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THERMAL ANALYSIS OF ANHYDROUS AND HYDRATED CHOLESTEROL
Application to gallstones

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Abstract

Cholesterol constitutes the major component of most gallstones. It was identified and determined, in gallstones, issued from eleven patients, by thermal analysis: differential scanning calorimetry (DSC), with the use of the melting temperature and enthalpy, thermogravimetry (TG), with the mass loss of water. Anhydrous cholesterol (ChA) was characterized by two endothermic peaks (polymorphic, melting) and cholesterol monohydrate (ChH) by two endothermic peaks (dehydration, melting), too. ChA needle and ChH plate crystals were observed under polarizing light microscopy. The numerous stones obtained from nine patients were cholesterol stones: the ChA was higher 45 and lower 96%. ChH was present in stones of three patients.

Keywords: anhydrous cholesterol, DSC, DTA, gallstones, hydrated cholesterol, melting, TG

Introduction

Biliary 'sludge' represents an early stage of gallstone formation and gallstones are bile concretions [1]. The categorizing of gallstones into three kinds: cholesterol, brown pigments and black pigment stones, was proposed at the National Institute of Health International Gallstone Workshop at 1982 [2].

Mixed and cholesterol stones are the main gallstones in western countries, their average value in cholesterol is 86.3% (range 41.7 to 100 %), and are characteristically multifaceted, mulberry shaped or ovoid and, on fracture, have varying degrees of pigmented centers, layers or shells. Brown pigment stones have a 11.4% cholesterol content (range 2.8 to 28.3%); they crush easily and have dark and light lamination on cross section. Black pigment stones tend to be small, resist manual crushing and pro-

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duce a shiny, glasslike surface on fracture. The average value in cholesterol is 0 to 9.7% [3]. A solitary stone is present in the 86.3% of cases [4].

Cholesterol monohydrate (ChH) constitutes the major component of most gallstones [5–8] and a major crystalline solid of many human atherosclerotic plaques [9]. Anhydrous cholesterol (ChA) can crystallize from model and native biles as filamentous crystals covered by a surface layer of lecithin molecules. During growth, filamentous crystals transformed via metastable intermediates into classical plate-like cholesterol monohydrate crystals [10]. The exact crystalline form of cholesterol which precipitates in the nucleus of forming and growing stone, is not known.

The main components (cholesterol, calcium bilirubinate, calcium carbonate) were characterized by infrared spectrometry and X-ray diffraction, in the 1950's. The IR spectrum provides a fingerprint for identification and is an accurate test for the determination of molecular structure. FTIR analysis was useful in characterizing cholesterol, and brown and black pigment gallstones [11, 12]. The applications of FTIR in the study of other calculi are very important [13].

Thermogravimetry (TG) can be used as a rapid and accurate method for cholesterol determination, in mixture with calcium oxalate and calcium carbonate in gallstones [14]. But other organic compounds (like bilirubine) can be present in gallstones, and decomposed in the same interval. The monohydrate form of cholesterol was determined by water mass loss [15] and X-ray diffraction [7, 16, 17]

The polymorphic transition phase of ChA in gallstones was analyzed by differential scanning calorimetry (DSC) [18].

In this study the authors present results obtained by thermal analysis, first, on ChA and ChH, and then the level of both cholesterol determined in gallstones from eleven patients.

Experimentals

Samples

ChA was obtained from Sigma – Aldrich Chimie and Prolabo. The solvents: ethyl alcohol and methyl alcohol were ACS grade reagents obtained from Carlo Erba.

