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THERMAL ANALYSIS AND MICROSCOPICAL CHARACTERIZATION OF CHOLESTEROL IN GALLSTONES

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Cholesterol constitutes the major component of most gallstones. It was identified and determined in gallstones by thermal analysis technique (DSC and TG-DTA), mainly by the use of the melting temperature (T_{onset}=145°C and T_{max}=149°C) and by DTG peak decomposition (T_{max}=364°C). Cholesterol anhydrous (ChA), which showed endothermic polymorphic peak, T_{max}=40°C, without mass loss, was differentiated from cholesterol monohydrate (ChH), which showed a broad endothermic peak, T_{max}=59°C, attributed to loss of water of crystallization (theoretical 4.45%). Morphological studies of gallstones were performed by optical microscopy and scanning electron microscopy (SEM). The stones consisted of a pigmented core with a variably-sized irregular central cavity, surrounded by a radially arranged deposits of plate-like ChH. The outer part of the stones showed ChA crystal arborescences. X-ray microanalysis gave a typical spectrum rich in C and O, and in some instances the presence of P, which was attributed to the presence of phospholipids. $CaCO_3$ was easily characterized by TG with the use of DTG decomposition peak at 674°C.

Keywords: *anhydrous cholesterol, hydrated cholesterol, gallstones, microscopy, thermal analysis*

Introduction

Gallstones are bile concretion [1]. Gallstone formation in the gallbladder is a common disease and constitutes a major health problem worldwide. The treatment of gallstones usually consists of the removal of both gallbladder and stones. Three major kinds of gallstones were described: pure cholesterol, brown pigment and black pigment stones (National Institute of Health International Gallstone Workshop 1982). Pure cholesterol and mixed stones are the main gallstones in western countries. The gallstones have an average cholesterol content of 86.3% (ranging from 41.7 to 100%), and are characteristically multifaceted, mulberry shaped or ovoid and, on fracture, have varying degrees of pigmented centers or layers [1].

Black pigment stones tend to be small, resist manual crushing and produce a shiny, glass-like surface on fracture. The cholesterol content of black pigment stones can range from 0 to 9.7%. Brown pigment stones have an average cholesterol content of 11.4% (ranging from 2.8 to 28.3%). The brown pigment stones are easily crushed and have dark and light laminations on cross section [2]. The 86.3% of cholesterol stones are present in the gallbladder alone [3]. When cholesterol levels are abnormally high, single crystals of the monohydrate form (ChH) tend to deposit, in bile, clumps of these crystals form gallstones [4]. Despite its importance in cholesterol gallstone formation, crystallization of cholesterol is poorly understood. It is widely accepted that cholesterol monohydrate is the crystalline form in the gallstones. However, recent results from Konikoff *et al*. [5], showed that cholesterol can crystallize from model and native bile as filamentous crystals covered by a surface layer of lecithin molecules. This kind of cholesterol could be anhydrous form (ChA). During growth, filamentous crystals are transformed via metastable intermediates into classical plate-like ChH crystals [5].

Some instrumental methods of analysis have been used to characterize and analyze gallstones, such as FTIR [6, 7], X-ray diffraction [8] and scanning electron microscopy [2]. The microchemical methods were also used since long time ago [9].

The exact crystalline form of cholesterol, which precipitates in the nucleus of forming and growing stone, is not known. Our aim is to determine the anhydrous or monohydrate state of cholesterol in gallstones, using thermal analysis methods, such as, differential scanning calorimetry (DSC) associated with thermogravimetry (TG).

ChH constitutes the major component of most gallstones. It is, also, a major crystalline solid of many human atherosclerotic plaques [4]. TG can be used as a

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method for the determination of cholesterol, calcium oxalate and calcium carbonate in gallstones. The individual mass losses of cholesterol occur at sufficiently different temperatures from the other components [10].

The direct analysis of cholesterol in gallstones was performed by the use of DSC transition phase. The polymorph transition at 38°C is reversible and reproducible, under heating and cooling. So, the method required no intermediate reactions and cholesterol can be determined directly in the powdered stone, with no other pretreatment or reagents [11].

