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CHOLIC ACIDS DETERMINATION FROM HEATS OF FUSION OBTAINED USING DIFFERENTIAL SCANNING CALORIMETRY. APPLICATION TO PHARMACEUTICAL PRODUCTS *

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ABSTRACT

The possibility of quantitatively analyzing some cholic acids by DSC is proposed using the heats of fusion. Some characteristic parameters of these analytical techniques have been evaluated for cholic, deoxycholic, chenodeoxycholic, ursodeoxycholic and lithocholic acids. The method has been applied to the analysis of two commercial drugs containing chenodeoxycholic and ursodeoxycholic acid, respectively. The results obtained are fully discussed.

INTRODUCTION

The increasing pharmacological interest in cholic acids and the extensive use of medicinal drugs containing such acids is well known. A reliable and simple method for the direct analysis of cholic acids in drugs is proposed here using the heats of fusion measured by DSC as analytical parameters.

Several colorimetric methods have been proposed, but they are not specific and require a very long operational time. A calorimetric method based on the heats of oxidation has been proposed [1] and will be compared with the one presently under discussion.

The enzymatic method described by Hurlock and Talalay [2], and applied by Iwata and Yamasaki [3], is specific and accurate but very expensive and time-consuming, while the procedure proposed here requires only a very short time and allows samples of reasonable mass to be used. A truly representative sample of the drug is obtained and no other reagent is required; only the sample under analysis is collected in a DSC capsule and accurately weighed. The DSC signal is proportional to the amount of cholic acid in the drug, and the peak temperatures can be used to identify the cholic acid.

MATERIALS AND METHODS

Cholic and deoxycholic acids were supplied by Merk (Lab.), while lithocholic acid was supplied by Fluka (Puriss.), and chenodeoxycholic and

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ursodeoxycholic acids were supplied by Giuliani S.p.A. Each was checked for purity by gas chromatography and DSC. The drug samples used were prepared by grinding and homogenizing the content of 10 capsules of each drug in a mortar and then weighing the selected quantity in an aluminum DSC pan using a Perkin-Elmer AR-2 electronic balance. The kit for the enzymatic analysis was supplied by Nyegard & Co.As. All other reagents were Merck. The DSC curves were obtained using a Perkin-Elmer DSC-2 instrument. The heating rate used was 2.5° C min^{-1}. The furnace atmosphere consisted of dry nitrogen at a flow rate of 100 ml min^{-1}. The spectrophotometric measurements were carried out using Bekman DK-2A and DU-2 spectrophotometers.

RESULTS AND DISCUSSION

The melting points of five of the more interesting cholic acids (corrected for the thermal inertia of the instrument) were analyzed by DSC and are summarized in Table 1, together with the corresponding values reported in the literature. The purity values of the standard used, obtained by DSC [8], were always higher than 99.8% and the results of many analyses on samples whose mass ranged between 2 and 4 mg for each acid show good reproducibility. In Table 2 the areas of the calorimetric peaks are collected in arbitrary units, with the corresponding quantities in mg. The heats of fusion (in kcal $mole^{-1}$) were obtained using indium to calibrate the instrument. Mean values and standard deviations ranging between 1 and 2% are also reported.

The heats of fusion may be divided into two groups: ursocholic, cheno-

TABLE 1

Melting points
(Sensitivity = 1 mcal sec^{-1} ; heating rate = 2.5°C min^{-1} ; nitrogen atmosphere; flow rate = 100 ml min^{-1}

Acid	Formula	M.W.	Found (°C ± 0.5)	Lit. values (°C)	Refs.
Cholic 3α, 7α, 12α-tri-hydroxycholanic	$C_{24}H_{40}O_5$	408.6	199.1	198	4,5,7
				200—201	6
Chenodeoxycholic 3α, 7α-dihydroxy-cholanic	$C_{24}H_{40}O_4$	392.6	164.5	162—164	6
				143	4,5,7
Deoxycholic 3α,12α-deoxy-cholanic	$C_{24}H_{40}O_4$	392.6	174.3	174—176	6
				176	4
				176—178	5,7
Ursodeoxycholic 3α, 7β-dihydroxy-cholanic	$C_{24}H_{40}O_4$	392.6	204.5	203	4,7
Lithocholic 3α-hydroxycholanic	$C_{24}H_{40}O_3$	376.6	186.0	186	4
				184—186	5—7

TABLE 2

Reproducibility of heats of fusion
(Sensitivity = 1 mcal sec^{-1}; heating rate = 2.5°C min^{-1}; nitrogen atmosphere; flow rate = 100 ml min^{-1})

