UDC 612.357.1 : 543.422.4

195. Goro Chihara, Kimiko Matsuo (née Shirakuma), Ayako Mizushima, Emiko Tanaka (née Kobayashi), Kumiko Arimoto, and Shigeko Sugano (née Fujii): Medical and Biochemical Application of Infrared Spectroscopy. VI.[1] Studies on Human Bile by Infrared Spectra and Some of its Clinical Application.

(*National Cancer Center Research Insititute*[*1])

Bile juice contains various bile acids, bile pigments, lipids, mucoic substances, inorganic substances, and various other substances, and each of them has been studied in detail. However, their chemical analysis is very complicated and there is no suitable method to be used. Routine clinical tests for bile now used are merely the color and turbidity of bile, and detection of bacteria.

The present authors carried out the analyses of bile through infrared spectrum and revealed that this is an effective means in the analysis of bile components[2] and for examining the pharmacological action of choleretics.[3]

In the present series of work, some considerations were made on the interpretation of the infrared spectrum of dried whole human bile and on fractions obtained on separation of bile, together with application of this method for clinical examinations.

I. Infrared Spectrum of Dried Whole Bile of Human Beings

Human bile juice was dried to a powder and its infrared spectrum was measured as a potassium bromide pellet. In spite of the bile being a complicated mixture, the infrared spectrum in general shows a fairly sharp absorptions.

About 600 spectra were measured with dried powder of human liver bile and gall-bladder bile, and their comparative examination showed that the spectra of human bile has definite absorptions, as long as there is no abnormalities in the liver, bile duct, or gall-bladder.

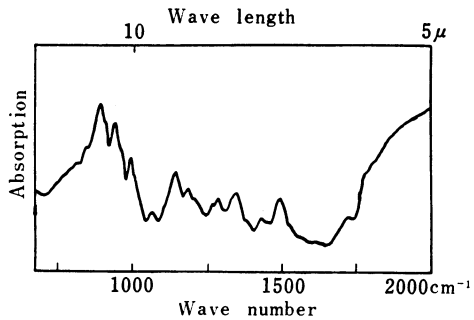

Fig. 1. Standard Infrared Spectrum of Dried Whole Gall-bladder Bile

Fig. 1 is the standard spectrum of dried whole gall-bladder bile of humans[*2] and absorptions are in the following positions[*3] : 1737(m), 1654(shoulder), 1605(vs), 1548(shoulder), 1459(m), 1442(w), 1403(s), 1379(shoulder), 1332(w), 1308(m), 1250(w), 1230~1212(m),

*1 Tsukiji 5-chome, Chuoku, Tokyo (千原呉郎, 松尾(旧姓白熊)君子, 水島綾子, 田中(旧姓小林)恵美子, 有本久美子, 菅野(旧姓藤井)滋子).

*2 A large number of standard gall-bladder bile was supplied by the kind of Dr. M. Okudaira of Tokyo Medical Examiner's office, which is gratefully acknowledged.

*3 s=strong, m=moderate, w=weak, v=very, br=broad.

1) Part V : This Bulletin, 8, 988 (1960).
2) G. Chihara, *et al.* : *Ibid.*, 8, 174 (1960).
3) *Idem* : *Ibid.*, 9, 939 (1961).

1194(shoulder-w), 1170(w), 1117(shoulder), 1109(shoulder-w), (1090)(br), 1079(s), (1062)(br), 1048(s), 1017(shoulder), 1001(shoulder-w), 976 (s), 969(shoulder), 953, (shoulder-w), 920(m), 903 (w), 871(w), 854(w), 806(w), and 732(w) cm^{-1}.

Even in the case of dried whole liver bile collected during surgical operation, the position of the absorptions was the same although the intensity of the absorptions was somewhat different.

The bile collected by duodenal sounding is generally unsuitable material since its spectrum is overlapped by the broad absorption of magnesium sulfate at around 1135 cm^{-1} of strong intensity. In this case, extraction of dried bile with methanol makes it possible to use the extract for analysis of bile acids as a fraction of total bile acids. Attempts were made to remove magnesium sulfate alone from bile juice but without avail.

II. Separation of Various Components from Bile Juice

Infrared spectrum of dried whole bile appears as the total sum of the absorptions of the constituent components of bile. Consequently, if each of the absorptions of whole bile can be co-ordinated to each bile component, variation of bile components can be known from the spectrum of the bile.

