SPECTROPHOTOMETRIC DETERMINATION OF BILE ACIDS; AN EVALUATION

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Dedicated to Professor Václav Horák on the occasion of his 70th birthday.

Bile acids can be determined by measuring absorbance in the ranges between 290 and 310 nm or between 370 and 390 nm of species which are formed when bile acids react in 77.8 wt.% sulfuric acid at 70 °C for 30 min. The absorption bands at about 300 nm correspond to a formation of allylic carbocations, those at about 380 nm to dienylic carbocations. The absorbance was found to be a linear function of concentration between 1 and 5 . 10^{-5} mol l^{-1}. Measurements of absorbance were compared for 11 bile acids. The method can be used for analysis of dilute solutions of these acids, e.g. in estimating their solubility.

Three types of analytical methods are used for determination of sterols, such as bile acids, cholesterol and some ketosteroids: (i) methods based on direct spectrophotometry; (ii) enzymatic methods with spectrophotometric finish, and (iii) chromatographic methods[1,2]. As chromatographic methods are often time-consuming, especially with respect to preliminary sample handling, enzymatic methods are often preferred[3] due to their selectivity. For cholesterol analysis the available methods have been recently evaluated[4]. When analysis of a large number of relatively pure samples is to be carried out, as needed, e.g. in studies of solubilities of these sterols or their partition[5] a simple direct spectrophotometric procedure seems so be most promising.

The Liebermann–Burchard color reaction for cholesterol[6,7], its modification by Tak et al. using of ferric ions as oxidizing agents[8,9], as well as the Hammarsten–Yamasaki reaction for bile acids[10,11] were described in the early decades. Occurence of absorption bands in the visible and UV region for solutions of bile acids containing varying

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concentration of sulfuric acid was observed relatively early[12]. More recently[13] absorption bands of bile acids in concentrated sulfuric acid after 2 h at room temperature and in 65 wt.% sulfuric acid after 15 min at 60 °C were reported. Even when the effects of the time of heating on the changes of absorption bands were observed, no attempt has been made to offer any interpretation for the presence of such absorption bands. Empirically, 65 wt.% sulfuric acid and heating were chosen for determination of bile acids, possibly in some mixtures[14 - 17] and on paper chromatograms[18].

More recently[19] presence of two absorption bands in sulfuric acid solutions of cholic and deoxycholic acid at 305 – 345 nm and 385 – 417 nm was recognized. The dependence of the changes in both the relative molar absorptivity and the rate of formation of these bands on sulfuric acid concentration was briefly reported, but no interpretation of the nature of the species or processes involved was proposed, apart from the conclusion that two species, which differ from the parent bile acid, are involved.

Alternatively, solutions of bile acids in 75 vol.% sulfuric acid were kept for 5 h at 50 °C and fluorescence measured[20,21]. This approach was used for determination of cholic, deoxycholic, chenodeoxycholic, and ursodeoxycholic acids in solubility studies[22].

The common limitation of the above mentioned optical methods resulted from the empirical choice of reaction conditions, which reflected the fact that the nature of the process yielding the chromogenic species is not fully understood. This is manifested by the statement in an extensive comparative study[23] of spectra obtained in solutions of steroids in concentrated sulfuric acid two hours after preparation: "To date, no definitive theory has been proposed which accounts for the many intricacies of steroid spectra in sulfuric (indeed any) acid."

Initially, Kalant[24] proposed formation of adducts involving two steroids molecules, whereas Linford[25 - 29] suggested formation of a single carbocation, which, nevertheless, absorbs at wavelengths shorter by 100 nm or more than shown by observed absorption bands. Japanese authors[30 - 52] did not investigate the behavior of bile acids, but rather of estrogens and ketosteroids. Based on analysis of reaction products they proposed correctly that absorbing species are polyenylic carbocations. On the other hand, they incorrectly concluded that the olefins, present in these solutions resulting from dehydration of alcohols, are oxidized by sulfuric acid, based on an increase of absorbance resulting from addition of selenic acid[39]. The role of sulfuric acid as the main oxidizing agent, proposed by the Japanese authors[30 - 52], cannot be reconciled with the absence of formation of an equimolar concentration of sulfurous acid and, by the production of the absorbing species in the presence of Brønsted and Lewis acids other than sulfuric acid (e.g. perchloric or $ZnCl_2$, $SbCl_5$, or $AlCl_3$). Similarly the nonlinear dependence of rate constants on concentration of sulfuric acid and the occurence of these oxidation processes producing absorbing species in solutions of sulfuric acid at concentrations as low as 50 wt.% (where SO_3 – a possible oxidant – is virtually absent) allows ruling out H_2SO_4 as the oxidizing agent.

