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Noninvasive methods to determine the critical micelle concentration of some bile acid salts

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Abstract

In this work the critical micelle concentrations (*cmc*) of four bile salts, sodium cholate, sodium glycocholate, sodium deoxycholate, and sodium glycodeoxycholate, are determined and presented. Three independent noninvasive methodologies (potentiometry, derivative spectrophotometry, and light scattering) were used for *cmc* determination, at 25 °C with ionic strength adjusted to 0.10 M with NaCl. Spectrophotometric and potentiometric studies of some bile salts were also executed at various ionic strength values, thus allowing the influence of the ionic strength on the *cmc* value of the bile salt to be assessed. A critical comparison of the *cmc* values obtained with data collected from the literature is presented. Furthermore, this work makes an evaluation of the conceptual bases of different methodologies commonly used for *cmc* determination, since variations in the results obtained can be related mainly to different intrinsic features of the methods used (such as sensitivity or the need to include tracers or probes) or to the operational *cmc* definition applied. The undoubted definition of the experimental bile salt concentration that corresponds to *cmc* (operational *cmc*) is essential since in the case of these amphiphiles the formation of micelles is not as abrupt as in the case of ordinary association colloids. The biphasic nature of their aggregation leads to a "round-shaped" variation of the experimental parameters under analysis, which makes difficult the evaluation of the *cmc* values and can be responsible for the different results obtained.
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Bile salts are the final products of the hepatic biotransformation of cholesterol and play important roles in the gastrointestinal absorption of lipo-soluble compounds and in their transport through the aqueous compartments of humans [1]. These amphipatic compounds are present in bile as mixed micelles that serve to transport additional cholesterol from the liver into the intestine [2,3].

The physiological and therapeutic properties of some bile salts [4] and of some structurally similar synthetic substances such as solubilizing agents [5,6] are essentially due to their ability to form both simple and mixed micellar aggregates, which facilitate the dissolution and transportation of lipo-soluble molecules. In this context, the critical micelle concentration (*cmc*)[1] is a fundamental parameter in the evaluation of the biological activity of bile salts.

The *cmc* of a surfactant is defined as the solute concentration at which micelles first appear in solution and,

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[1] *Abbreviations used:* cmc, critical micelle concentration; R, Rayleigh scattering band; RP, reversed-phase.

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in practical terms, is related to appreciable changes in such phenomena as light scattering, surface tension, or solubilization of other organic molecules [6–8]. These changes in the usual behavior of the substances can be explained by the existence of micellar aggregates in solution and are the bases of the analytical processes that are used to determine the *cmc*. The model usually applied to describe bile salt association invokes a stepwise aggregation mechanism [8], where the *cmc* appears over a concentration range, rather than at a sharp value.

Many different techniques are used for determination of bile salt *cmc*, such as surface tension [1,4,8–12], reversed-phase (RP) high-performance liquid chromatography [7], small-angle X-ray scattering [8,13], electron paramagnetic resonance (EPR) [4,9,11], nuclear magnetic resonance (NMR) [2,8,9], light scattering and refractometry [4,8–11,14–16], microcalorimetric titration [9,14], spectrophotometry [3,4,8,9,17], and fluorescence probes [2,9,18,19].

In Table 1 the *cmc* values described in the literature for various bile salts (sodium cholate, sodium glycocholate, sodium deoxycholate, and sodium glycodeoxycholate) are listed.

Analysis of the values shown in Table 1 reveals that results obtained for the same micellar system differ dramatically, not only between methods used but also within a given method. Furthermore, the methodologies used for the determination of the *cmc* can be divided into two groups according to whether it is necessary to use exogenous molecular probes or tracers. These probes usually consist of substances that are poorly soluble in water; their solubilities increase by micellar incorporation, which allows the determination of the *cmc* value. Unlike the methods that involve only micelles, the methods that use probes originate, at least, a second equilibrium in solution between the detergent and the molecule that is used as indicator, which can disturb the hydrophobic interactions between the detergent molecules. Hence, this class of methods is usually described as invasive. By noninvasive we mean, obviously, that no probes or tracers were involved in the methodologies presented; therefore the micellar system is composed only of the detergent. Although it has been demonstrated with sodium lauryl sulfate [7] that if the probe is fairly insoluble in water the disturbance will be minimal, this factor should not be neglected.

In this work the critical micelle concentrations of four bile salts, sodium cholate, sodium glycocholate, sodium deoxycholate, and sodium glycodeoxycholate, were determined by three different noninvasive techniques: potentiometry, derivative spectrophotometry, and light scattering. The experiments were performed at 25 °C with an ionic strength of 0.10 M in NaCl, unless otherwise stated.