ChA was recrystallized in dry methanol, it presented needle-like crystals. ChA recrystallized in the mixture ethanol–water (95–5), led to cholesterol hemiethanolate (Ch_{heth}) with plate-like crystals [16]. This sample was immersed in bidistilled water during 48 h, and then, ChH was isolated by filtration and washed repeatedly with bidistilled water. It was slightly and quickly dried under vacuum at room temperature. The wet monohydrate plate-like crystals were placed on a microbalance pan and periodically weighed. When constant mass was observed, it was assumed that all excess water had evaporated from crystal surfaces. The dried sample study was made by thermal analysis. Needlelike ChA and platelike ChH crystals were typified under polarizing light microscopy. Karl Fisher determination of water gave 4.34% for ChH (4.45% theoretical), 1.00% for Ch_{heth} and 0% for ChA, (with a standard deviation of 0.11%).

Eleven patients gallstones were stocked in closed boxes. Those, corresponding to the first eight patients, are 15 years old (used, mainly, in the development of the thermal technics), the last three, less than three months old. The stones were totally tested, when their mass was lower than 20 mg. Otherwise, the calculi were cut and analysis was carried out on these chips.

Thermal analysis

DSC Setaram 92 (Scientific and Industrial Equipment, Setaram S.A., 69300–Caluire/ France) was used, with 2°C min^{-1} heating rate, from room temperature to 570°C, under static air. The sample mass (10–20 mg) was put in aluminium crucibles.

DTA-TG (simultaneous differential thermal analysis – thermogravimetry) Setaram 92, was used, with 5°C min^{-1} heating rate, from room temperature to 850°C, under reconstituted air (oxygen 22 - nitrogen 78%) sweeping of 0.5 L h^{-1}. Thermocouples were Platinel®. The sample mass (10–20 mg) was put in platinum crucibles. Calcium oxalate monohydrate was used in Thermogravimetry for the mass loss standardization.

In DSC and DTA-TG temperature standardization was made by the melting of benzoic acid, tin, lead, zinc and aluminium. Enthalpy standardization was made with these standards. The indicated temperatures (°C), of the presented cholesterol and gallstones, were attributed to the maximum (T_{max}) and the extrapolated (T_{onset}) of each peak. Temperature accuracy was 0.1°C in DSC, and 1°C in DTA-TG.

Results and discussion

Anhydrous cholesterol (ChA)

Anhydrous cholesterol (ChA) was recrystallized from dry methanol. The needlelike crystals were isolated by filtration and dried by air sweeping at room temperature and analysed by thermal technics. The DSC curve (heating from 20 until 170°C) showed a first small endothermic peak (polymorphic transition) at about 40°C and a second one (melting) at about 150°C (Fig. 1). By TG, a high stability (no mass loss) is observed from 20 to 200°C. This confirmed the dryness of the crystals. The DTG (derivative thermogravimetry) peak maximum is at about 365°C, with a mass loss of 55% at this temperature. The ChA sample, submitted to two runs, with successive heating (presence of an endothermic melting peak) and cooling (presence of an exothermic crystallization peak), showed during the second run, decreased temperatures and enthalpies. A low degradation and a lack of crystallization can explain these variations. Therefore, to avoid some errors in gallstone thermal analysis, the stones were studied without thermal pretreatment.

The results, obtained on two ChA samples (Prolabo and Sigma):15 tests by DSC and 14 tests by DTA-TG, are in Table 1. Temperature (T in °C), enthalpy (ΔH in J g^{-1}) of polymorph and fusion, by DSC, maximum of the DTG (derivative thermogravimetry) peak (in °C), decomposition rate (% min^{-1}), by TG, are given in this Table.

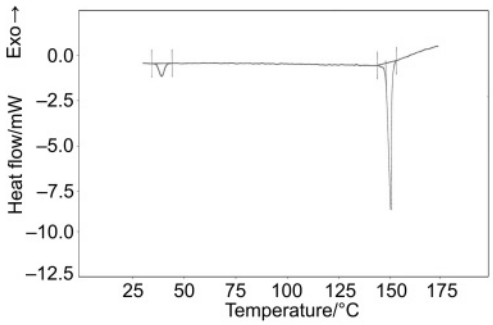

Fig. 1 DSC curve of anhydrous cholesterol (heating rate 2°C min^{-1} under static air, sample mass 8.9 mg; 1st endothermic peak T_{max}=39.7°C and T_{onset}=37.2°C, ΔH_p=7.16 J g^{-1}; 2nd endothermic peak T_{max}=150.9°C and T_{onset}=149.0°C, ΔH_f=54.1 J g^{-1})