By following changes of the absorbance at various wavelengths with time as a function of concentration of sulfuric (and in some case perchloric) acid, it was possible to distinguish the effects of acidity on the rates of formation of absorbing species and on the established equilibria. Comparison of the behavior of various sterols then assisted in identification of some of the processes involved. An investigation along these lines indicated[53 - 56] that formation of absorbing species from bile acids in 60 – 90 wt.% sulfuric acid follows kinetics which in some cases is second order, but in most cases first order in sterol. Two types of main absorption bands are formed: these in the range between 290 and 310 nm correspond to allylic carbocations those between 370 and 390 nm to dienylic carbocations. The structure of the absorbing species has been confirmed by UV spectra of olefins, formed on dilution of the acidic reaction mixtures. Small bands in the 410 – 430 nm region were attributed to trienylic carbocations. Formation of an absorption band in the 350 nm range was observed only for compounds which can yield 3,5-dications (such as chenodeoxycholic acid, hyodeoxycholic acid, and cholesterol[57]).

In most of the studied cases, allylic carbocations are converted into dienylic carbocations and these eventually into trienylic carbocations. In some cases competetive processes occur, resulting in a formation of isomeric polyenylic carbocations absorbing at slightly different wavelengths.

Both equilibrium and rate constants formed depend on the acidity of the solution. Plots of values of both equilibrium and rate constants on acidity function H_0 (defined as $-\log a_{H^+}$) indicate that the rate determining step is accompanied in some cases by one, but in most cases by two acid-base equilibria. As the pK_a values of individual acid-base equilibria differ, the role of acid-base reactions is manifested by different dependences of rate constants on acidity for individual carbocations (Fig. 1) and hence of the absorbance at a given wavelength on concentration of sulfuric acid.

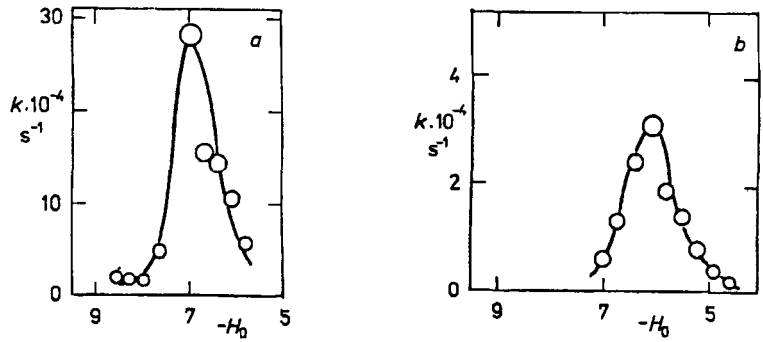
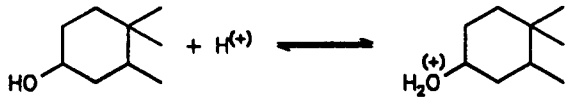


FIG. 1

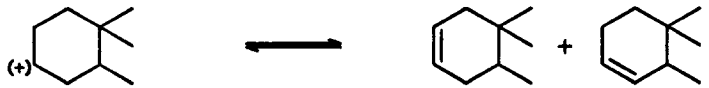
Dependence of the first order rate constant of the formation of the allylic carbocation absorbing at 310 nm (*a*) and of the dienylic carbocation absorbing at 380 nm (*b*) on Hammett acidity function (H_0) for the reaction of 1 . 10^{-4} mol l^{-1} chenodeoxycholic acid at 25 °C in solutions containing varying concentrations of ethanol and sulfuric acid

The nature of the reaction yielding absorbing species can be interpreted by the following sequence of processes, depicted here for the formation of allylic carbocations in ring A of the steroid:



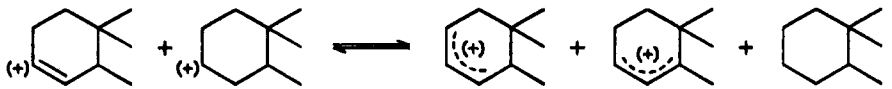
(A)

(B)



(C)