In the study of gallstones, we observed, in the range from 35 to 120°C, some little transition phases. One of them could be attributed to water. So, we have suggested the use of DSC technique, in studying the transition solid–liquid phases at about 150°C.

The results of thermal analysis of ChA and ChH, and microscopical characterization as well, performed on gallstones obtained from thirty patients, are presented in this paper.

Experimental

Thermal analysis

Samples

ChA was obtained from Sigma–Aldrich Chimie S.A.R.L. (38299 Saint Quentin, Fallavier/France) and Prolabo (94126 Fontenay Sous-Bois/France). The solvents (ethyl alcohol and methyl alcohol) were ACS grade reagents and obtained from Carlo–Erba (27106 Val de Reuil/France).

ChA was recrystallized in dry methanol and it gave needle-like crystals. ChA recrystallized in ethanol:water (95:5), afforded cholesterol hemiethanolate (Ch_{heth}) with plate-like crystals [12].

ChH was obtained by immersing Ch_{heth} in bidistilled water for 48 h, followed by filtration, washing by bidistilled water and drying under vacuum at room temperature. The wet monohydrate plate-like crystals were placed on a balance pan and periodically weighed. When constant mass was observed, it was assumed that all excess water had evaporated from crystal surfaces. Needle-like ChA and plate-like ChH crystals were typed under polarizing light microscope. The result obtained by Karl Fischer determination of water in ChH was mean value±SD=4.34±0.11%, while the theoretical value of water in ChH is 4.45%.

Gallstones from thirty patients were preserved in closed boxes. The majority of them were 15 years old. The whole stone was analyzed, when its mass was less than 20 mg. Otherwise, the calculi were cut and analysis was done on these chips.

Instruments and methods

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 (weighing 10–20 mg) was put in aluminum 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%) flow of 0.5 L h^{-1}. Thermocouples were Platinel^R. The sample (weighing 10–20 mg) was put in platinum crucibles. Calcium oxalate monohydrate was used in TG for the mass loss standardization.

In DSC and DTA-TG, temperature standardization was made by the melting of benzoic acid, tin, lead, zinc and aluminum. Enthalpy standardization, using melting, was made with these standards.

The indicated temperatures (°C), in the presented curves of cholesterol and gallstones, were attributed to the maximum (T_{max}) and the extrapolated (T_{onset}) of each peak.

Morphological studies

A drop of ChA dissolved in dry methyl alcohol was deposit on a glasslide and allowed to dry, then examined using a Dialux 20 microscope, Leitz, France.

Cholesterol gallstones, 4 to 12 mm in diameter (N=18) were obtained from patients without acute cholecystitis, who underwent elective cholecystectomy for biliary colic. Cholesterol content of the stones averaged 71.6±0.5% (of the wet mass), as determined on a Folch extract [13] using the 'test combination' CHOD-PAP method (Boeringher–Mannheim, Germany). The stones were rinsed in phosphate buffer saline (PBS) 0.1 M pH 7.4, then stored at 4°C until analysis. They were then hemisected using a clean razor blade and examined with a stereomicroscope Wild M3 (Wild Leitz Ltd., Heerbrugg, Switzerland), equipped with a WILD MPS46/52 Photoautomat system.

Scanning electron microscopy (SEM) was performed with a JSM 35 (JEOL-Europe, Croissy-Sur-Seine, France) instrument (600 nm of resolution), and X-ray probe microanalysis was performed using a Tracor 524 instrument (Tracor, United Kingdom).

To determine the element composition, freshly hemisected stones were mounted on a graphite specimen holder using graphite glue (to avoid metallic contamination) and then coated with carbon to minimize electrostatic charges [14].

Results and discussion

The following temperatures are given:

- T_{max} corresponds to the temperature of the peak maximum,
- T_{onset} corresponds to the extrapoleted temperature of the intersection of the 2 tangents (baseline and peak),
- ΔH is the variation of enthalpy obtained by a linear integration of the curve area.

ChA

The DSC curve (from room temperature to 170°C) of ChA showed the following peaks: a small endothermic peak (polymorph transition) at about T_{max}=40°C and a second endothermic peak (melting) at about T_{max}=150°C.

