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ANALYTICAL COMPARISON OF CALORIMETRIC, ENZYMATIC AND CHEMICAL METHODS FOR THE QUANTITATIVE DETERMINATION OF CHOLIC ACIDS *

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ABSTRACT

Several methods for the determination of cholic acids have been examined using pure cholic acid as standard. Some analytical aspects have been checked and evaluated. Two methods based on DSC were considered using the detection and measurement of the heats of oxidation and heats of fusion, respectively. The results were compared with those obtained both by the enzymatic method using 3α-hydroxysteroid dehydrogenase as reagent and by a chemical method based on the Pettenkofer reaction. Operative procedures of the last method have been tested and improved with respect to the Irvin procedure in some cases.

The discussion is reported in terms of precision, sensitivity and possible fields of application for the examined method.

INTRODUCTION

In recent years, the interest in cholic acids has been increasing in gastroenterology [1] and in pharmacology [2]. Therefore the analysis of cholic acids in different matrices is increasingly required, particularly for the determination of the lithogenic index [3]. The use of drugs containing cheno- and ursodesoxycholic acids in the therapeutic treatment of cholelithiasis requires simple and reliable analytical techniques as an alternative to more complex methods (i.e. the gas chromatographic or the radioimmunological methods). Among the newly proposed techniques are those involving DSC. An analytical comparison of one DSC technique [4] useful for biological samples, another recently proposed DSC technique [5] more suitable for pharmaceutical samples, and the enzymatic method commonly used for biological samples [6] is carried out here. Finally, the comparison has been enlarged to include a chemical method [7] offering quite simple operations and cheap execution. Moreover, some operative parameters were checked and/or verified with respect to the previously reported working conditions.

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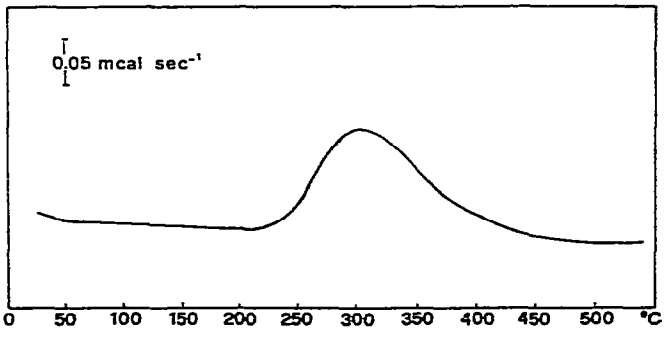
CALORIMETRIC DETERMINATION BY DSC

In the method recently proposed [4], exactly the same quantity of aluminum oxide (~20 mg) is introduced into two pans. To the first pan, an accurately known volume of a methanolic solution of the acid is added by microsyringe and the same volume of pure methanol is added to the second pan. The two pans are then placed in an oven at 60°C until the methanol is completely removed. Immediately afterwards, both pans are introduced into a DSC furnace. The result is an exothermic peak between 220 and 450°C, the area of which is proportional to the quantity of acid present (Fig. 1). In Table 1 are collected the results of a series of six cholic acid standards in methanol at concentration of 2.50, 4.00, 5.00, 6.00, 7.50, and 10.00 mmole l^{-1}, each one repeated three times. The inert material used to dilute the sample is one of the classic materials used to prepare TLC layers. Therefore, the separation of several bile acids can be performed by thin layer chromatography, and their quantitative determination carried out by removing the layer corresponding to the spot and placing it in the DSC sample pan while the reference pan is filled with the same quantity of acid-free aluminium oxide obtained from the same plate. Experimental features of the TLC—DSC technique were the subject of a previous work [4]; experimental details are given here for cholic acid DSC analysis. When separation by TLC is unnecessary, whenever a high quantity of acid has to be analysed, i.e.