From the variation of the corresponding apparent pK_a (pK_{app}), the spectral characteristics or the light-scattered values with different concentrations of bile salt, it was possible to determine the *cmc* value of the aforementioned bile salts.

The potentiometric and the spectrophotometric studies were also executed at various ionic strength values (0, 0.05, 0.10, 0.15, and 0.20 M in NaCl), thus allowing the influence of this parameter in the pK_{app} values and in the bile salts' *cmc* to be assessed.

The processes described in this paper are quick and simple and require no sample purification, tracers, or probes. The values obtained with the three noninvasive techniques used are in good agreement and are close to the literature values determined by other methods, in which the operational *cmc* has the same definition.

In addition to proposing the three noninvasive methods, this paper gathers some literature data so that a comparison of the published *cmc* values can be made, which can reveal enormous differences in the research results available.

Materials and methods

Reagents and solutions

The bile acids (sodium salts) were obtained from Sigma and used as received. All other chemicals were from Merck (grade *pro analysis*); solutions were prepared with double-deionized water (conductivity less than 0.10 μS/cm). Bile acid solutions were prepared by rigorous dilution of a stock solution of known concentration value that has been determined by conductimetric titration with HCl 0.10 M (Merck; *Titrisol*) added by means of a Crison 2031 piston buret and using a Crison Micro CM 2202 conductivity meter. The ionic strength was adjusted to 0.10 M with NaCl, unless otherwise stated.

Potentiometric determination of acidity constants

All potentiometric measurements were carried out with a Crison 2002 pH meter and 2031 buret controlled by a personal computer which was also used for data manipulation. The electrode assembly was made up of an Orion 900029/4 AgCl/Ag reference electrode and a Russel SWL glass electrode. System calibration was performed by the Gran [32] method with regard to hydrogen ion concentration, using strong acid/strong base titration [HCl (0.001 M)/NaOH (≈0.02 M)] with solutions whose ionic strengths were adjusted to be identical to that of the titrand. Titrations were always carried out under a nitrogen atmosphere at 25 °C in a double-walled glass cell.

Acidity constants for the bile salts were obtained by titrating 20.00 ml of aqueous solutions of the bile salts (0.8–30 mM) with HCl (0.10 or 0.01 M). Determinations

Table 1
Critical micelle concentration values (mM) for some bile acids reported in the literature

Method[a]	Experimental conditions	NaC[a]	NaGC[a]	NaDC[a]	NaGDC[a]
Pot	25 °C; pH 8.5; in Sudan III [17]	9.0			
	25 °C [20]	11.0	4.0		
	25 °C; pH 10.0; $I = 0.15$ M (NaCl) [21]	2.3			
LS	25 °C [10]		13.8		
	25 °C [22]; $I = 0$ M				2.12
	$I = 0.15$ M (NaCl)				1.10
	$I = 0.50$ M (NaCl)				0.74
	pH 9; $I = 0.10$ M (NaCl) [1]			2.5–3	
S	25 °C; tracer: naphthalene [23][b]	1.6; 2.0			
	pH 7; $I = 0.15$ M (NaCl); tracer: OT Orange [24][b]	7.8		2.3	
	30 °C; pH 7.8 (borate); assay with cholesterol [3][b]; $I = 0$ M	19		4.8	
	$I = 0.05$ M (NaCl)	14		3.6	
	$I = 0.10$ M (NaCl)	11		3.0	
	$I = 0.15$ M (NaCl)	10		2.6	
	$I = 0.20$ M (NaCl)	8		2.4	
	25 °C; alkylbenzenes [25][b]	12–15			
	20 mM sodium phosphate buffer (pH 7)[b,c] [26]	12.6			
EPR	25 °C; pH 7.8 (borate); $I = 0.06$ M (NaCl) [4,11]				
	5-NS	5.0		2.0	
	16-NS	8.0		3.0	
ST	25 °C [10]		12.9		
	$I = 0.15$ M (NaCl) [1]	7.5			
	25 °C [22]; $I = 0$ M				2.12
	$I = 0.15$ M (NaCl)				1.10
	$I = 0.50$ M (NaCl)				0.74
	pH 9.0 [8] $I = 0.001$ M (NaCl)	8.32		2.70	
	$I = 0.005$ M (NaCl)	6.76		2.40	
	$I = 0.01$ M (NaCl)	5.89		1.70	
	$I = 0.05$ M (NaCl)	3.98		1.29	
	$I = 0.10$ M (NaCl)	3.31		1.00	
	$I = 0.50$ M (NaCl)	2.63		0.79	
	pH 8 [27]; $I = 0$ M	13	12	10	
	$I = 0.15$ M (NaCl)	11	10	3	
TM	[28]	27.4		8.7	
C	30 °C; pH 7.9 (K_3PO_4) [9]	18.4		5.3	
	study conducted at different temperatures: 13–15; 40 °C [14]	15.0		5.0	
		15.5		6.2	
RPLC	room temperature; pH 7 (K_3PO_4) [7]	13.80	12.02	5.25	4.26
O	variable temperatures and electrolyte concentrations [29]	19.7		13.0	
NMR	pH 8.0–8.4 [2]	16.0			
F	pH 8.0–8.4 [2]	13.5	11.0		
	study conducted at different temperatures: 25 and 35 °C [19,30][d]	6.2; 12.8		2.4; 6.5	
		6.3; 14.1		3.4; 6.9	
CE	20 mM sodium phosphate buffer (pH 7)[c] [26]	12.8			
VC	[31]		9.0		
FTTR/ATRS	[31]		9.0		