In DTA, these peaks were also present at T_{max}=44 and 152°C (Fig. 1a) and, several small exothermic and endothermic deformations until 450°C. In TG, a high stability (no mass loss) was observed from 20 to 200°C. This observation confirmed the dryness of the crystals. The DTG peak maximum was at about 360°C, with a mass loss of about 55%, at this temperature.

The reproducibility of melting temperatures of the two standard samples of ChA, made by DSC and DTA, is shown in Table 1. The DSC technique gives a better reproducibility in T_{max} than DTA (SD=0.61 instead of 0.79 for the 1st sample, SD=0.84 instead of 0.93 for the 2nd sample). The reproducibility of T_{onset} is better than T_{max}, whatever the used technique (DSC or DTA).

For a recrystallized ChA, the values of temperature (T in °C) and enthalpy (ΔH in J g^{-1}) of polymorph and fusion for DSC and the values of the maximum DTG (derivative thermogravimetry) peak (°C) and decomposition rate (% min^{-1}), for DTG, are shown in Table 2. T_{onset} presented a better reproducibility than T_{max}.

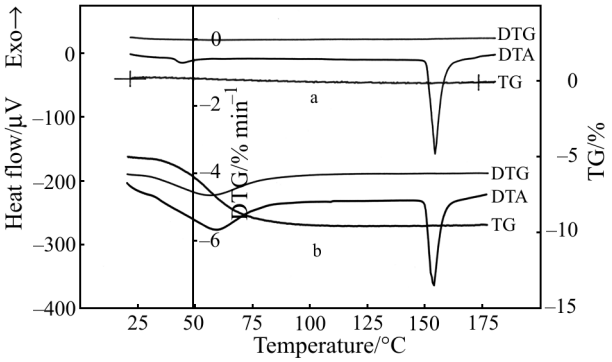

Fig. 1 TG, DTG and DTA curves of cholesterol. a – ChA, sample mass 17.7 mg; endothermic peaks: T_{max}=44 and 152°C; mass loss 0% from ambient temperature to 170°C; b – ChH, sample mass 21.3 mg; T_{max}=58°C; endothermic peaks: T_{max}=59 and 152°C; mass loss 4.46% (theoretical 4.45%) from ambient temperature to 100°C and 0% from 100 to 170°C)

The ChA sample, subjected 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 lack of crystallization can explain these variations. Therefore, to avoid some errors in gallstone thermal analysis, the stones were studied without thermal pretreatment.

Cholesterol in gallstones can be identified by its melting temperature and, if necessary, by the DTG maximum peak temperature. Its quantification can be made by the use of the melting transition enthalpy of the pure ChA.

Table 1 Reproducibility of endothermic melting peak temperatures of the two standard samples of ChA, made by DSC and DTA

		T_{max}/°C		T_{onset}/°C	
		DSC	DTA	DSC	DTA
Sigma	mean value±SD	151.3±0.61	151.8±0.79	149.1±0.38	148.9±0.35
Prolabo	mean value±SD	150.0±0.84	152.0±0.93	148.1±0.50	147.7±0.55

DSC – differential scanning calorimetry; DTA – differential thermal analysis; 8 runs on each sample; SD – standard deviation

Table 2 DSC and DTG peaks of ChA recrystallized standard

	DSC peaks						DTG peaks	
	polymorph temperature/°C		melting temperature/°C		enthalpy/J g^{-1}			
	T_{onset}	T_{max}	T_{onset}	T_{max}	ΔH_p	ΔH_f	T_{max}/°C	rate*/% min^{-1}
mean value±SD	36.9±0.71	39.6±0.72	148.8±0.90	151.1±1.10	7.0±0.33	54.6±1.79	366.0±9.28	7.36±0.35

15 tests were performed on recrystallized ChA; heating rate: DSC 2°C min^{-1}; TG-DTA 5°C min^{-1}; *rate of decomposition; DTG – derivative thermogravimetry; *p* – polymorph; *f* – fusion; SD – standard deviation

ChH

The preparation and analysis of ChH was described by our group earlier [15].