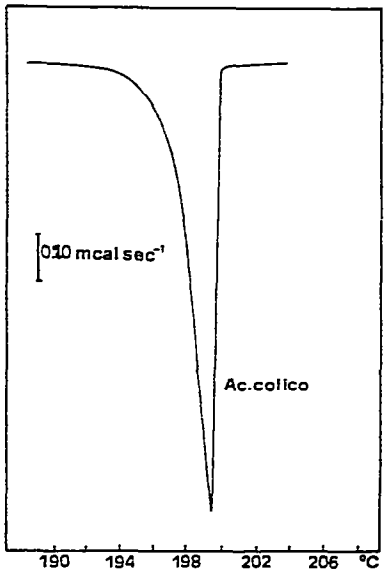


Fig. 1. Calorimetric analysis by the heats of oxidation. DSC curves: 100 mg of cholic acid as standard; sensitivity, 0.05 mcal sec^{-1}; heating rate, 10°C min^{-1}; atmosphere, dynamic oxygen; flow rate, 100 ml min^{-1}.

Fig. 2. Calorimetric analysis by heats of fusion. DSC curves: 3.76 mg cholic acid as standard; sensitivity, 1 mcal sec^{-1}; heating rate, 2.5°C min^{-1}; atmosphere, dynamic nitrogen; flow rate, 100 ml min^{-1}.

TABLE 1

Calorimetric method (DSC) (oxidative transitions). Regression of the results obtained in the determination of standards of cholic acid in methanolic solution. Regression straight line expressed as $Y = A + BX$, where X is the concentration of the standard in mmole l^{-1} and Y is the area of the DSC peak, in arbitrary units. R indicates the correlation coefficient. For each X value, the Y values found are reported together with those obtained from the regression straight line, the standard error on Y, absolute and normalized regarding to the value of Y (standard relative error).

X	Y	Y (Regr.)	σ_Y	σ_Y/Y
2.50	1.50	1.60	0.06	0.04
	1.70			
	1.50			
4.00	2.30	2.50	0.04	0.02
	2.70			
	2.50			
5.00	3.10	3.10	0.04	0.01
	2.90			
	3.30			
6.00	3.90	3.69	0.03	0.01
	3.50			
	3.80			
7.50	4.50	4.59	0.04	0.01
	4.70			
	4.60			
10.00	6.20	6.09	0.07	0.01
	6.00			
	6.00			

Regression: $Y = A + BX$

Pr	Value	Std.err.
A	0.098	0.088
B	0.599	0.013
R	0.9957	0.0231

Residual std. err.	0.144
% Variation coeff.	4.0
Student's Test	43.0
p	<0.001

for drugs control, the DSC technique can be used by the determination of the heat of fusion rather than the heat of oxidation [5]. This method, which is easier to perform, consists of introducing into a DSC pan an accurately known weight of the sample and measuring the area of the fusion peak between 140 and 210°C (Fig. 2). A series of measurements, obtained from different weights of cholic acid ranging from 1.00 to 6.70 mg l^{-1}, is shown in Table 2.

ENZYMATIC METHOD

The enzyme used is 3α-hydroxysteroid dehydrogenase obtained from *Pseudomonas testosteroni* as described by Marcus and Talalay [8]. The

TABLE 2

Calorimetric method (DSC) (heat of fusion). Regression of the results obtained in the determination of weighed standards of cholic acid. Regression straight line expressed as $Y = A + BX$, where X is the quantity in mg and Y the heat in mcal. R indicates the correlation coefficient. For each X value, the Y values found are reported together with those obtained from the regression line, the standard error on Y, absolute and normalized regarding to the value of Y (standard relative error).