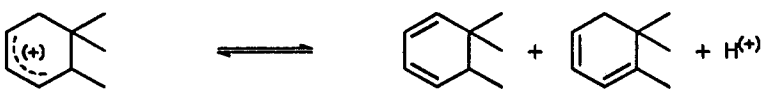
followed, e.g. by



(D)

A_1 A_2

A_1 and A_2 are allylic carbocations absorbing at about 310 nm. The disproportionation (*D*) involves a hydride transfer from the olefin to the carbocation in a reaction where the carbocation is the oxidizing agent. Shift of the equilbrium (*A*) to the right with increasing acidity results in an increase in the rate of formation of allylic carbocations, shift of equilibrium (*C*) in a decrease. Either the hydride transfer (*D*) is the rate determining step and the reaction follows second order kinetics, or the cleavage of a C–H bond in (*C*), resulting in first order kinetics.

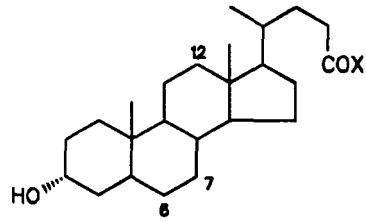


(E)

Allylic carbocations A_1 and A_2 formed in reaction (*D*) can undergo acid-base equilibria, and the resulting olefins can be further oxidized by present carbocations to form dienylic carbocations, absorbing in the 370 – 390 nm range. Reactions of the type (*A*), (*C*), and (*E*) are acid-base equilibria, the presence of which is manifested by the dependence of measured rate and equilibrium constants on acidity.

The optimum conditions for the determination of individual bile acids would involve for each compound: *a*) finding the most suitable concentration of sulfuric acid and *b*) finding such period of time, when the absorbance is highest, the formation of the absorbing species fastest and the absorbance remains practically constant over a period of time. Such optimum conditions can be found either for the formation of an allylic carbocation absorbing at 310 nm or for that of a dienylic carbocation that absorbs at about 380 nm. The optimum conditions, in particular the sulfuric acid concentration, are in principle different for individual sterols.

When facing the problem of developing an analytical method for the determination of 11 bile acids *I* – *XI*, for the majority of which the dependence of absorbance at characteristic wavelengths on time and sulfuric acid concentration has been unknown, such project would involve considerable expenditure of time. Hence another approach was adopted, in which a single concentration of sulfuric acid (77.8 wt.%) and reaction



	Acid	6	7	12	X
I	Lithocholic	H	H	H	OH
II	7-Oxolithocholic	H	=O	H	OH
III	Hyodeoxycholic	α-OH	H	H	OH
IV	Chenodeoxycholic	H	α-OH	H	OH
V	Ursodeoxycholic	H	β-OH	H	OH
VI	Deoxycholic	H	H	α-OH	OH
VII	Hyocholic	α-OH	α-OH	H	OH
VIII	Cholic	H	α-OH	α-OH	OH
IX	Ursocholic	H	β-OH	α-OH	OH
X	Glycodeoxycholic	H	H	α-OH	$NHCH_2COOH$
XI	Taurodeoxycholic	H	H	α-OH	$NHCH_2SO_3H$

time of 30 min was used at a single temperature (70 °C) for the determination of all bile acids studied. Under conditions used this resulted for some bile acids in a predominant formation of an allylic carbocation absorbing at about 310 nm, for others in that of a dienylic carbocation which absorb at about 380 nm. For analytical purposes the absorbance at the maximum of the predominant band was measured.

EXPERIMENTAL

Materials

Bile acids were commercial samples (*I* and *IV*: Fluka, Buchs; *III*, *VI*, and *VIII*: Sigma, St. Louis; *VII*, *X*, and *XI*: Calbiochem, La Jolla or gifts (*II* and *IX* by Gipharmex, Milan and *V* by Alfa–Wasserman, Bologna); they were used as received either as acids (*I* to *V*, *VII*, and *IX*) or sodium salts (*VI*, *VIII*, *X*, and *XI*). Analytical grade sulfuric acid (96 wt.%, ρ = 1.835 g cm^{-3}) was obtained from Carlo Erba, Milan.

To prepare aqueous stock solutions, bile acids were converted into sodium salts or alternatively the stock solution contained 50 vol.% (38 wt.%) aqueous ethanol. All solutions were prepared daily fresh.