An endothermic peak at 59°C, due to water volatilization, and another one at 152°C, due to melting, were shown by DSC for the prepared ChH (Fig. 1). The comparison of thermal analysis curves of ChA and ChH, by TG, is shown in Fig. 1. ChH lost mass (4.46%) at less than 100°C. The only bounded water was present in ChH. The free water had gone out, before the DTA-TG heating. Five runs of DSC gave an average value $m\pm SD=58.0\pm3.5$°C for the water evolving T_{max} endothermic peak. For the melting, the values were as follows: 148.0±0.19°C for the T_{onset} and 152.0±0.52°C for the T_{max}. Five other runs were done by TG-DTA, the mass loss was 4.52±0.20% from 20 to 170°C.

Wada *et al*. [16] observed a wide endothermic peak attributed to moisture, another sharp one at 70°C and a slight decrease in melting of the hydrated form (146°C). Several small endothermic peaks were observed by using a closed pan, under elevated pressure [12, 17].

ChH samples stored for long time at room temperature and/or under vacuum, showed smaller (area and temperature) DSC endothermic peaks and TG mass losses. ChH seems to lose water of crystallization under air sweeping at room temperature. A recent isolated gallstone was crushed, then immediately studied by TG-DTA and another stone, issued from the same patient, was powdered and stored for 24 h in dried atmosphere at room temperature. We noted that the first endothermic peak attributed to water was missing in the second case (dry storage conditions); the water mass loss was decreased from 4.40% to nearly less than 0.15%. The characterization of ChH will be difficult if the gallstones are crushed and dried. We observed, also, that ChA could be hydrated again when placed in a moist condition.

Analysis of gallstones

As shown in Fig. 2, each stone consisted of a pigmented central core with a variably-sized irregular central cavity, surrounded by radially arranged deposits of plate-like cholesterol hydrate, (Fig. 2: ChH). In this pigment-rich central core ChH crystals are embedded in pigment spherules deposits (Fig. 2: P). The outer part of stones showed cholesterol crystal arborescences (Fig. 3) similar in shape to that observed for recrystallized pure ChA (Fig. 4).

SEM studies confirm the presence of plate-like ChH in the central part of the stones (Fig. 5) and of needle-like ChA in the outer areas (Fig. 6).

With the aim of characterizing the cholesterol in gallstones, and with the use of its melting, it was 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. From the observed T_{max} and T_{onset} values of cholesterol in the studied gallstones, we suggest the probable melting temperature interval to characterize the presence of cholesterol in gallstones: 144 to 152°C for T_{max}

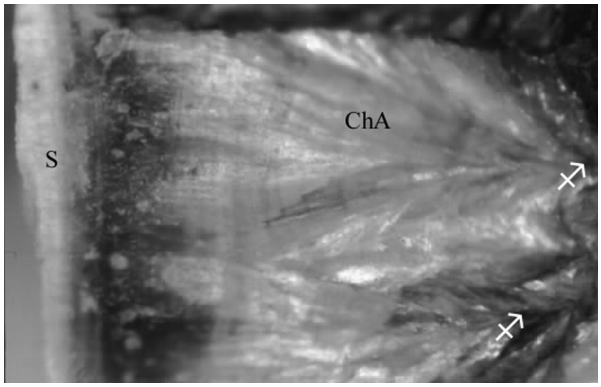

Fig. 3 Outer part of a cholesterol gallstone: needle-like cholesterol anhydrous (ChA) are growing from pigment-rich areas (crossed arrows) to the surface

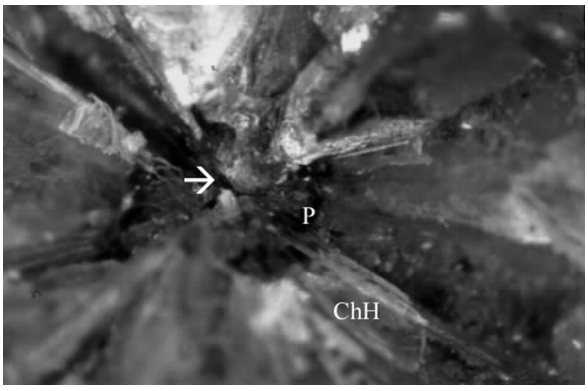

Fig. 2 Central area of a cholesterol gallstone, cholesterol is present as plate-like cholesterol monohydrate (ChH) merging from a central cavity (arrow) surrounded by pigment deposits (P)