X	Y	Y (Regr.)	σ_Y	σ_Y/Y
1.00	19.8	20.8	1.8	0.087
1.20	21.2	25.4	1.7	0.067
1.40	29.7	30.0	1.6	0.054
3.00	66.1	66.8	1.0	0.016
3.50	76.8	78.3	1.0	0.013
3.56	82.9	79.7	1.0	0.013
3.76	88.0	84.3	1.0	0.012
4.15	95.9	93.3	1.1	0.012
4.39	102.1	98.8	1.2	0.012
4.40	100.2	99.0	1.2	0.012
6.70	145.8	152.0	2.3	0.015

Regression: $Y = A + BX$

Pr	Value	Std.err.
A	−2.22	2.37
B	23.0	0.63
R	0.9966	0.0275

Residual std.err.	3.4
% Variation coeff.	4.4
Student's Test	36.3
p	<0.001

enzyme can be used for the quantitative analysis of the 3α-hydroxysteroids and particularly of the cholic acids. The scheme is

$$3\alpha\text{-hydroxysteroid} + NAD^+ \underset{pH = 5.0}{\overset{\text{3α-hydroxysteroid dehydrogenase, pH = 9.0}}{\rightleftharpoons}} 3\text{-chetosteroid} + NDAH + H^+$$

By the use of pH 9 buffer and hydrazine, which reacts with the ketone formed, the reaction is completely shifted to the right and then an NADH quantitative spectrophotometric determination can be done at 340 nm. Details are given in a previous paper [9]. Table 3 gives the results of a series of cholic acid standards at concentrations of 1.00, 1.50, 2.00, 3.00, 4.00, and 5.00 mmole l^{-1}, each one repeated three times.

CHEMICAL METHOD

The method, described by Irvin et al. [7], is based on the Pettenkofer reaction which takes places between cholic acids and freshly distilled phurphurol at 65°C in sulphuric acid solution. Then, after rapid cooling, glacial

TABLE 3

Enzymatic method (U.V.) Regression of the results obtained in the determination of standards of cholic acid in methanolic solutions. Regression straight line expressed as $Y = A + BX$, where X is the concentration of the standard in mmole l^{-1} and Y the absorbance value at $\lambda = 340$ nm. R indicates the correlation coefficient. For each X value, the Y values are reported together with those obtained from the regression straight line, the standard error on Y, absolute and normalized regarding to the value of Y (standard relative error).

X	Y	Y (Regr.)	σ_Y	σ_Y/Y
1.00	0.197	0.198	0.005	0.025
	0.193			
	0.191			
1.50	0.297	0.301	0.004	0.014
	0.301			
	0.297			
2.00	0.414	0.404	0.004	0.009
	0.404			
	0.428			
3.00	0.600	0.609	0.003	0.005
	0.612			
	0.608			
4.00	0.813	0.815	0.004	0.005
	0.813			
	0.803			
5.00	1.050	1.021	0.006	0.006
	0.992			
	1.030			

Regression: $Y = A + BX$

Pr	Value	Std. err.
A	—0.008	0.007
B	0.206	0.002
R	0.9991	0.0108

Residual std.err.	0.013
% Variation coeff.	2.4
Student's Test	92.5
p	<0.001

acetic acid is added and spectrophotometric measurements are carried out [7] at 620 or 660 nm. These wavelengths do not correspond to particular points of the spectrum, but fall in a critical portion where the absorbance notably changes with the wavelength, as shown in Fig. 4.

A preliminary study of the working conditions was necessary. Using cholic acid as standard, selected absorbance values of the final solution are reported in Figs. 5 and 6 as function of the reaction time and the reading time. Figures 4—6 indicate that the best working conditions are: $\lambda = 750$—760 nm, reaction time = 15 min, reading time = 1 h.

The absorption spectra of other acids, such as deoxycholic, chenodeoxycholic, ursodeoxycholic and lithocholic acids, show neither maxima nor shoulders in the range 770—790 nm, while the maxima observed before 700 nm are affected by the absorption of the reactants which also change significantly with the phurphurol solution over short periods. However, the results