[a] Abbreviations: Pot, potentiometry; LS, light scattering studies; S, solubilization assay; EPR, electronic paramagnetic resonance; ST, surface tension; TM, theoretical models; C, calorimetry; RPLC, reversed-phase high performance liquid chromatography; O, osmotic activity measurements; NMR, nuclear magnetic resonance; F, fluorescence probes; CE, capillary electrophoresis; VC, viscometry, FTTR/ATRS, Fourier transform infrared attenuated total reflection spectroscopy; NaC, sodium cholate; NaGC, sodium glycocholate; NaDC, sodium deoxycholate; NaGDC, sodium glycodeoxycholate.

[b] The concentration of the solubilized tracer or compound was determined by UV/Vis spectrophotometry.

[c] Using 2-naphthalenemethanol as a tracer for micelle formation.

[d] First and second *cmc* values.

were performed at the following ionic strengths (adjusted with NaCl): 0.10, 0.15, and 0.20 M. System calibration was always performed before and after each determination by titrating HCl with NaOH [33]. The characteristics of the glass electrode, as the “constant” and slope (S) of the modified Nernst equation [34], were similar below and above *cmc* [35,36]. Calculations were performed with data obtained from at least four independent titrations, each with more than 20 points, and the experimental titration data were analyzed using the computer program Superquad [37]; for bile salts the range of $-\log[H^+]$ used was 4.0 to 6.0 and for the

conjugate bile salts it was 3.5 to 5.0 [38]. The model that best fits the experimental data assumes only one equilibrium in solution, corresponding to the protonation of the carboxylic group of these molecules. The errors reported for pK_{app} in this work were calculated by the method of Albert and Serjeant [39], in which the errors are calculated as the maximum difference between the logarithm of the average of the antilogarithms of the calculated pK_{app} values and their individual values.

Spectrophotometric determinations

All absorption spectra were recorded in the range from 200 to 350 nm with a Hitachi U-2000 dual-beam spectrophotometer with scan rate of 800 nm/min, using quartz cells with 1-cm path length that were thermostated at 25 °C. Measurements were made with bile salt solutions below and above their *cmc*: 1.0×10^{-4}; 5.0×10^{-4}, 1.0×10^{-3}, 3.0×10^{-3}, 5.0×10^{-3}, 1.0×10^{-2}, 1.5×10^{-2}, 2.0×10^{-2}, and 4.0×10^{-2} M for cholate and glycocholate and 5.0×10^{-4}, 1.0×10^{-3}, 2.0×10^{-3}, 3.5×10^{-3}, 4.0×10^{-3}, 5.0×10^{-3}, and 7.0×10^{-3} M for deoxycholate and glycodeoxycholate. The values of the *cmc* were obtained from the shifts in wavelength maximum (λ_{max}) and the increases in absorbance maxima (A_{max}) induced by changes in bile salt concentration.

Spectrofluorimetric determinations (light scattering)