Acid	Sample wt. (mg)	Area (arbitrary units)	Heat of fusion (kcal $mole^{-1}$)		
				Mean	S.D.
Cholic	4.40	35.42	9.32	9.48	0.09
	4.15	33.90	9.45		
	4.39	36.08	9.52		
	3.76	31.08	9.57		
	3.56	29.28	9.52		
Chenodeoxycholic	4.11	23.52	6.36	6.27	0.10
	5.54	31.87	6.40		
	7.15	39.82	6.19		
	3.04	16.78	6.14		
	5.87	32.97	6.25		
Deoxycholic	4.22	24.20	6.38	6.70	0.18
	4.18	25.99	6.92		
	4.73	28.88	6.79		
	4.00	24.24	6.74		
	4.34	25.98	6.66		
Ursodeoxycholic	3.37	9.21	7.60	7.51	0.11
	5.26	14.12	7.46		
	4.04	10.93	7.52		
	5.01	13.22	7.33		
	4.47	12.30	7.65		
Lithocholic	4.43	37.23	8.98	9.08	0.06
	3.32	28.28	9.09		
	4.09	34.84	9.10		
	4.41	37.43	9.06		
	3.65	31.41	9.18		

TABLE 3

Regression lines: analytical forms $y = a + bx$ (y in mcal, x in mg)

Acid	Range (mg)	a (mcal)	σ_a (mcal)	b (mcal mg^{-1})	σ_b (mcal mg^{-1})	R	CV%
Cholic	1.5—7.0	−2.22	2.37	23.01	0.63	0.997	4.5
Chenodeoxycholic	1.0—7.0	−0.81	0.96	15.92	0.24	0.999	2.8
Deoxycholic	1.0—7.0	−0.55	0.60	17.09	0.26	0.997	3.7
Ursodeoxycholic	0.5—6.0	−0.65	0.60	19.08	0.18	0.999	1.6
Lithocholic	1.0—6.5	−1.83	1.88	24.19	0.57	0.998	1.9

The significance level is $P < 0.001$ in all cases.

TABLE 4

Comparison of two medicinal drugs

Drug containing			
Chenodeoxycholic acid		Ursodeoxycholic acid	
Chenodeoxycholic acid	71.4%	Ursodeoxycholic acid	83.3%
Corn starch	26.6%	Starch	10.0%
Aerosil	1.4%	Magnesium stearate	3.3%
Magnesium stearate	0.6%	Precipitated silica	3.3%

deoxycholic and deoxycholic acids whose heats of fusion range between 6.5 and 7.5 kcal mole^{-1}, and cholic and lithocholic acids, whose heats of fusion are about 9 kcal mole^{-1}.

Regression lines were calculated using samples of 0.5—7.0 mg mass. The results are summarized in Table 3. A good correlation was found between the quantities of the standards and the measured heats (R = 0.997 — 0.999).

The proposed method is therefore useful for the quantitative analysis of the examined cholic acids. In particular, the "a" intercept values being very close to the "σ_a" values, emphasize that the calibration curves, allowing for experimental errors, pass through the origin. From the CV% values a good reproducibility of the measures can be observed between 2% for the cheno-deoxy-, ursodeoxy- and lithocholic acids, and 4% for the cholic and deoxycholic acids.

The method was then applied to the analysis of chenodeoxycholic and ursodeoxycholic acids in two medicinal drugs prepared as capsules and sold in Italy and other countries. Their compositions are compared in Table 4. All the data used to calculate the calibration curves (regression lines in Table 3)

TABLE 5

Calorimetric determination of chenodeoxycholic acid

Experimental values vs. values calculated from the regression line (y in mcal, x in mg, y(regr.) = —0.81 + 15.92 x)

x	y	y(regr.)	σ_y	σ_y/y (%)
1.08	16.5	16.4	0.7	4.6
1.58	24.2	24.4	0.7	2.7
1.72	26.6	26.6	0.6	2.4
2.10	31.4	32.6	0.6	1.8
2.53	38.1	39.5	0.5	1.3
2.82	45.7	44.1	0.5	1.1
3.04	47.5	47.6	0.5	1.0
4.11	66.5	64.6	0.5	0.7
5.45	82.6	86.0	0.6	0.7
5.54	89.2	87.4	0.6	0.7
5.87	93.3	92.7	0.7	0.8
7.15	112.6	113.0	1.0	0.9

TABLE 6

Calorimetric determination of ursodeoxycholic acid
Experimental values vs. values calculated from the regression line (y in mcal, x in mg, y(regr.) = —0.65 + 19.08 x)

x	y	y(regr.)	σ_y	σ_y/y (%)
0.66	12.0	11.9	0.5	4.1
0.75	13.6	13.7	0.5	3.5
2.08	39.0	39.0	0.3	0.8
2.48	45.9	46.7	0.3	0.6
2.52	47.3	47.4	0.3	0.6
2.65	49.4	49.9	0.3	0.6
3.37	65.2	63.7	0.3	0.5
4.04	77.4	76.4	0.3	0.5
5.01	93.6	95.0	0.6	0.5
5.26	100.0	99.7	0.5	0.5

for these two acids are collected in Tables 5 and 6, respectively.