Normal bile, showing standard absorptions, was separated into bile acids, bile lipids, proteins, polysaccharides, and bile pigments by the route shown in Chart 1. The precipitate obtained on addition of a large amount of methanol to bile juice chiefly contains mucoic substances (proteins and polysaccharides) and extraction of its filtrate with ether gives the whole lipids in the ether layer and chiefly bile acid fraction in the methanol layer. These substances were identified by comparison of their infrared spectra with those of fundamental substances that constitute bile acid such as sodium glycocholate and lecithin.

The infrared spectrum of each of the separated fractions is more distinct than that of the dried whole bile and many observations are gained as to the constituent components of each fraction. Based on these facts, a method for interpreting the infrared spectrum of dried whole bile was established.

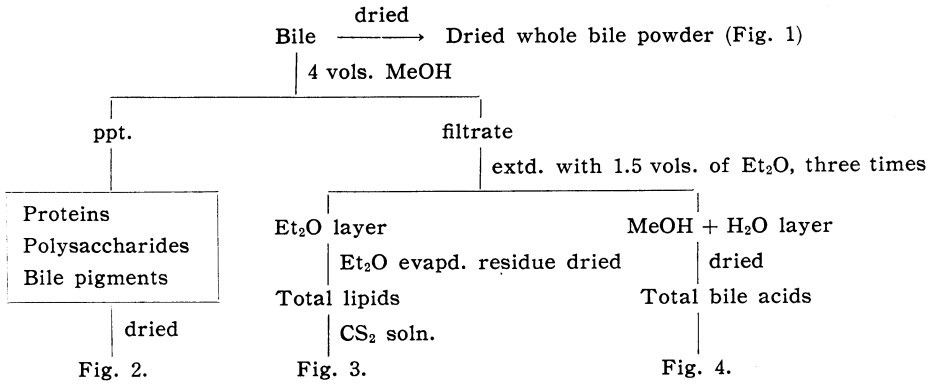

Chart 1. Separation of Various Components from Bile

III. Infrared Spectra of Mucoic Substances

The precipitate obtained on addition of a large volume of methanol to bile juice contains chiefly mucoic substances (proteins and polysaccharides). The precipitation is complete by the addition of ca. 4 volumes of methanol (usually 8 cc. of methanol to 2 cc. of bile) and further addition of methanol no longer produces precipitation. The infrared spectrum of the powder obtained on drying this precipitate is shown in Fig. 2. The

absorption bands are found at 1653±10(s), 1520±2(s), 1441(m), 1393(w), 1333(w), 1309(w), 1235(m), 1159(w-m), 1112(w), 1065(s, broad), 927(w), 890(w-m), and 675(m) cm^{-1}. These bands may be interpreted as the overlapped absorptions of proteins[4] and polysaccharides[5] from the facts already well known. From the intensity ratio of the absorption of proteins at around 1650 and 1520 cm^{-1} to that of polysaccharides at around 1065 cm^{-1}, a semi-quantitative consideration is possible but this intensity ratio is not necessarily constant.

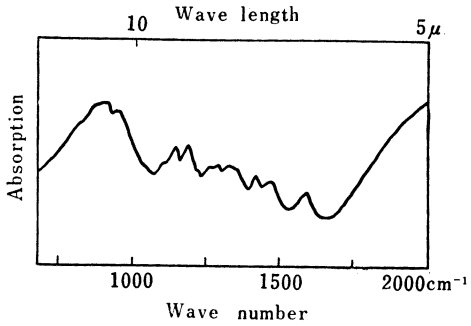

Fig. 2. Infrared Spectrum of the Precipitate formed on Addition of Methanol to Bile

This fraction is generally small in the bile and its spectrum does not affect the spectrum of dried whole bile except for being the background of absorption at around 1000~1100 cm^{-1}. However, in abnormal condition, such as the depletion of bile acids, this absorption appears in strong intensity in the spectrum of dried whole bile and gives some useful clinical clue. The quantity of bilirubin in bile is very small and it does not appear in the spectrum except in special cases.

IV. Infrared Spectrum of Total Lipids of Bile

Addition of methanol to bile, removal of the precipitate thereby produced by filtration, and extraction of the filtrate with ether (usually three 12 cc. portions of ether for 8 cc. of the filtrate) results in extraction of total lipids into the ether layer. Evaporation of ether from this extract and infrared spectrum of the residue as carbon disulfide solution gives the spectrum indicated in Fig. 3 (A). The position of its main absorptions is as follows: 1740(vs), 1377(m), 1241(s), 1170(m), 1090(s), 1062(s), 969(s), 920(w), 874(w), 821(m), 765(w), and 720(w) cm^{-1}.