Procedure

A stock solution of the bile acid (1 ml) was added to 3 ml of 96 wt.% sulfuric acid solution, placed in a vial, located in an ice/salt bath, to avoid local overheating and uncontrolled reaction conditions: final solutions contained between 1 . 10^{-5} and 5 . 10^{-5} mol l^{-1} sterol in 77.8 wt.% sulfuric acid and 0% or 5.7 wt.% ethanol. The solutions were homogenized by shaking and placed in a thermostat at 70 °C, for 30 min. After the given time interval the spectra were recorded for each compound on a Hitachi 220 S spectrophotometer in the range 200 – 500 nm, using as the reference a sulfuric acid solution of the same composition as the studied solution.

RESULTS AND DISCUSSION

For each compound the absorbance was measured for at least five concentrations of a bile acid in the range between 1 . 10^{-5} and 5 . 10^{-5} mol l^{-1} (even when an extension of the study to both lower and higher concentrations is possible) after 30 min, when the absorbance became practically independent of time. In each case a linear dependence of the measured absorbance – either corresponding to allylic or dienylic carbocations – was observed (Table I). This table also includes the values of the wavelength used and statistical data obtained in linear regression. Figure 2 illustrates why for cholic acid the temperature of 70 °C and t_1 = 30 min was chosen.

For compounds *I*, *II*, *X*, and *XI* the species predominating in a solution containing 77.8 wt.% sulfuric acid at 70 °C after 30 min were allylic carbocations absorbing at 290 – 310 nm, for all other compounds the predominating species were dienylic carbocations, absorbing at 370 – 390 nm.

The values of regression coefficients (Table I) indicate that all the plots of absorbance as a function of sterol concentration are linear and that the standard error of estimate is sufficiently low. Hence measurements of absorbance due to individual

polyenylic carbocations are well suited for determination of studied sterols in dilute solutions. The variations of molar absorptivities with structure of bile acid are relatively small (Table I). This differs considerably from the procedures in which fluorometric finish is used[22], where the slopes of the dependence of fluorescence intensity on concentration strongly depends on the structure of the bile acid. The molar absorptivities of allylic carbocations are somewhat lower than those of the dienylic carbocations. As a first approximation an average value of log ε = 3.9 for allylic and 4.2 for dienylic carbocations could be used even for the analysis of an unknown sterol, but more reliable analytical results can be obtained only when a calibration curve is constructed for each bile acid individually.

TABLE I
Spectrophotometric data (λ in mm, ε in $dm^3\ mol^{-1}\ cm^{-1}$) for the reaction of bile acids in 77.8 wt.% sulfuric acid at 70 °C after 30 min

Compound	λ	log ε	Intercept	r^a	s^b
I	308	3.62	−0.004	0.9977	0.009
II	294	4.00	0.006	0.9996	0.013
III	383	4.29	0.004	0.9993	0.015
IV	382	4.23	0.012	0.9980	0.031
V	382	4.29	−0.002	0.9997	0.015
VI	385	4.18	−0.004	0.9996	0.0009
VII	389	4.39	0.002	0.9998	0.0007
VIII	373	4.03	0.002	0.9998	0.006
IX	390	4.37	0.003	0.9998	0.003
X	309	3.96	0.006	0.9970	0.028
XI	309	3.97	−0.003	0.9992	0.017

[a] Correlation coefficient; [b] standard estimate of error, defined as $s = \{[\sum_{i=1}^{n} (\bar{y}_i - y_i)^2]/(n-2)\}^{1/2}$.

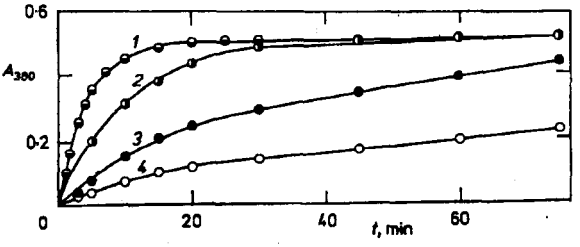


FIG. 2
Dependence of the change in absorbance at 380 nm with time on temperature for 5 . 10^{-5} mol l^{-1} cholic acid in 77.8 wt.% sulfuric acid of the dienylic carbocation. Temperature (°C): *1* 80; *2* 70; *3* 60; *4* 50

The present study indicates the suitability of the direct spectrophotometric method based on formation of absorbing allylic and dienylic carbocations in sulfuric acid solutions for determination of a wide variety of bile acids in dilute solutions.

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