Table 1 DSC and DTG peaks for two ChA samples. DSC (heating rate 2°C min^{-1}, 15 tests), TG (heating rate 5°C min^{-1}, 14 tests), *SD*=standard deviation, *RSD*=relative standard deviation, *p*=polymorph, *f*=fusion

	DSC peaks						DTG peak	
	Polymorhic temperature/°C		Melting temperature/°C		Enthalpy/J g^{-1}		T_{max}/ °C	Rate/ % min^{-1}
	T_{onset}	T_{max}	T_{onset}	T_{max}	ΔH_p	ΔH_f		
Mean	36.9	39.6	148.8	151.1	7.0	54.6	366	7.36
SD	0.706	0.718	0.900	1.096	0.325	1.79	9.28	0.346
RSD /%	1.92	1.82	0.60	0.73	4.64	3.27	2.54	4.70

The identification of cholesterol in gallstones, could be made by the use of cholesterol melting temperature, and, if necessary, by the DTG maximum peak temperature. Its quantification would be made by the use of the melting transition enthalpy of the purum ChA (mean =54.6±1.79 J g^{-1}).

Cholesterol hydrate (ChH)

The preparation of ChH was presented in the part 'Samples'. DTA and TG curves obtained on ChA, Ch_{heth} and ChH are shown in Fig. 2.

Ch_{heth} curves show a small endothermic peak at about 37°C with a regular increasing mass loss until 107°C (4.06%). This could be attributed to a partial loss of volatilized water and ethanol. In fact, a wet sample presented a larger peak and a higher mass loss. The endothermic peak at 110°C represents the alcohol volatilization [16]. Ch_{heth} in contact with water does not produce an endothermic peak at 110°C. The obtained ChH shows a peak at 59°C due to water volatilization. Only bounded water is present in ChH. The free water has gone out, before DTA-TG heating.

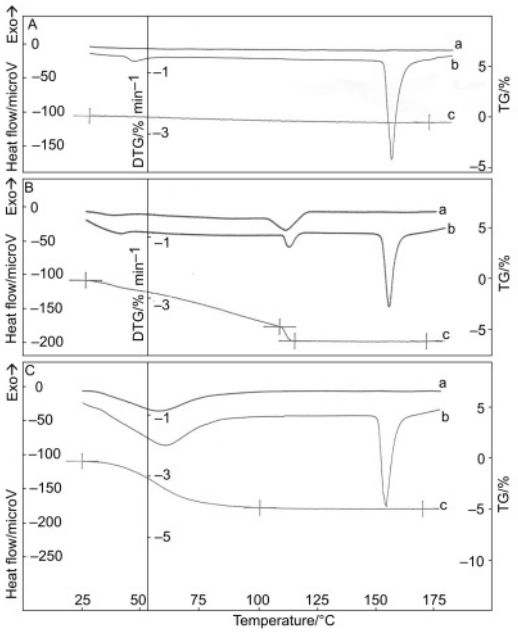

Fig. 2 Thermal analysis of cholesterol (DTG; a – DTA; b – TG; c – heating rate 5°C min^{-1} under 0.5 L h^{-1} air sweeping) A=ChA, sample mass 17.7 mg, DTA – 1st endothermic peak T_{max}=44°C, 2nd endothermic peak T_{max}=152°C and T_{onset} =147.5°C; TG – mass loss, from room temperature to 170°C=0 %. B=Ch_{heth}, sample mass 16.0 mg, DTA – 1st endothermic peak T_{max}=37°C, 2nd endothermic peak T_{max}=110°C, 3rd endothermic peak T_{max}=151°C and T_{onset}=148°C; TG – mass loss, from room temperature to 107°C=4.06 %, from 107°C to 113°C=1.31%, from 113°C to 170°C=0%. C=ChH, sample mass 20.3 mg; DTA – 1st endothermic peak T_{max}=59°C, 2nd endothermic peak T_{max}=152°C and T_{onset}=148°C; TG – mass loss, from room temperature to 100°C=4.46%, from 100°C to 170°C=0%

