively narrow PA range. In a second set of experiments a proton affinity relative scale is given also for keto bile acid derivatives, detected as metabolites in man and easily prepared from the corresponding hydroxyl derivatives.[3] Rationale of the differences found for the various compounds and comparison with the data available in solution are discussed, also supported by theoretical calculations.

Results and discussion

The electrospray ionization mass spectrum (ESI-MS) of a bile acid (HBA) in negative-ion mode is characterized by the presence of the deprotonated molecular ion $[BA]^-$ of the proton-bound dimer formed by two deprotonated molecular ions loosely bonded to a proton $[(BA) \cdots H \cdots (BA)]^-$ and by little fragmentation. If two different bile acids (HBA_1, HBA_2) are simultaneously introduced into the mass spectrometer formation of two homo proton-bound dimers and of the hetero proton-bound dimer $[BA_1 \cdots H \cdots BA_2]^-$ are observed, as shown in Fig. 1. Mass selection of this latter

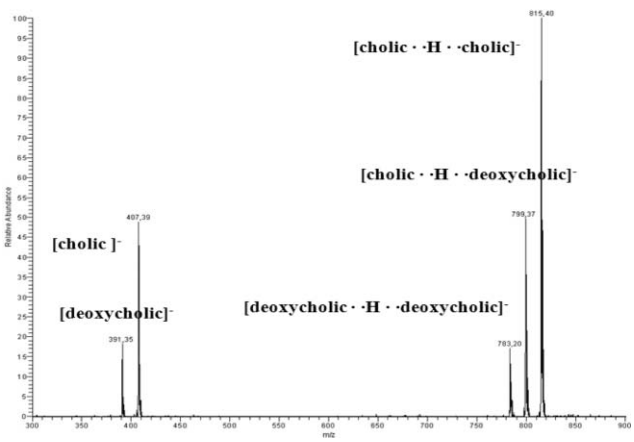


Fig. 1 ESI-MS mass spectrum in negative-ion mode of an equimolar mixture of cholic and deoxycholic acids. Homo proton-bound dimers are observed at m/z 783 and 815, respectively, the hetero proton-bound dimer is detected at m/z 799.

Table 1 Results of the MS/MS dissociation of proton-bound dimers. The relative intensity of the different anions has been established by using palmitic acid as reference (value 1)

Acid	Anion rel. intensity	PA/kJ mol^{-1}[a]
phocaecholic	60	
4-nitrobenzoic	50	1373
cholic **2**	28	
2,4,6-trimethylbenzoic	16	1418
deoxycholic **4**	10	
4-methoxybenzoic	3	1426
chenodeoxycholic **3**	2	
ursodeoxycholic **6**	1.8	
palmitic	1	1459[b]
lithocholic **5**	0.9	
cholanic **1**	0.8	

[a] Proton affinities from reference 12. [b] Obtained by theoretical calculations.

ionic species, followed by fragmentation according to Scheme 2, estimates the relative intrinsic bond strength toward the proton of BA_1^- *vs.* BA_2^-.[10,11]

A typical MS/MS experiment for a mixture of lithocholic and chenodeoxycholic acids is shown in Fig. 2. The proton bound-dimer at m/z 767 is mass-selected and submitted to collision-induced dissociation. The chenodeoxycholate anion (m/z 391) is lost preferentially since its proton affinity is lower than that of the lithocholate anion (m/z 375).

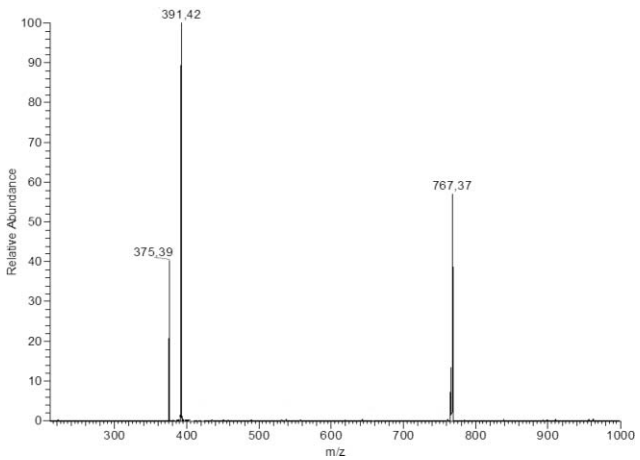


Fig. 2 Fragmentation of mass-selected proton bound-dimer (m/z 767) of lithocholic and chenodeoxycholic acid affording lithocholate (m/z 375) and chenodeoxycholate (m/z 391) anions.