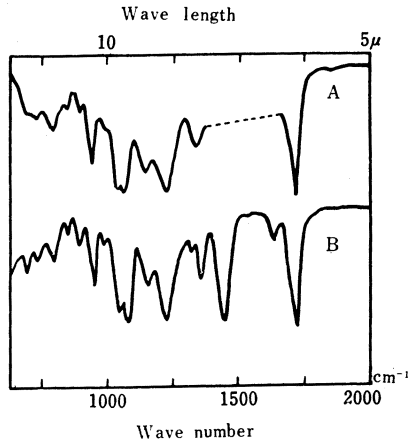

Fig. 3. Infrared Spectra
(A) Total lipids of bile
(B) Lecithin

4) M. Beer, G.B.B.M. Sutherland, K.N. Tanner, D.L. Wood: Proc. Roy. Soc., A, **249**, 147 (1958).
5) S.A. Barker, E.J. Bourne, M. Stacey, D.H. Wiffen: J. Chem. Soc., **1954**, 171.

Interpretation of this spectrum can be made by the same method as that used for total lipids of serum,[6,7] although in normal bile, absorption of lecithin is very intense in the spectrum of total lipids and the whole spectrum is similar to that of lecithin itself (Fig. 3 (B)). In cholelithiasis and other abnormalities, absorption of cholesterol[8] may appear especially strong.

This fraction appears in the spectrum of dried whole bile as each of the absorptions indicated in Fig. 3 (A) and the absorption at 1738 cm^{-1} is suitable as the key band. It is possible to make semi-quantitative estimation of the amount of lipid in the bile by the relative intensity of this absorption.

V. Infrared Spectrum of Bile Acids

The methanolic solution left after extraction of total lipids with ether chiefly contains bile acids. This fraction was dried to a powder and its infrared spectrum was measured by the potassium bromide pellet method. The spectrum is shown in Fig. 4 (A) and absorptions are present in the following positions*4 : 1643 (vs) (TG), 1603 (vs) (G), 1548 (shoulder) (GT), 1459 (shoulder) (GT), 1447 (m) (GT), 1405 (s) (G), 1381 (w) (TG), 1332 (w) (GT), 1309 (m) (G), 1248 (w) (GT), 1219 (s) (T), 1196 (w) (TG), 1170 (w), 1115 (m) (GT), 1080 (s) (GT), 1045 (s) (TG), 1017 (shoulder) (GT), 1001 (w), 980 (s) (GT), 948 (m) (GT), 924 (shoulder) (GT), 917 (m) (GT), 901 (w) (GT), 859 (m) (GT), and 742 (w) (T) cm^{-1}.

This spectrum can be interpreted as the overlapped absorptions, with sodium glycocholate as the main component, with a small amount of sodium taurocholate and other compounds. The absorptions of sodium glycocholate (Fig. 4 (B)) are all present in the infrared spectrum of this fraction and are of sufficient intensity to accept it as the main component.

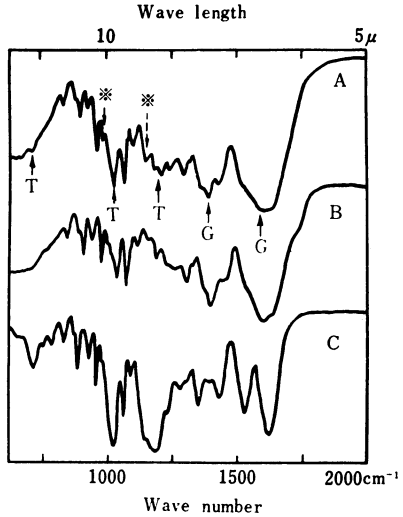

Fig. 4. Infrared Spectra
(A) Bile acid fraction of bile
(B) Sodium glycocholate
(C) Sodium taurocholate

However, there are several absorptions (1219, 1170, 1001, and 742 cm^{-}) in the spectrum of this fraction which are not present in the spectrum of sodium glycocholate and an inversion of intensities (1080 and 1045 cm^{-1}). These absorptions, except for those at 1170 and 1001 cm^{-1}, are thought to be due to the contamination of absorptions of sodium taurocholate (Fig. 4 (C)).

*4 G=sodium glycocholate, T=sodium taurocholate.

6) G. Chihara, *et al.* : This Bulletin, **8**, 222 (1960).
7) N.K. Freeman : Ann. N. Y. Acad. Sci., **69**, 131 (1957).
8) G. Chihara, S. Yamamoto, H. Kameda : This Bulletin, **6**, 52 (1958).