For Ch_{heth} and ChH samples, if stocking a long time at room temperature, and/or under vacuum, dessication takes place with endothermic peaks and smaller mass losses. The results of numerous runs made on these two samples and on ChA are shown in Table 2.

Another preparation of cholesterol hydrate was made. ChA was put, for 14 days at room temperature, in a moisture atmosphere. The increasing mass was 9.75%. By DSC, a wide endothermic peak appeared at T_{max}=53.9°C.

Wada *et al*. [7] observed the wide endothermic peak attributed to moisture, another sharp at 70°C and a slightly decreasing of melting temperature (148°C) for the anhydrous form and for the hydrate (146°C). Several endothermic peaks were observed by using a closed pan, under elevated pressure [15, 16].

So, ChA can rehydrate when placed in a moisture atmosphere. ChH seems to lose crystallization water under air sweeping at room temperature. The characterization of ChH will be difficult if gallstones are crushed and dried.

Table 2 DTA peaks and TG *mass loss* for ChA, Ch_{heth} and ChH (heating rate 5°C min^{-1}, *SD=standard deviation, RSD*=relative standard deviation; all the samples had the same curves by DSC)

ChA (14 tests)	Polymorphic temperature/°C			Melting temperature/°C		Mass loss from 20 to 170°C/%
	T_{max}			T_{onset}	T_{max}	
Mean	43			148	152	~0
SD	1.82			0.73	0.71	
RSD/%	4.22			0.50	0.46	
Ch_{heth} (10 tests)	Water and ethanol volatilization temperature/°C	Ethanol volatilization temperature/°C		Melting temperature/°C		Mass loss from 20 to 170°C/%
	T_{max}	T_{max}	T_{onset}	T_{onset}	T_{max}	
Mean	40	108	110	148	151	5.15
SD	4.97	1.18	1.64	0.81	1.05	0.22
RSD/%		1.10	1.49	0.55	0.69	4.30
ChH (5 tests)	Water volatilization temperature/°C			Melting temperature/°C		Mass loss from 20 to 170°C/%
	T_{max}			T_{onest}	T_{max}	
Mean	58			148	152	4.52
SD	3.49			0.19	0.52	0.20
RSD /%				0.13	0.34	4.37