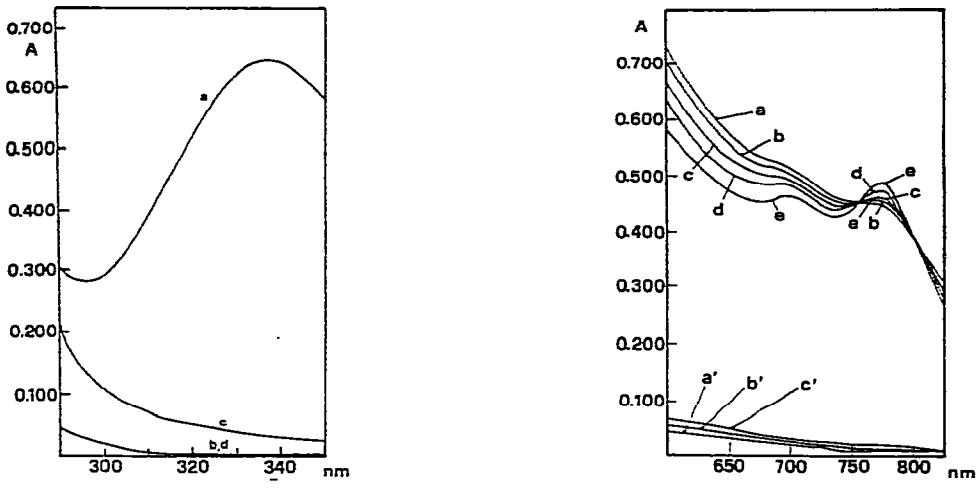


Fig. 3. Enzymatic analysis. Absorption spectra vs. water at room temperature; optical pathway 1.00 cm. Cholic acid standard, 3.00 mmole l^{-1} in methanol. a, Methanolic standard + Sterognost-3α; b, methanolic standard + hydrazine; c, methanol + Sterognost-3α; d, methanol + hydrazine.

Fig. 4. Spectrophotometric analysis by the chemical method. Absorption spectra at room temperature of sample (a, b, c, d, e) and of the blank reagents (a', b', c') at different reading times: a = 2 min; b = 12 min; c = 27 min; d = 47 min; e = 65 min; a' = 2 min; b' = 27 min; c' = 65 min. Ethanolic cholic acid standard, 0.55 mmole l^{-1}, reference, H_2O; optical pathway, 1.00 cm; reaction time, 15 min.

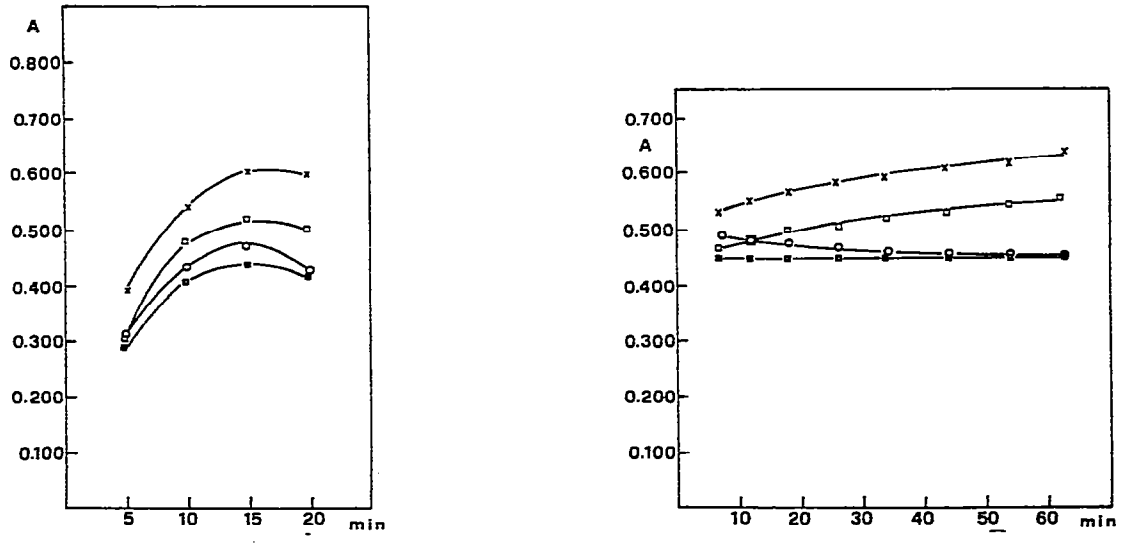


Fig. 5. Spectrophotometric analysis by the chemical method. Absorbances as function of the reaction time at 65°C at four different wavelengths: ■, λ = 755 nm; ○, λ = 775 nm; □, λ = 660 nm; ×, λ = 620 nm. Ethanolic cholic acid standard, 0.55 mmole^{-1}; reference, H_2O; optical pathway, 1.00 cm; reading time 1 h.