The spectrofluorimetric determinations were performed with a Shimadzu RF5001-PC, with light intensity attenuated using a Polaroid HNP'B filter. In all determinations the slit width was 5 nm, and the excitation and emission wavelengths were 400 nm. Measurements were made with bile salt solutions below and above their *cmc*: 5.0×10^{-4}, 1.0×10^{-3}, 3.0×10^{-3}, 5.0×10^{-3}, 7.0×10^{-3}, 8.0×10^{-3}, 9.0×10^{-3}, and 1.0×10^{-2} M for cholate, 6.0×10^{-3}, 7.0×10^{-3}, 8.0×10^{-3}, 9.0×10^{-3}, 1.5×10^{-2}, 2.0×10^{-2}, 3.0×10^{-2}, and 3.5×10^{-2} M for glycocholate, 5.0×10^{-4}, 1.0×10^{-3}, 1.5×10^{-3}, 2.0×10^{-3}, 4.0×10^{-3}, 5.0×10^{-3}, 5.5×10^{-3}, and 6.0×10^{-3} M for deoxycholate, and 5.0×10^{-4}, 7.0×10^{-4}, 1.0×10^{-3}, 1.5×10^{-3}, 3.0×10^{-3}, 4.0×10^{-3}, 5.0×10^{-3}, and 6.0×10^{-3} M for glycodeoxycholate. The reported data are the average of eight independent measurements.

Results

Potentiometric determinations

The values for the calculated pK_{app} of cholate and glycocholate at concentrations below and above their *cmc* and for different ionic strengths are presented in Table 2. No data are reported for deoxycholate and glycodeoxycholate due to their low solubility in water, specifically at pH values lower then their pK_{app}. From the data in Table 2, it can be seen that for bile salt concentrations below their *cmc* the values of the pK_{app} are constant and independent of bile salt amount, whereas above the *cmc* the apparent pK_{app} values increase with an increase in bile salt concentration and tend to level off at high concentrations. The region in which the pK_{app} values start to increase corresponds to the formation of small aggregates of bile salts, and as their concentration increases so does their average size.

A plot of pK_{app} vs log (bile salt concentration) yields two intersecting straight lines (Fig. 1). The horizontal

Table 2
Values for the calculated pK_{app}s of different sodium cholate (NaC) and sodium glycocholate (NaGC) concentrations (M), at 25.0 ± 0.1 °C and variable ionic strength (I), with sodium chloride being the electrolyte used

$I = 0.20$ M		$I = 0.15$ M		$I = 0.10$ M	
NaC	pK_{app}	NaC	pK_{app}	NaC	pK_{app}
0.0008	4.63 ± 0.05	0.0008	4.66 ± 0.04	0.0008	4.69 ± 0.05
0.0010	4.63 ± 0.05	0.0010	4.66 ± 0.05	0.0010	4.69 ± 0.05
0.0100	4.73 ± 0.06	0.0108	4.77 ± 0.04	0.0100	4.77 ± 0.05
0.0151	4.82 ± 0.05	0.0162	4.88 ± 0.05	0.0151	4.90 ± 0.05
0.0201	4.90 ± 0.05	0.0216	4.96 ± 0.06	0.0201	4.96 ± 0.04
0.0232	4.94 ± 0.06	0.0270	5.01 ± 0.05	0.0232	4.99 ± 0.05
0.0290	4.99 ± 0.06	—	—	0.0290	5.04 ± 0.06
NaGC	pK_{app}	NaGC	pK_{app}	NaGC	pK_{app}
0.0003	3.59 ± 0.04	0.0008	3.64 ± 0.05	0.0003	3.68 ± 0.03
0.0008	3.59 ± 0.04	0.0016	3.64 ± 0.05	0.0008	3.68 ± 0.05
0.0095	3.67 ± 0.05	0.0040	3.64 ± 0.05	0.0099	3.69 ± 0.04
0.0143	3.73 ± 0.05	0.0048	3.64 ± 0.05	0.0149	3.76 ± 0.05
0.0192	3.77 ± 0.05	0.0056	3.64 ± 0.06	0.0199	3.83 ± 0.05
0.0239	3.81 ± 0.05	0.0120	3.70 ± 0.06	0.0239	3.87 ± 0.04
0.0280	3.83 ± 0.06	0.0160	3.75 ± 0.06	0.0280	3.89 ± 0.06
—	—	0.0201	3.80 ± 0.05	—	—
—	—	0.0281	3.86 ± 0.06	—	—

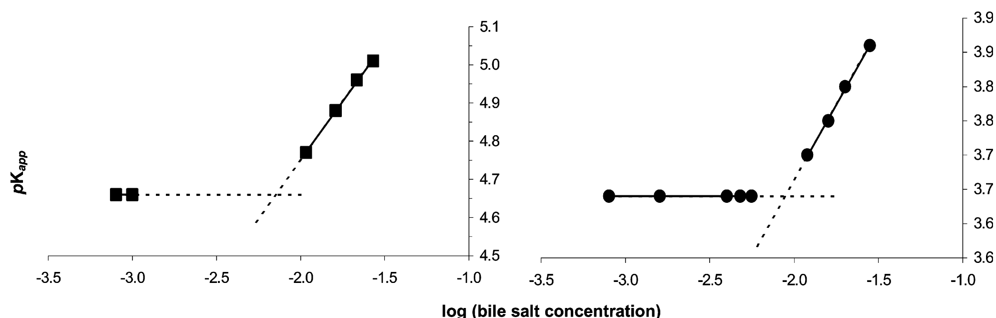

Fig. 1. Representation of pK_{app} vs log (bile salt concentration) for sodium cholate (squares) and sodium glycocholate (circles) ($I = 0.15$ M in NaCl at 25.0 ± 0.1 °C).