Based on the relative abundances of the fragment ions originating from the MS/MS decompositions of a large number of combinations of different bile acids, it was possible to infer the qualitative order of proton affinities as: (higher) **1** > **5** > **3** > **4** ≫ **2** (lower). The pertinent results are collected in Table 1. The bile acids investigated have been compared with carboxylic acids (reference acids) of known proton affinity such as 4-nitrobenzoic, 4-methoxybenzoic, 2,4,6-trimethylbenzoic acids[12] and with the PA calculated for palmitic acid, see experimental section for details. The proton affinity range of the reference compounds is difficult to extend due to the absence of suitable reference bases that possess, in addition, a high molecular weight.[13] The measurements were carried out by comparing pairs of bile acids, of reference

Table 2 Results of the MS/MS dissociation of proton-bound dimers. The relative intensity of the different anions has been established by using palmitic acid as reference (value 1)

Acid	Anion rel. intensity	PA/kJ mol^{-1} [a]
2,4,6-trimethylbenzoic	18	1418
3,7,12-triketo **10**	14	
3,12-diketo **9**	7	
3,7-diketo **8**	6	
3-keto **7**	2	
palmitic	1	1459[b]
cholanic **1**	0.8	

[a] Proton affinities from reference 12. [b] Obtained by theoretical calculations

acids and of bile acids with reference acids of similar proton affinity.[14] Palmitic acid was mixed with cholanic, lithocholic, ursodeoxycholic, chenodeoxycholic and 4-methoxybenzoic acid, respectively, assigning to its anion the relative intensity value of 1. 4-Methoxybenzoic acid (anion relative intensity 3, as obtained from previous measurements) was then used as reference in mixtures with deoxycholic and 2,4,6-trimethylbenzoic acids, respectively. 2,4,6-Trimethylbenzoic acid was used with cholic acid. Cholic acid was used in mixtures with 4-nitrobenzoic and phocaecholic acids, respectively. Additional checks were carried out by further crossing: lithocholic with cholanic acid; 4-methoxybenzoic acid with ursodeoxycholic and chenodeoxycholic acids *etc*.

The experiments indicate a PA higher than 1459 kJ mol^{-1} for bile acids lacking of hydroxyl functions or with one hydroxyl group, whereas bile acids with two hydroxyl substituents lay in the range 1418–1459 kJ mol^{-1}. The set of the dihydroxylated bile acids has been completed with ursodeoxycholic acid **6**, the 7β-hydroxyl epimer of chenodeoxycholic acid, the two compounds have a similar proton affinity.

The overall picture of the relative acidity of the bile acids has been extended to keto-cholanic acids, a group of molecules characterized by the presence of a single keto function, 3-keto-cholanic acid **7**, by the occurrence of two carbonyl groups in position 3,7 (**8**) and 3,12 (**9**) and by the 3,7,12-triketo-cholanic acid derivative (dehydrocholic acid, **10**), Scheme 1. As shown in Table 2 the keto cholanic acids also follow the trend: the higher number of substituents the lower the PA value, with all the derivatives grouped in a narrower range (1418–1459 kJ mol^{-1}) with respect to the hydroxyl derivatives **2–6**.

From a comparison of the data reported in Tables 1 and 2 the position of deoxycholic **4** and cholic **2** acids is peculiar and deserves some comment. Deoxycholic acid exhibited a PA value bracketed between that of 4-methoxybenzoic acid (1426 kJ mol^{-1}) and that of 2,4,6-trimethylbenzoic acid (1418 kJ mol^{-1}), much lower than the corresponding dihydroxyl homologous **3** and **6**. A likely explanation may be found in the role played by the hydroxyl group in position 12, able to stabilize the carboxylate anionic function by distal hydrogen bond assistance. Support to this assumption may be found in DFT calculations carried out at the GGA/DNP level of theory, using the DMol^3 module of Materials Studio system of programs.[15,16] The equilibrium geometries of chenodeoxycholic acid and its anion, and deoxycholic acid and its anion in both hydrogen-bonded and extended conformations, have been obtained. According to the calculations, the anion of **4**

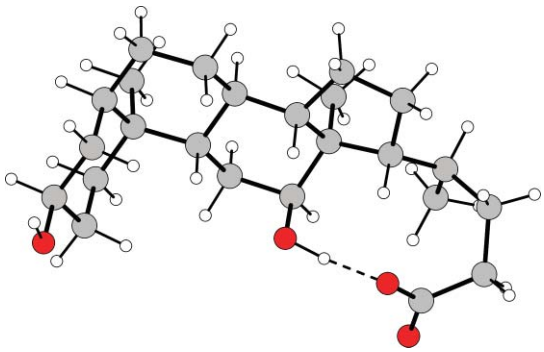


Fig. 3 Optimized geometry of the dehoxycholate anion, showing the intramolecular hydrogen bond (dashed line).

can be stabilized 'in vacuum' by the formation of an intramolecular O–H · · · O hydrogen bond, as shown in Fig. 3. Actually, the energy of this conformation is some 46 kJ mol^{-1} (11 kcal mol^{-1}) lower than the energy of the 'extended' conformation *i.e*. without any intermolecular interaction; moreover, the acid/anion difference in energy for deoxycholic acid is quite low compared to the calculated difference for the same couple of **3**: 1392.14 and 1463.08 kJ mol^{-1}, respectively.