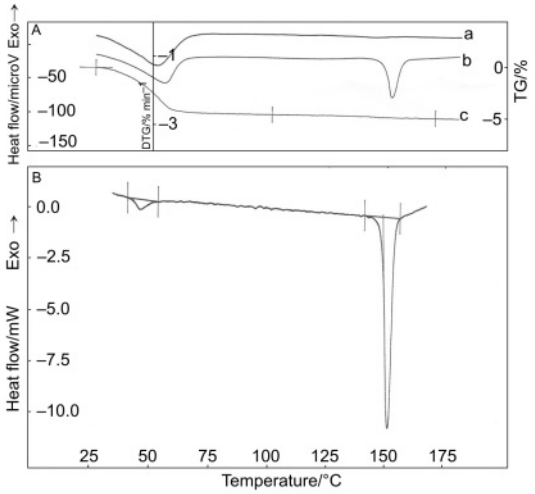

Fig. 3 Thermal analysis of gallstones. A=patient N°11; a – DTG; b – DTA; c – TG; heating rate 5°C min^{-1} under 0.5 L h^{-1} air sweeping, sample mass 10.5 mg; DTA – 1st endothermic peak T_{max}=55°C, 2nd endothermic peak T_{max} =149°C and T_{onset}=145°C; TG – mass loss, from room temperature to 100°C=4.35%, from 100°C to 170°C= 0.14%. B=patient N°5, heating rate 2°C min^{-1} under static air, sample mass 21.3 mg; DSC – 1st endothermic peak T_{max}=45.4°C and T_{onset}=42.2°C, ΔH_p=3.62 J g^{-1}; 2nd endothermic peak T_{max}=151.1°C and T_{onset} =148.3°C, ΔH_f=51.8 J g^{-1}

Analysis of gallstones

With the aim of characterizing the cholesterol in gallstones by the use of its melting, it is necessary to point a probable temperature interval of melting. It is well known, that the presence of impurities in a crystalline organic compound, decreases the melting temperature. So, we suggest an interval of the melting peak temperature of cholesterol in gallstones. The choice of the probable interval was made from every analysed stone: 144 to 152°C for T_{max} and 139 to 149°C for T_{onset}.

We recall that ChH would be at the start of the gallstone crystal formation. The differentiation between anhydrous and hydrated forms, cannot be applied only on the endothermic melting peak, because at a higher temperature than 100°C, only anhydrous form will be present. So, the quantitative analysis of the cholesterol will be expressed in ChA %.

The repetability of the ChA determination was made on stones issued from the same patient:

– 10 tests were run on 10 small stones. Each stone (9.0 to 23.3 mg) was totally analysed by DSC;

– 10 tests were run on one stone (about 1 cm length), powdered and dried at room temperature. The homogeneous sample, studied by DSC, was 8.8 to 16.3 mg.

The results are in Table 3.

Table 3 Repeatabilities of ChA determination in gallstones by DSC (The stones are issued from the same patient; heating rate 2°C min^{-1}, *SD*=standard deviation, *RSD*=relative standard deviation)

Samples	Melting peak	T_{max}/°C	T_{onset}/°C	ChA/%
10 tests on 10 stones	Mean	149.8	144.1	77.8
	SD	0.947	0.770	3.80
	RSD/%	0.63	0.53	4.88
10 tests on one powdered and dried stone	Mean	148.9	146.0	92.9
	SD	0.853	0.900	1.70
	RSD/%	0.57	0.62	1.83

A small variation appears in the values of T_{max} (1°C deviation) and T_{onset} (1.9°C deviation). RSDs are good in these two cases (0.5 to 0.6%). But, the repeatability of the ChA determination is better for the homogeneous powdered sample (1.83%), than the 10 stones tested separately (4.88%). In this last case, some minor components can be present in the stones. Gallstones must be analysed without physical modification, even if some small variations can appear in the results. All stones had a dry appearance, except one, mixed with bile. This stone, after dessication in absorbent paper, presented 4.50% as a mass loss, corresponding to ChH evolved water.

We recall that the differentiation between ChA and ChH, by thermal technics, based on the presence of the small polymorphic peak at about 40°C for ChA, and the water volatilization at about 58°C for ChH, accompanied with about 4.5% of mass loss from 20 to 170°C. In this paper, we will present, only, the results concerning the cholesterol in the biliary stones. In Table 4, are, for each patient, the number of stones, geometry, length, color, expression in ChA (%).

We recall that the stones issued from the eight first patients, are about 15 years old, and stocked in closed boxes. The tests, done on these stones were used in the development of the thermal analysis. The stones of the patients N°9, 10 and 11 are ChH.

Tests were done on central and peripherical fragments of several stones. Table 4 shows some variations in several stones extracted from the same patient. Indicated results are corresponding to several tests done on each stone, and/or on several stones obtained from the same patient. ChA (%) was determined by DSC, with the use of the melting enthalpy. The water determination (mass loss from room temperature to about 170°C), was made by TG). In some cases, a mass loss slightly higher than the theoretical value of 4.45% can be attributed to free water. In these cases, we deduced ChH (%) from ChA (%) by the use of the relation ChH (%)=1.0445ChA (%).