Considering the mass range it is possible to see that the standard deviation on the measured heats is always lower than 2% when the mass of the sample is greater than about 2 mg. Determination of the acids present in the two medicinal drugs was therefore carried out on samples whose mass was greater than 2 mg. The results obtained are collected in Tables 7 and 8, together with the corresponding results obtained from analysis of the same samples by the enzymatic method, with 3α-hydroxysteroid dehydrogenase [2,3,9] reported as reference.

It is seen that, even if the reproducibility is good, when considering the analysis of the ursodeoxycholic acid in the drug there are losses of about 10% with respect to the nominal value stated by the manufacturers.

Recovery tests on the cholic acids present in the two drugs under examin-

TABLE 7

Enzymatic and calorimetric determinations on drugs containing 71.4% chenodeoxycholic acid (values in mg)

Calorimetric method				Enzymatic method			
Calcd.	Found	Mean	S.D.	Calcd.	Found	Mean	S.D.
2.86	2.89			124.3	123.3		
	2.89				124.8		
	2.84				124.1		
	2.85	2.87	0.02		125.6	123.6	1.6
	2.88				124.8		
	2.89				120.9		
	2.84				121.7		
% Difference between calcd. and mean values: +0.3				% Difference between calcd. and mean values: —0.6			

TABLE 8

Enzymatic and calorimetric determinations on drugs containing 83.3% ursodeoxycholic acid (values in mg)

Calorimetric method				Enzymatic method			
Calcd.	Found	Mean	S.D.	Calcd.	Found	Mean	S.D.
3.33	3.07			116.8	106.8		
	2.83				101.4		
	3.11				106.9		
	3.05	3.00	0.10		109.9	105.8	2.8
	2.93				102.2		
	3.09				105.7		
	2.94				107.8		
% Difference between calcd. and mean values: —9.9				% Difference between calcd. and mean values: —9.4			

ation were carried out using both the calorimetric and enzymatic methods. The results are collected in Tables 9—12. For the chenodesoxicholic acid, percent differences from the means of the recoveries were obtained which do not notably differ from the reproducibility of the measures. On the contrary, for the ursocholic acid these differences are more remarkable and, in general, are negative (losses). Considering that the reference values used in

TABLE 9

Recovery on drugs containing 71.4% chenodeoxycholic acid using the calorimetric method (values in mg)

Found in 4.00 mg of drug	Added standard	Found	Recovery (%)	Difference % of recovery	Mean value of recoveries (%)	Difference % of mean recovery
2.87	1.13	4.20	105.0	+5.0		
	1.13	4.08	102.0	+2.0		
	1.13	4.01	100.3	+0.3	101.8	+1.8
	1.13	3.99	99.8	—0.2		
	1.13	4.07	101.8	+1.8		
2.87	2.35	5.09	97.5	—2.5		
	2.35	5.34	102.3	+2.3		
	2.35	5.41	103.6	+3.6	100.8	+0.8
	2.35	5.21	99.8	—0.2		
	2.35	5.25	100.6	+0.6		
2.87	2.66	5.57	100.7	+0.7		
	2.66	5.50	99.5	—0.5		
	2.66	5.50	99.5	—0.5	99.9	—0.1
	2.66	5.54	100.2	+0.2		
	2.66	5.51	99.6	—0.4		

TABLE 10

Recovery on drugs containing 71.4% chenodeoxycholic acid using the enzymatic method (values in mg)

Found in 87.0 mg of drug	Added standard	Found	Recovery (%)	Difference % of recovery	Mean value of recoveries (%)	Difference % of mean recovery
61.2	39.3	104.0	103.5	+3.5		
	39.3	105.2	104.7	+4.7	103.0	+3.0
	39.3	101.3	100.8	+0.8		
61.2	62.8	120.9	97.5	−2.5		
	62.8	123.3	99.4	−0.6	100.1	+0.1
	62.8	128.4	103.5	+3.5		
61.2	78.5	137.8	98.6	−1.4		
	78.5	139.4	99.8	−0.2	99.2	−0.8
	78.5	138.5	99.1	−0.9		

the recovery tests are not calculated, but result from the means of quadruplicate analyses carried out on the drug, the losses observed in the recoveries can be roughly attributed to only the added quantities. In such a way losses of about 7—8%, with respect to the added quantities, can be observed using the calorimetric method (unless the 0.58 mg addition is so small that the