Fig. 4 Cholesterol anhydrous, recrystallized from dry methanol. At the end of needle-like crystals (crossed arrows) new needle-like crystals grow to from arborescent structures

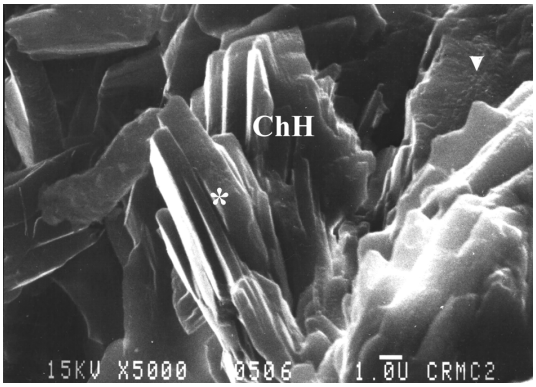

Fig. 5 SEM: plate-like cholesterol monohydrate crystals (ChH) are piled up. Pigment deposit (arrowhead) can be observed on the large surface of some of these crystals

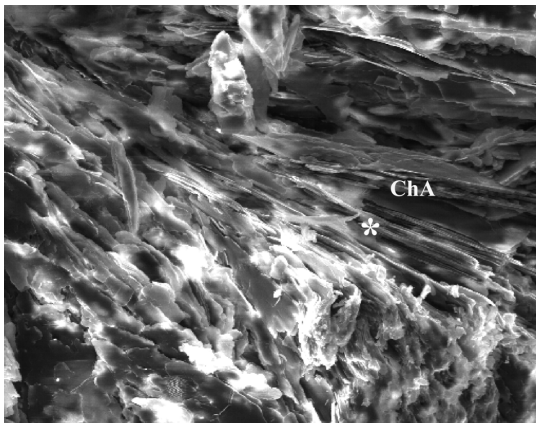

Fig. 6 SEM: needle like cholesterol anhydrous (ChA), radiating from the central core, are present in the outer part of the cholesterol stones

and 139 to 149°C for T_{onset}. The reproducibility was observed on 10 tests done on 10 stones issued from the same patient. The results were, mean±SD= 149.8±0.94°C for T_{max} and 144.1±0.76°C for T_{onset}.

Considering the first endothermic peak makes the differentiation between ChA and ChH, in gallstones. A small and narrow peak at about 40°C characterizes the anhydrous form. A wide peak at about 60°C may be due to water evolving. In addition, ChH done by TG, showed a mass loss at 150°C of 4.45%. Only in recent stones, stored at very low temperature, this mass loss was noted. Even, this value can be more in wet stones. In old stones, and in stones stored at high temperature, the mass loss was very low comparing to the theoretical 4.45%. Figure 7 shows the thermal analysis of two different stones (ChA and ChH). The characterization and determination of cholesterol were made by DSC [patient No. 5, endothermic peaks: polymorph (T_{onset}=42.2 and T_{max}=45.4°C, ΔH_p=3.62 J g^{-1}) and melting (T_{onset}=148.3 and T_{max}=151.1°C, ΔH_f=51.8 J g^{-1}); content of ChA found in this gallstone 94.8% expressed as ChA] and differentiation between ChA and ChH was made by TG-DTA [patient No. 15, endothermic DTA peaks: T_{max}=55 and 150°C; TG mass loss=4.35% from ambient temperature to 100°C and 0.14% from 100 to 170°C; content of ChH found in this gallstone 84.0% expressed as ChA].

D'Ascenzo *et al*. [11] showed the possible determination of cholesterol in gallstones, only by the use of the polymorph transition enthalpy at 40°C, using DSC technique. We think that the determination accuracy would be better if we consider the melting enthalpy instead of the polymorph transition enthalpy.