Fig. 6. Spectrophotometric analysis by the chemical method. Absorbances as function of the reading time at room temperature at four different wavelengths: ■, λ = 755 nm; ○, λ = 775 nm; □, λ = 660 nm; ×, λ = 620 nm. Ethanolic cholic acid standard, 0.55 mmole l^{-1}; reference, H_2O; optical pathway, 1.00 cm; reading time, 15 min.

TABLE 4

Chemical method (Vis.). Regression of the results obtained in the determination of standards of the cholic acid in ethanolic solution. Regression straight line expressed as $Y = A + BX$, where X is the concentration of the standard in mmole l^{-1} and Y the value of the absorbance at $\lambda = 755$ nm. R indicates the correlation coefficient. For each X value, the Y values found are reported together with those obtained from the regression straight line, the standard error on Y, absolute and normalized regarding to the value of Y (standard relative error).

X	Y	Y (Regr.)	σ_Y	σ_Y/Y
0.120	0.086	0.095	0.009	0.096
	0.084			
	0.098			
0.300	0.231	0.236	0.007	0.031
	0.229			
	0.231			
0.500	0.402	0.393	0.006	0.009
	0.408			
	0.423			
0.750	0.603	0.589	0.006	0.009
	0.603			
	0.562			
1.000	0.741	0.786	0.007	0.009
	0.827			
	0.766			
1.250	0.981	0.982	0.010	0.010
	0.955			
	1.011			

Regression: $Y = A + BX$

Pr	Value	Std.err.
A	0.006	0.0104
B	0.785	0.014
R	0.9976	0.0174

Residual std.err.	0.023
% Variation coeff.	4.4
Student's Test	57.3
p	<0.001

from several tests carried out on these acids show that the analysis can also be performed by this method reading in the range 700—800 nm and particularly at 750—760 nm, even if some working conditions are not so good as for cholic acid. The results of a series of triplicate determinations on standard ethanolic solutions of cholic acid at 0.120, 0.300, 0.500, 0.750, 1.000 and 1.250 mmole^{-1} are given in Table 4.

DISCUSSION

The analytical results of chemical cholic acid assay and those of the enzymatic and calorimetric methods can be compared on the basis of the

TABLE 5

Parameters determined from the results of the cholic acid analyses. A and B are the coefficients of the linear regressions $Y = A + BX$ (where Y is the observed absorbance or DSC area or fusion enthalpy and X is the concentration in mmole l^{-1} or the weight of the standard in mg). R is the correlation coefficient, p is the level of significance and represents the probability that the correlation is casual, CV is the remaining variation coefficient estimated as the percent ratio between the standard remaining deviation of the regression and the medium observed value. The operative concentration range is reported for each method.

Parameters	Method			
	Chemical	Enzymatic	Calorimetric oxidation	Calorimetric melting
A	0.001 ± 0.010	−0.008 ± 0.007	0.098 ± 0.088 ua	−2.22 ± 2.37 mcal
B	0.785 ± 0.014 l mmole^{-1}	0.206 ± 0.002 l mmole^{-1}	0.599 ± 0.014 l ua mmole^{-1}	23.0 ± 0.6 mcal mg^{-1}
R	0.998 ± 0.017	0.999 ± 0.010	0.996 ± 0.023	0.996 ± 0.028
p	<0.001	<0.001	<0.001	<0.001
CV(%)	4.4	2.4	4.0	4.5
Range	0.120 − 1.250 mmole l^{-1}	1.00 − 5.00 mmole l^{-1}	2.50 − 10.00 mmole l^{-1}	1.00 − 6.70 mg

data given in Tables 1—4. A summary of the parameters used for the comparison is given in Table 5. It should be noted that all the tested methods show a satisfactory correlation coefficient, although the best numerical value was given by the enzymatic method.