line corresponds to bile salt concentration below the *cmc* and the values of pK_{app} remain constant; above the *cmc* the reported values of pK_{app} increase with bile salt concentration and must correspond to apparent acidity constants, as they were calculated under the assumption that all bile salt was in the free form.

However, the bile salt forms do aggregate, thus reducing the concentration of free bile salt and, what is more important, preventing hydrogen ions from binding to the bile salts in the micelles. The point of intersection of the two straight lines must thus correspond to the concentration at which the free bile salt molecules start to aggregate, viz. the *cmc*. The *cmc* values obtained with this approach are included in Table 3.

Spectrophotometric determinations

Spectrophotometry can be used for cmc determination either by the widespread dye inclusion or, in the context of this study, without the use of molecular probes, by the analysis of an intrinsic feature of the system that varies, such as ε, λ_{max}, or derivative absorbance [40]. Derivation can be a useful tool, since it promotes better resolution of the bands and increases the signal to noise ratio.

The absorption and the first derivative spectra of the bile acid salts are very similar throughout the series studied. As an example, the spectra obtained for glycodeoxycholate in the range 200 to 300 nm are depicted in Fig. 2.

The main feature observed is one band at 210 nm that has been used to determine bile salt concentration by

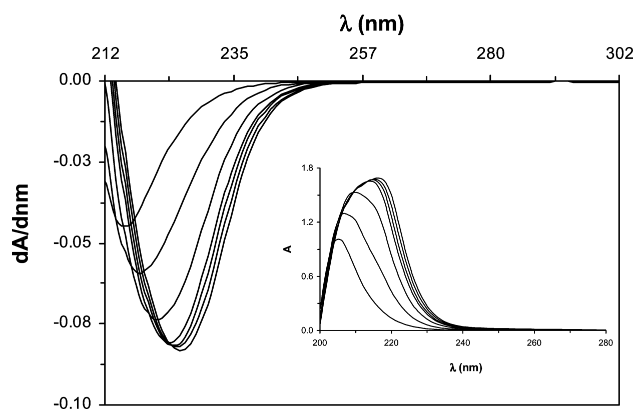

Fig. 2. The absorption (A) and the first derivative spectra (dA/dnm) of sodium glycodeoxycholate at different concentrations ($I = 0.10$ M in NaCl at 25.0 ± 0.1 °C) and in the range from 200 to 300 nm with scan rate of 800 nm/min.

chromatography [41,42]. A plot of absorption at λ_{max} vs bile salt concentration reveals two intercepting straight lines (Fig. 3), and it must be pointed out that for each line the experimental points obtained obey the Lambert–Beer law; furthermore, the line corresponding to the higher concentrations has practically a null slope for cholate and glycocholate. The breakdown of the Lambert–Beer law at specific concentrations of bile salts means that above this concentration some type of interaction must take place between the free ions; although for higher concentrations the Lambert–Beer law is also verified, the observation of a smaller extinction coefficient means that the aggregates that are formed hinder absorption. From the Lambert–Beer law, $A_{obs}/b = \varepsilon_1 c_1$, where b stands for the optical path for the monomeric

Table 3
Critical micelle concentration values (mM) for sodium cholate (NaC), sodium glycocholate (NaGC), sodium deoxycholate (NaDC), and sodium glycodeoxycholate (NaGDC), obtained in this work, at 25 °C

Method	Experimental conditions	NaC	NaGC	NaDC	NaGDC
UV/Vis	$I = 0.10$ M (NaCl)	6.10	11.90	2.56	2.20
Potentiometry	$I = 0.05$ M (NaCl)	6.30	9.06		
	$I = 0.10$ M (NaCl)	7.30	9.44		
	$I = 0.15$ M (NaCl)	7.24	8.86		
	$I = 0.20$ M (NaCl)	6.85	5.91		
Light scattering	$I = 0.10$ M (NaCl)	5.90	11.60	2.35	2.23