Cholesterol can be identified, in DSC, by using melting temperature or, if necessary, by the DTG decomposition peak. The results of cholesterol stones, obtained from 30 patients, are shown in Table 3. All contained cholesterol. Melting temperatures were in the intervals: 139 to 149°C for T_{onset} and 144 to 152°C for T_{max}. DTG peak temperature was at mean±SD= 363.6±8.04°C.

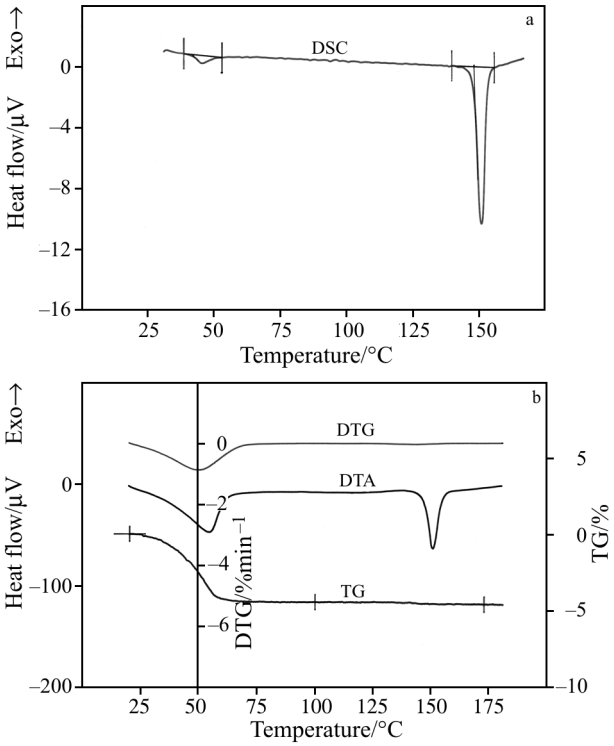

Fig. 7 a – DSC curves of gallstone (patient No. 5). Sample mass 21.3 mg; endothermic peaks: polymorph (T_{onset}=42.2 and T_{max}=45.4°C, ΔH_p=3.62 J g^{-1}) and melting (T_{onset}=148.3 and T_{max}=151.1°C, ΔH_f=51.8 J g^{-1}). The content of ChA found in this gallstone was 94.8% (expressed as ChA). b – TG, DTG and DTA curves of a gallstone (patient No. 15); sample mass 10.5 mg; DTG peak T_{max}=54°C; endothermic peaks: T_{max}=55 and 150°C; mass loss 4.35% from ambient temperature to 100°C and 0.14% from 100 to 170°C. The content of ChH found in this gallstone was 84.0% (expressed as ChA)

Table 3 The results of thermal analysis of cholesterol stones obtained from 30 patients

Characterization of cholesterol	DSC endothermic melting peak*		DTG peak** decomposition
	T_{onset}/°C	T_{max}/°C	T_{max}
mean value±SD	145.4±1.72	148.9±1.61	363.6±8.04
determination of cholesterol	DSC endothermic peaks*		DTG peak** decomposition
	ΔH_p/J g^{-1}	ΔH_f/J g^{-1}	rate/% min^{-1}
mean value±SD	4.1±1.42	41.9±6.62	6.36±1.33

*DSC – differential scanning calorimetry, Heating rate=2°C min^{-1}; 52 tests, ** TG-DTA – simultaneous thermogravimetry-differential thermal analysis, DTG – derivative thermogravimetry, Heating rate=5°C min^{-1}; 25 tests, SD – standard deviation, *p* – polymorph, *f* – fusion; sample mass (either by DSC or TG-DTA) on 77 tests: mean value±SD=19.8±6.3 mg. Only the ΔH_f was used in cholesterol determination

The stones issued from 30 patients were analyzed quantitatively. Enthalpy changes of fusion (median value±SD=41.9±6.62 J g^{-1}) were used for cholesterol determination. 29 gallstones contained more than 43% cholesterol (minimum 43.3 and maximum 98.4%) expressed as ChA in gallstone with mean±SD=76.8±12.1%. Median values of ΔH_p and DTG peak decomposition rate were, 4.1 J g^{-1} and 6.36% min^{-1}, respectively.