TABLE 11

Recovery on drugs containing 83.3% ursodeoxycholic acid using the calorimetric method (values in mg)

Found in 4.00 mg of drug	Added standard	Found	Recovery (%)	Difference % of recovery	Mean value of recoveries (%)	Difference % of mean recovery
3.00	0.58	3.65	102.0	+2.0		
	0.58	3.65	102.0	+2.0		
	0.58	3.65	102.0	+2.0	101.5	+1.5
	0.58	3.61	100.8	+0.8		
	0.58	3.60	100.6	+0.6		
3.00	1.84	4.66	96.3	−3.7		
	1.84	4.75	98.1	−1.9		
	1.84	4.62	95.5	−4.5	97.1	−2.9
	1.84	4.77	98.6	−1.4		
	1.84	4.70	97.1	−2.9		
3.00	2.54	5.42	97.8	−2.2		
	2.54	5.35	96.6	−3.4		
	2.54	5.32	96.0	−4.0	96.7	−3.3
	2.54	5.36	96.8	−3.2		
	2.54	5.34	96.4	−3.6		

TABLE 12

Recovery on drugs containing 83.3% ursodeoxycholic acid using the enzymatic method (values in mg)

Found in 70.0 mg of drug	Added standard	Found	Recovery (%)	Difference % of recovery	Mean value of recoveries (%)	Difference % of mean recovery
51.1	39.3	84.8	93.8	−6.2		
	39.3	85.6	94.6	−5.4	95.4	−4.6
	39.3	88.3	97.7	−2.3		
52.0	58.9	104.8	94.5	−5.5		
	59.9	107.2	96.7	−3.3	95.4	−4.6
	58.9	105.5	95.1	−4.9		
51.1	78.5	126.0	97.6	−2.4		
	78.5	122.5	94.1	−5.9	95.6	−4.4
	78.5	123.3	95.1	−4.9		

randomly distributed errors of the method overcome the systematic error), while losses of about 7—10% take place when using the enzymatic method. These losses are in good agreement, within the reproducibility of the measures, with those given in Table 8.

Interferences due to the other substances present in the examined drugs can explain these losses. In particular, absorption phenomena may be suggested as the main cause of losses of the same order of magnitude observed with two different methods of determination, i.e. calorimetric and enzymatic.

CONCLUSION

The method proposed for the analysis of cholic acids by determination of the heats of fusion appears to be suitable for application, especially with regard to its simplicity and speed. The losses observed when the method is applied to the analysis of ursocholic acid in the drug are, unexpectedly, of the same order as those observed using the enzymatic method on the same samples and, as a first hypothesis, can be assigned to the interferences of other substances present. Future studies could be directed at characterizing these interferences which, because they are so similar in two such different methods, can be attributed in a first approximation to absorption phenomena.

The data required for comparison of the determination using the heats of fusion and that using the heats of oxidation are summarized in Table 13. The sensitivity ratio between the method using the heats of fusion and that using the heats of oxidation is 1 : 30 to 1 : 40.

It can be concluded that the oxidation method is more useful for the analysis of cholic acids in biological samples, after chromatographic separation, because of its higher sensitivity. On the other hand, the heats of fusion

TABLE 13

Heats of oxidation and heats of fusion

Oxidation: sensitivity = 0.05 mcal sec^{-1}; heating rate = 10°C min^{-1}; = oxygen atmosphere; flow rate = 100 ml min^{-1}; apparatus = Du Pont model 990 DSC.

Fusion: Sensitivity = 1 mcal sec^{-1}; heating rate = 2.5°C min^{-1}; nitrogen atmosphere; flow rate = 100 ml min^{-1}; apparatus = Perkin-Elmer DSC 2.

Acid	Oxidation		Fusion	
	Temp. * (°C)	Heat of oxidn. ** (kcal $mole^{-1}$)	Temp. (°C)	Heat of fusion (kcal $mole^{-1}$)
Cholic	237	—270.2	199.1	9.48
Chenodeoxycholic	220	—267.0	164.5	6.27
Deoxycholic	234	—247.8	174.3	6.70
Ursodeoxycholic			204.5	7.51
Lithocholic	218	—262.0	186.0	9.08

* Approximate value graphically estimated as the starting peak temperature; no correction was applied for thermal delay of the apparatus

** Evaluated graphically.

method is suggested for the analysis of cholic acids in the drugs because it is directly applicable without any previous separation and/or extraction treatment.

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