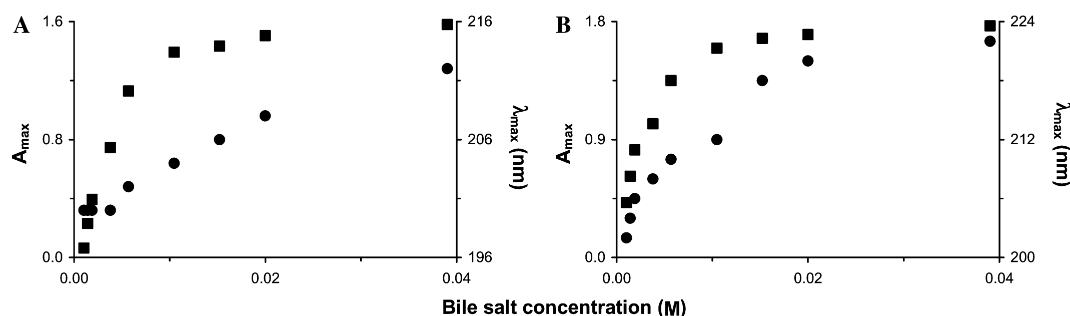

Fig. 3. Representation of A_{max} (■) or λ_{max} (●) vs sodium cholate (A) or sodium glycocholate (B) concentrations.

form of the bile salts, and after the *cmc*, $A_{obs}/b = \varepsilon_1 c_1 + \varepsilon_2 c_2$ where $\varepsilon_1 c_1$ remains constant; as aggregation will bury some of the chromophores in the micelle, $\varepsilon_1 > \varepsilon_2$, and thus A_{obs} above the *cmc* will increase much slower or will remain practically constant (if $\varepsilon_2 \approx 0$). The concentration at which both lines intercept corresponds to the *cmc*.

A plot of λ_{max} vs bile salt concentration shows no variation up to a certain value of concentration, after which the value of λ_{max} starts to shift to lower energies (Fig. 3); again it is for cholate and glycocholate that the larger shifts are observed. However, the key observation is that the concentration for which the change in behavior is observed is the same for plots of A_{obs} and of λ_{max} vs bile salt concentration. The observed shift above the *cmc* must be a consequence of the burring of the chromophoric groups in a nonpolar environment, as the micelles start to form. The values of the *cmc* obtained by both approaches are included in Table 3.

Spectrofluorimetric determinations (light scattering)

The intensity of the Rayleigh scattering band (R) was used as an indication of the size of the particles in solution, and a representation of R vs bile salt concentration is depicted in Fig. 4. The value of concentration at which the two straight lines intercept corresponds to the *cmc* value (Table 3). This technique provides an easy method to determine *cmc* values and allowed the obtainment of data that could be compared with literature results using this technique; no attempts were made to extract molecular weights and aggregation numbers from the data obtained.

Dependence of the cmc values on ionic strength

The effect of counterion concentration on the *cmc* values of bile salts has been studied by different authors [3,8,13,43] and a general conclusion is that the addition of an inert electrolyte, which may alter the ionic strength of the solution, will promote an increase of the micellar aggregates of the bile salts [8,13]. According to the literature [8,12,44] the effects of counterion on the *cmc* of bile salt micelles are mainly mediated by progressive neutralization of the ionic charges. As the *cmc* is an index of the balance between the opposing forces of hydrophobic interaction and ionic repulsion, reduction of the latter allows micelle formation to occur at a lower concentration, which means a decrease in *cmc*.

In Table 4 the variations of the *cmc* values are shown for sodium cholate and sodium glycocholate as the concentration of sodium chloride is increased at 25 °C and pH 7.0. It can be observed in Table 4 that for cholate the results obtained by spectrophotometry show only a small variation with the increase of NaCl concentration from 0 to 0.20 M. These results contradict the well-described decrease in *cmc* with counterion concentration and can result from difficulties in resolving the "round-shaped" variation in the spectral characteristics observed. Nevertheless, a constancy or a small increase in *cmc*,