The old stones consisted of ChA only. On the other hand, ChH was characterized, mainly in the central part of the stone, for the recent eight gallstones.

We can also note that after the decomposition of cholesterol at about 400°C char is formed. Its slow combustion under air flow was observed by TG-DTA. From 25 runs, the results were as follows for T_{max} of the exothermic combustion peak, mean±SD= 476.5±9.84°C.

In some cases, a mass loss, slightly higher than the theoretical value of 4.45%, was observed. The increased value could be due to added free water. So, we deduced the ChH (%) from the ChA (%) by the use of the relation:

$$\text{ChH (\%)}=\text{ChA (\%)}\cdot 1.0445.$$

In TG (Fig. 8) and for six samples, an additional step, at about 700°C, was shown (characterizing $CaCO_3$ decomposition to CaO and CO_2). $CaCO_3$ was characterized by DTG with a decomposition peak (mean value±SD=673.9±14.9°C , for 8 runs) and a mass loss from about 600 to 800°C by TG. In Fig. 8a (patient No. 18), mass loss was 2.89% from 600 to 800°C ($CaCO_3$ content was 6.9%); in Fig. 8b (patient No. 26) the mass loss 11.11% was higher ($CaCO_3$ content was 25.9%). $CaCO_3$ was present at the beginning in gallstones and not produced from decomposition of calcium bilirubinate (this was confirmed by chemical analysis of $CaCO_3$ in the stones).

X-ray microanalysis performed on the ChH and on ChA crystals (Fig. 9) gave the same typical spectrum (Fig. 9a) with only C and O. Analysis performed on pigment spherules revealed always a strong pike for calcium, and in some instances the presence of phosphor (Fig. 9b), which can be attributed to the presence of phospholipids in these parts of the stone.

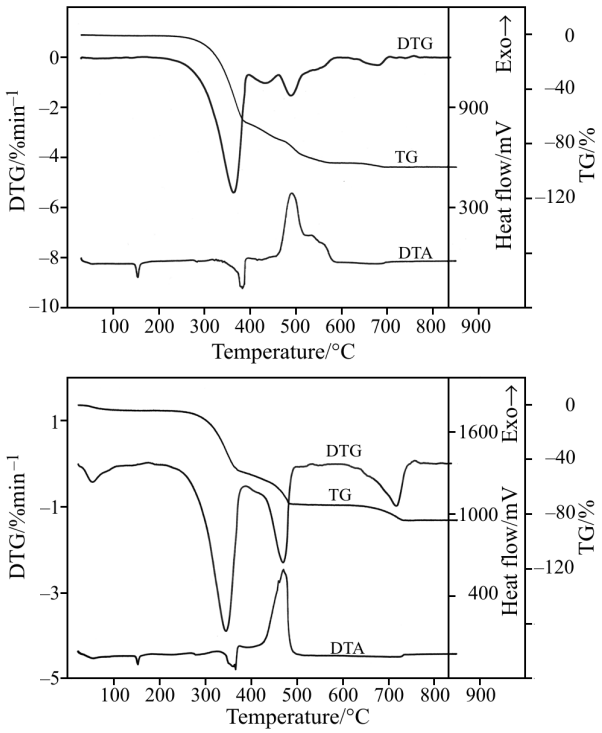

Fig. 8 TG, DTG and DTA curves of 2 gallstones. a – (patient No. 18; sample mass 20.7 mg; DTG peak T_{max}=362 and 430°C (unknown), 487.5 and 673°C; DTA endothermic melting peaks: T_{max}=150°C [the temperature of the other peaks is not indicated]; TG mass loss 0.79% from ambient temperature to 170°C and 2.89% from 600 to 800°C. The content of ChA found in this gallstone was 77.30% (expressed as ChA) and $CaCO_3$ content was 6.9%). b – (patient No. 26; sample mass 16.2 mg; DTG peaks T_{max}=51, 341, 467 and 715°C; DTA endothermic peaks: T_{max}=60 and 150°C [the temperature of the other peaks is not indicated]; TG mass loss 4.14% from ambient temperature to 170°C and 11.11% from 550 to 800°C). The content of ChH found in this gallstone was 68.9% (expressed as ChA) and $CaCO_3$ content was 25.9%

Moreover, infrared spectroscopy [18–21] has revealed that calcium in cholesterol stones may be present as carbonate, phosphate and bilirubinate.