Also the acidity of cholic acid is remarkable high, its PA value being bracketed in between that of 2,4,6-trimethylbenzoic acid and 4-nitrobenzoic acid (1373–1418 kJ mol^{-1}). A possible explanation may be found taking into account two converging effects: i) the presence of three hydroxyl groups on the molecule and ii) the distal intermolecular hydrogen bond assistance, as in the case of deoxycholic acid. Note that phocaecholic acid (3α,7α,23-trihydroxy-5β-cholan-24-oic acid),[17] a natural bile acid isolated from the bile of snakes, seals and other marine mammals, characterized by the presence of a hydroxyl substituent on the lateral chain at position C(23) is even more acidic, the value being comparable to that of 4-nitrobenzoic acid, Table 1. The strong electron-withdrawing effect of the OH group on the lateral chain and the hydrogen bond effect with the carboxylate function, Fig. 4, should explain these findings both in the gas-phase and in solution.[17]

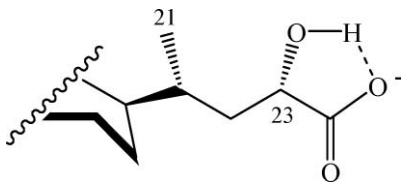


Fig. 4 Hydrogen bond effect of the anion of phocaecholic acid.

The acidity of bile acids in solution has been re-examined in an attempt to clarify the conflicting data accounted in the literature.[4] In the classical review on bile acid chemistry and physiology the pK_a reported for cholic, deoxycholic and chenodeoxycholic acid was 4.98, 5.30 and 5.88 respectively.[1] Other more recent studies indicate very similar values for cholic, chenodeoxycholic and deoxycholic acids.[4,17,18] The results obtained in the gas-phase suggest that the mentioned acids should have a significant difference also in solution, due to the strong intramolecular interaction of the hydroxyl group in position 12 with the carboxylate anion, occurring with cholic and deoxycholic acids.

Conclusions

The most abundant mammalian bile acids have been ordered with respect to anion proton affinity in the gas-phase, bypassing the occurrence of self-aggregation and micellar formation characterizing the solution-phase. The intrinsic differences found for the various bile acids have been explained with the help of theoretical calculations. The role of specific hydroxy functions, able to form intramolecular hydrogen bonds, have been highlighted. Further studies on the gas-phase properties of bile acids toward metal cation affinities are underway.

Experimental section

The bile acids use in this study are commercially available derivatives or easily prepared from the corresponding hydoxyl derivatives by simple oxidation. Full characterization of **1–10** may be found in the literature.[17–19] ESI mass spectra were obtained using a LCQ Duo (ThermoQuest, San Jose, CA, USA), in negative-ion mode, by introducing a 1 : 1 mixture of two selected bile acids dissolved in methanol (10^{-3} M). Instrumental parameters: capillary voltage –10 V, spray voltage 4.50 kV, capillary temperature of 150 °C, mass scan range was from *m/z* 100 to 1000 amu, for 30000 ms scan time; N_2 was used as sheath gas. MS/MS mass spectra of hetero proton-bound dimers were performed upon isolation of $[BA_1 \cdots H \cdots BA_2]^-$ ionic species and application of normalized collision energies from 10 to 25% of the instrument maximum. The samples were injected into the spectrometer by a syringe pump at a constant flow rate of 8 μL min^{-1}. The measurements were carried our by comparing couples of bile acids, of reference acids and of bile acids with reference acids of similar proton affinity.

The fully optimized geometry of deoxycholic and chenodeoxycholic acids, and their anions, besides the proton affinity of the palmitic acid, have been obtained by density functional theory (DFT) calculations. The proton affinity of the palmitic acid is the negative of the enthalpy change of the following reaction in gaseous phase (at standard conditions):

$$\text{palmitate}^- (g) + H^+ (g) \rightarrow \text{palmitic acid (g)}$$

This quantity has been have been evaluated according to the approximate formula:

$$PA = \Delta Eel + \Delta ZPV E + \Delta Evib(T) + 5/2RT$$

where ΔEel, ΔZPV E, ΔEvib are the differences between the total electronic energy, the zero point vibrational and the temperature-dependent portion of vibrational energy of the base molecule and its protonated form at 298 K, respectively. ΔEvib has been considered negligible compared to the zero point energy ZPE. All DFT calculations were carried out at the GGA/DNP level of theory, using the DMol³ module of Materials Studio system of programs (Materials Studio Version 4.4, Accelrys Software Inc., 2008).[13,14]

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