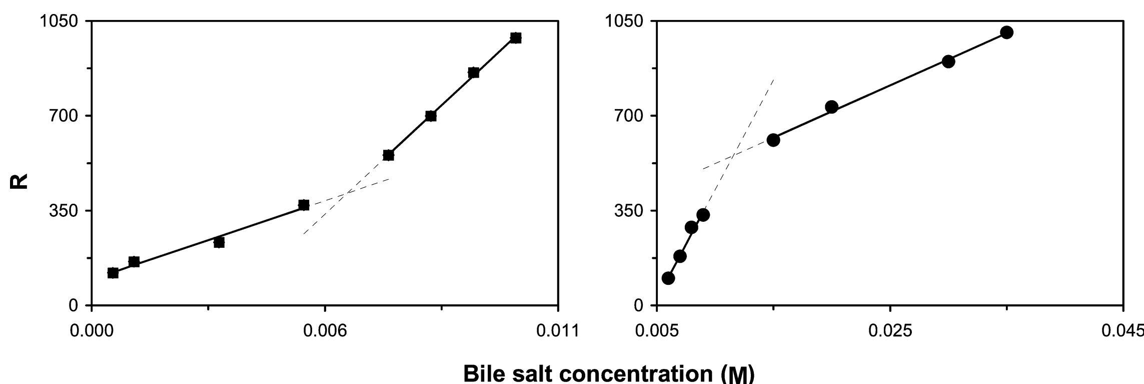

Fig. 4. Representation of R (Rayleigh scattering band) vs sodium cholate (squares) or sodium glycocholate (circles) concentrations.

Table 4
Critical micelle concentrations values (M) of sodium cholate and sodium glycocholate obtained by potentiometry and UV/Vis spectrophotometry at 25.0 ± 0.1 °C as a function of the ionic strength (I), with sodium chloride being the electrolyte used

I (M)	Spectrophotometry[a]	Potentiometry	
	Cholate	Cholate	Glycocholate
0	5.3×10^{-3}	—	—
0.05	5.6×10^{-3}	—	—
0.10	6.0×10^{-3}	7.30×10^{-3}	9.44×10^{-3}
0.15	6.1×10^{-3}	7.24×10^{-3}	8.86×10^{-3}
0.20	5.9×10^{-3}	6.85×10^{-3}	5.91×10^{-3}

[a] *cmc* values are means of at least two independent determinations at pH 7.0.

determined using a spectral shift technique, with NaCl concentration up to 0.7 M, was described for sodium taurocholate and sodium taurodeoxycholate [45]. The results from potentiometry show the well-known decrease in cmc from 0.10 to 0.20 M NaCl. The potentiometric experiments were not conducted at a NaCl concentration lower than 0.10 M, given the inconstancy of the ionic strength throughout the titrations under such experimental conditions.

Discussion

As can be observed in Table 1, results obtained for the *cmc* determination of the bile salts studied are diverse, even within the same methodology. As an example, the *cmc* values that were obtained for sodium cholate in this work have the same order of magnitude as those described in studies using EPR [4,11], spectrophotometry with OT orange as tracer [24], surface tension studies [1,8], and the first *cmc* values determined using a fluorescence probe [19,30]. However, the results disagree with others, depending on the experimental methodology that was used and on the definition of the operational *cmc* applied.

Therefore, the great discrepancies observed in some cases (Table 1), caused not only by the differences in the analytical processes but also by the experimental apparatus, explain some of the comments to the studies in which they are mentioned, allowing for an objective criticism and for a comparison with the results obtained in this work.

In the work presented by Shaw et al. [7], the *cmc* values were determined by RP-HPLC and the results obtained were compared with those determined by solubilization of a lipophile compound (20-methylcolanthrene) in a bile salt solution according to the latter's concentration. In this case, the graphical representation of the tracer concentration in relation to the bile salt's concentration in solution does not originate two well-defined straight lines, and it is difficult to determine with precision the intersection of the lines that would correspond to the *cmc*. Therefore, it was necessary to define an *operational cmc*, which would correspond to the concentration value of the bile salt in which the duplication of the straight line value obtained for diluted solutions occurs. The same approach was applied in other studies [14,18,23,28].

A different *cmc* definition was applied in the works carried out by Kawamura et al. [4,11], in which the *cmc* values were determined by EPR on the basis of the rotational correlation time values (τ_1), according to the bile salt concentration. In this case, the value of the operational *cmc* was defined as the minimal concentration from which deviations in the τ_1 value start to appear according to the bile salt concentration, which, for diluted concentrations, is a linear function of the bile salt concentration. This fact explains, at least to some extent, the reason by which, with this process of analysis, lower *cmc* values are obtained.

The τ_1 value increases abruptly at the *cmc* value in the case of dodecyl sulfate [4], a feature that is not observed with the bile salts. It is also said that $d\tau_1/dc_0$, where c_0 is the detergent concentration in the *cmc* region, is higher for the dihydroxy bile salts than for the trihydroxylics. This fact suggests that the cooperative effect in the formation of the micelles is higher for dihydroxy bile salts than for trihydroxylics.