All methods have extremely good significance levels and yield a no correlation probability below 0.1%, i.e. a confidence level over 99.9%.

With regard to the precision of the examined methods, the CV values indicate that the enzymatic method has the greatest accuracy compared with the chemical and calorimetric methods which are more or less the same.

The sensitivity of the chemical and enzymatic methods can be directly compared by the angular coefficients of the regression straight lines. This comparison, just about the operating conditions, can be made because both methods refer to the same measurement unit (the absorbance of a 1.00 cm optical pathway). On the other hand, the calorimetric methods make use of area measurements which are not comparable with the absorbances, so that direct comparison between the angular coefficients of the regression straight lines is not possible. However, sensitivity can be compared when all the coefficients are made homogeneous, i.e. by dividing them by the full range of the utilized scales. Assuming they have a value of 1.00 for the full range of absorbances and a value of 6.00 as a full range of the surface oxidation measurements, the values 0.785, 0.206, and 0.100 will be respectively obtained for the chemical, enzymatic, and calorimetric methods. The sensitivity comparison with the calorimetric methods is not significant because it refers to the determination of a weight instead of concentration. Thus we can come to the conclusion that the chemical method is more sensitive than the enzymatic method but less precise. It therefore follows that the calorimetric technique, the precision of which is comparable with that of the chemical method, is as far as the oxidative reaction is concerned, less sensitive than the enzymatic method. Actually, on the basis of precision and sensitivity, we do not think that it would be advisable to suggest one method rather than another since the numerical values do not differ sufficiently. Various considerations would lead to the final choice between the three methods. The enzymatic assay is sensitive to the 3α-OH— group of bile acids and so it has an equal sensitivity toward the group of acids (cholic, deoxycholic, . . . etc.); obviously that is a great advantage for those assays, mostly clinical, where the quantitative determination of the bile acid group is requested. The method is highly selective and it is suitable for biological fluids without any previous separation [10]. The enzymatic method is rapid and therefore time-saving, but it requires expensive reactants. The drawback lies in proper storage and in the reconstitution. The sensitivities of the chemical and calorimetric methods are a function of the bile acids assayed [4,5,7]. These methods are not sufficiently selective and are affected by the chemical environment and the samples must therefore be subjected to a preliminary separation and purification whenever they do not show good purity characteristics. However, they do not require expensive or harmful reactants as does the chemical technique, but the latter, on the other hand, is very sensitive. The calorimetric techniques are fast and easy to carry out, if the right equipment is available, and, when applied to biological samples, they

are also very selective because they can be combined with TLC.

Chemical and calorimetric methods are recommended where the work is on pure samples or at least on samples of definite composition.

MATERIALS AND EQUIPMENT

Cholic acid standards were purchased from Merck (LAB). Ursodeoxycholic acid was supplied by Giuliani s.p.a. and other acids by Aldrich. The purity specifications of each sample were checked by gas chromatography. Sulphuric acid, glacial acetic acid, ethanol, and methanol 99.5% minimum purity were purchased from Merck. The phurphurol, supplied by Merck, was freshly distilled when required. The enzyme used was produced by Nyegard Co. As. (Sterognost-3α). The aluminium oxide used for calorimetric analysis was H and T and was supplied by Merck.

Beckman DK-2A and DU-2 instruments were used for the spectrophotometric measurements. The DSC curves were obtained with a Du Pont 990 DSC using an oxygen flow of 100 ml min^{-1} at a temperature gradient of 10°C min^{-1} for the oxidative decomposition method and a Perkin-Elmer DSC-2 with a dry nitrogen flow of 100 ml min^{-1} at a temperature gradient of 2.5°C min^{-1} for the heat of fusion method.

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