Conclusions

These results, obtained by thermal analysis, show the presence of cholesterol, in the greater part of the stones obtained from 11 patients. The choice of ChA determination is based on the melting enthalpy. We think that the gallstones must be tested in their

Table 4 ChA determination of gallstones issued from 11 patients

Patient N^o	Number of stones	Length/cm	Geometry	Color exterior	Color centre	ChA/%
1	1	>0.5	elongated	white	white	70 to 75
	2	<0.5	elongated	white	white	
2	1	1.5	elongated	black	white	90 to 98 nucleus
3	1	1	spherical	white	dark-red	
	3	0.5	pyramidal	white	dark-red	87 to 89
	~10	<0.5	pyramidal	white	black	
4	1	1.5	elongated	white	white	0
	–	–	–	–	–	–
	1	>0.5	pyramidal	grey	white	
	2	0.5	pyramidal	grey	white	9 to 10
	~10	<0.5	pyramidal	grey	grey	
5	3	1	spherical	dark-red	white	91 (external) and 98 (nucleus)
6 (a)						77 to 78
7	1	1.5	pyramidal	grey	grey	16 (external) and 44 (nucleus)
	1	1	elongated	grey	grey	
	1	0.5	pyramidal	grey	grey	
8	1	1.5	elongated	grey	grey	93
	–	–	–	–	–	–
	2	0.5	pyramidal	white	dark-red	89 (external) and 66 (nucleus)
9	1	1.2	pyramidal	grey	grey	49 to 67 (b)
10	~15	1	pyramidal	white	dark-red	91 to 96 (c)
	–	–	–	–	–	–
	~30	0.3	pyramidal	white	dark-red	67.5 to 82 (d)
11 (f)						84 to 94.5 (e)

(a) The sample was supplied under grey homogeneous powdered form; (b) 51 to 70, (c) 95 to 100, (d) 70.5 to 86, (e) 88.5 to 99, expressed in ChH (%); (f) the sample was supplied by several white fragments

physical form, and not after crushing, worse after drying at 80 or 110°C. Results would be better in sensibility, but the composition may be slightly different from the gallstones analysed, as received.

ChH seems to be unstable. It gives ChA by losing its bounded water. Water of hydration presumably diffuses through the anhydrous materials and escapes from the edges of the crystals [8]. In Finland, it was observed that ChA was, by far, the most abundant compound in gallstones [17], in opposite in the western countries ChH is the most important component.

The identity between needle – anhydrous structure and plate – cholesterol monohydrate was observed by optical microscopy. The transition to the anhydrous form can be speeded up by increasing the drying temperature [16]. The gallstones issued from the eight first patients were 15 years old, and therefore, the presence of only ChA would be explained by a possible volatilization of water during this stocking. The three latter gallstones were ChH stones.

Nucleated bile is typified by platelike ChH crystals, that agglomerate via mucin glycoproteins to form cholesterol gallstones. It was suggested that crystalline cholesterol, in bile, may not be completely mature or hydrated initially, but undergoes a series of transformations to become thermodynamically stable monohydrate plates [10]. It was shown that the bovine gallblader mucin accelerated ChH crystal growth in supersatureted model bile [19]. Cholesterol crystals bind directly to mucin, whereas calcium salts and pigments deposit on APF/CBP and ApN bind to the mucin [20]. Biliary proteins are assumed to play an important role in cholesterol gallstone formation [21].

We consider that the stones obtained from nine patients are cholesterol stones: the level is higher 45 and lower 98% in ChA.

Thermal analysis (DSC and TG technics) can characterize the type of cholesterol (anhydrous or hydrated), and determine the cholesterol content in gallstones as received. This text would be considered as a continuation of the earlier study on urinary calculi by thermal analysis [22].

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