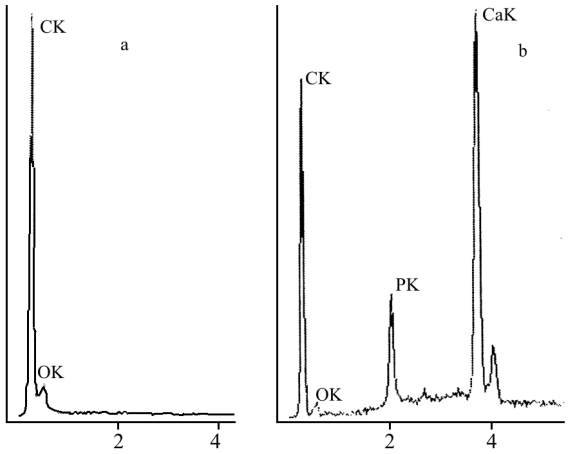

Fig. 9 EDAX analysis of cholesterol: panel a – analysis was done on either ChH or ChA (* in Figs 4 and 5), only C and O are detected. Panel b – analysis was performed on the pigment area, Ca and to less extent P were detected with C and O

Topographic localization

In some cases, thermal analysis tests were done on either central or peripheral fragments of the stone. The results of the indicated part of the stone were nearly identical to that obtained from several trials done on each stone or on several stones obtained from the same patient (Fig. 10).

The ChH seems to be clearly defined, but unstable. It transforms to ChA by losing its water of hydration. Water of hydration presumably diffuses through the anhydrous materials and escapes from the edges of the crystals [22].

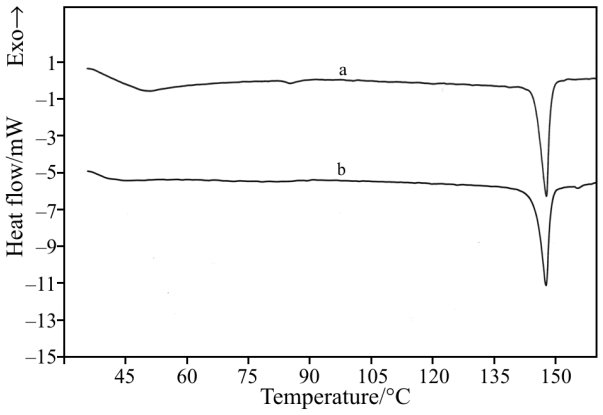

Fig. 10 DSC curves of a gallstone (patient No. 26); a – ChH white core, sample mass 14.3 mg, endothermic peaks (T_{max}=50.9 and 147.8°C, ΔH_f=36.8 J g^{-1}, 67.4% of ChA); b – ChA white periphery, sample mass 11.8 mg, endothermic peaks (T_{max}=about 47 (very weak) and 147.7°C, ΔH_f=43.3 J g^{-1}, 79.3% of ChA)

The gallstones extracted from the first eight patients were 15 years old, and therefore, the presence of only ChA would be explained by the volatilization of water during this long time. It seemed that in the bile, the ChA is the start of the filamentous, helical then tubular microstructure crystallization [5]. The plate-like cholesterol crystals appear to grow from apical areas of the tubes [5] and then agglomerate, via mucin glycoproteins, to form cholesterol gallstones [1, 23, 24].

Conclusions

Two types of cholesterol were characterized in gallstones. The central part of the gallstones consisted mainly of ChH, while ChA were mainly found in the periphery of the stone. The topographic distribution of these two types of cholesterol gallstones may be due to their environmental growth and/or storage conditions after surgery.

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