In another study (Balduci et al. [28]), the determination of the *cmc* values was conducted from theoretical models, considering that the micellization of bile salts is a two-step process. As the concentration approaches the *cmc*, bile salt molecules form dimers or slightly larger aggregates. These primary micelles are stabilized mainly by hydrophobic interactions wherein the apolar surfaces of adjacent molecules, are partially sequestered from the solvent, whereas the polar groups tend to remain accessible to, and solvated by, the aqueous solution. Upon a further increase in concentration, the primary micelles tend to combine into larger aggregates, stabilized mainly by intermolecular hydrogen bonding between some of the hydroxyl groups. Position and orientation of the hydroxyl groups play important roles in the aggregation process. In the primary aggregation step, hydrophilic "islands" on a generally hydrophobic surface reduce the stabilization provided by the hydrophobic interaction. In contrast, the secondary aggregation is essentially due to the hydrogen bond stabilization provided by properly oriented hydroxyl groups.

The two-step process outlined above is reflected in a theoretical model proposed by decomposing ΔG_M into its two major contributions, $\Delta G_M = \Delta G_1 + \Delta G_2$, where ΔG_1 represents the free energy change associated with the essentially hydrophobic interaction, responsible mainly for primary aggregation, and ΔG_2 accounts for the hydrogen bonding contribution, responsible mainly for secondary aggregation.

The *cmc* values of some of the bile salts described in this work are presented in Table 3. Although the values presented are similar to other results presented in the same table, determined by different analytical processes, some variation in the values can be attributed to presuppositions in the elaboration of the theoretical model. These include the following assumptions: the micellar solution in the *cmc* region is a monodisperse solution, micelles do not interact with each other, and the aggregation number is always the same for all the studied bile salts.

On the other hand, variations of the *cmc* values obtained can reflect the sensitivity of the analytical method used for the determination. In the work by Kratohvil and DelliColli [22], in which the *cmc* values of taurocholique and taurodeoxycholique acids, determined by nephelometry, are compared with the values obtained by determining the surface tension, it was concluded that the latter values are higher because the determination of the surface tension is not a sensitive enough process to detect the presence of small aggregates, such as dimers. It is also said that the cooperative effects in the formation of aggregates with dihydroxy bile salts are higher than those with trihydroxylics [46].

From the examples mentioned above, one can conclude that the *cmc* values for bile salts are not always comparable, which can be due to the possibility of micellar aggregation being a continuous phenomenon and, therefore, it being impossible to define a single *cmc* value; consequently, it is necessary to define an operational *cmc* value. It should be stated that in the methodologies that we present (potentiometry, derivative spectrophotometry, and light scattering) the operational *cmc* is defined as the bile salt concentration corresponding to the minimal concentration in which a change in the response of the measured or calculated property occurs. This definition might explain, at least to a certain extent, the similarity of the values obtained by other techniques, for example EPR, and the fact that these values are lower than those determined by methodologies in which the definition of operational *cmc* may differ. This problem is clearly pointed out in a work in which the *cmc* was determined in solubilization studies with a neutral tracer, naphthalene, in sodium cholate solutions [23]. The concentration of the solubilized tracer was determined by spectrophotometry. The results obtained allow for the calculation of three different *cmc* values for sodium cholate (1.6×10^{-3}, 2.0×10^{-3}, and 3.0×10^{-3} M), according to the experimental points used for the definition of the two lines that show the solubility of naphthalene in relation to the concentration of bile salt in solution.

Concluding remarks

The cooperative effects in the formation of micellar aggregates, which are more noticeable with dihydroxy bile salts than with trihydroxylics, could explain the fact that the higher discrepancy in the *cmc* values presented in the literature happens precisely with the latter, because of the difficulty in defining a single *cmc* value and because the results correspond to different definitions of operational *cmc*.

On the other hand, it becomes clear that the results obtained with trihydroxy bile salts by some methods, such as calorimetry, NMR, or osmotic activity measurements, are systematically higher, which can be due to the low sensitivity of the analytical processes used to detect the presence of small aggregates such as, for example, dimers, therefore engendering less valid conclusions.

From the results presented in this work, in the determination of the *cmc* values, we can infer that potentiometry is a valid alternative for the determination of *cmc* in detergents that present acid or basic characteristics. Also, it should be admitted that this concept might also be applied to other techniques used in the determination of pK_{app} values such as, for example, nuclear magnetic resonance or UV/Vis spectrophotometry.

Along with potentiometry, the other methods for *cmc* determination described in this work (spectrophotometry and light scattering) proved to be simple, effective, and noninvasive and did not require the use of tracers or further sample manipulation.

Finally, a careful analysis of the literature on bile salt *cmc* determination shows the omission of details of experimental conditions in some papers, such as pH values, temperature, ionic strength, and operational *cmc* definition used. These parameters influence the *cmc* values and should always be specified to enable valid comparisons between the results of separate studies.

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