The chief difference between absorptions of sodium glycocholate and sodium taurocholate is the presence or absence of absorptions of carboxyl ion (ν_{as} COO^- 1603 cm^{-1}, ν_s COO^- 1405 cm^{-1}) and of sulfonyl ion (ν_{as} SO_3^- 1219 cm^{-1}, ν_s SO_3^- 1045 cm^{-1}, ν CS 742 cm^{-1}), and there is no great change in other regions, except for the absorption at 1309 cm^{-1}.

Consequently, the absorptions at 1219 and 742 cm^{-1} in the infrared spectrum of bile acid fraction of humsn bile and the stronger intensity of absorption at 1045 cm^{-1} than that at 1080cm^{-1}, in reverse of those of sodium glycocholate, may be taken to be due to sodium taurocholate present in the bile. Absorption of taurocholate at 1219 cm^{-1} and that of glycocholate at 1405 cm^{-1} are suitable as the key bands in the quantitative consideration of the constitution of conjugate amino acid from infrared spectrum.

It should be noted that, although the amount of deoxycholate has been believed to be larger than cholate in human bile acids, the present series of work has revealed that the bile acid contained in normal human bile is almost wholly cholic acid. The infrared spectrum of the bile acid fraction described above agrees approximately with the absorptions of conjugate cholate and the absorption of conjugate deoxycholate cannot be recognized as the main component, Absorptions other than conjugate cholate are the very weak absorptions (1001 and 1170 cm^{-1}), with parallel change in the intensity. Absorptions of this fraction appear in the infrared spectrum of dried whole bile.

The results described in the above four sections were obtained with a dozen kinds of bile showing standard absorptions and had reliable reproducibility.

VI. Interpretation of the Infrared Spectrum of Dried Whole Bile and Some of its Clinical Application

From the results described in the foregoing sections, the absorptions in the infrared spectrum of dried whole bile can be assigned as shown in Table I.

Table I. Assignment of Infrared Absorptions of Dried Whole Bile

Wave No. (cm^{-1})	Assignment	Wave No. (cm^{-1})	Assignment	
1737 m	lipid (lecithin, triglyceride)[a]	(1090) br	lecithin	polysaccharide[a] (broad background)
1654 v.s. (should)	cholate (G T), protein[a]	1079 s	cholate (G T)	
1605 v.s.	glycocholate (bilirubin)[a]	(1062) br	lecithin	
1548 v.s. (should)	cholate (G T), protein[a]	1048 s	cholate cholesterol (G T), deoxycholate (G)	
1459 m	lipid	1017 should	cholate (G T)	
1442 w	cholate (G T)	1001 w	deoxycholate	
1403 s	glycocholate[a]	976 s	cholate (G T)	
1379 should	lipid	969 should	lecithin	
1332 w	cholate (G T)	953 w	cholate (G T)	
1308 m	cholate (G), deoxycholate (G)	920 m	cholate (G T)[a]	
1250 w	lecithin,[a] bilirubin, protein	903 w	cholate (G T)	
1230~1215 m	taurocholate[a]	871 w	cholate (G T)	
1194 w	cholate (G T)	854 w	cholate (G T)	
1170 w	triglyceride	806 w	cholate (G T)	
1117 should	cholate (G T)	732 w	taurocholate[a]	
1109 should	deoxycholate (G)			

a) Key band, G=Glycocholate, T=Taurocholate

The absorptions marked with *a*) are those which can be adopted as the key band for the particular substance. By comparing the intensity of these bands with those shown in Fig. 1, some assumption can be made on their constituent components.

The intensity of the band at 1737 cm^{-1} corresponds to the quantity of lipids (lecithin) in the bile, that of absorptions at 1654 and 1548 cm^{-1} to proteins, that of absorptions at 1605 and 1403 cm^{-1} to glycocholate, that of absorptions at 1230, 1048, and 732 cm^{-1} to

taurocholate, that of absorptions at 976 and 920 cm^{-1} to cholate, that of absorptions at 1250 and (1605) cm^{-1} to bilirubin, and the broad background absorption in the region of 1000~1100 cm^{-1} to polysaccharides (mucoic substances).

Interpretation of the infrared spectrum of dried whole bile, together with that of the spectrum of each fraction of the bile, is valuable to clinical tests. Detailed report on the work in this region will be made elsewhere but a few representative examples will be given for clinical application of the infrared spectral analysis of bile.

Infrared spectrum of dried whole bile is especially effective in the detection of abnormal bile and the spectra shown in Fig. 5 are such examples.

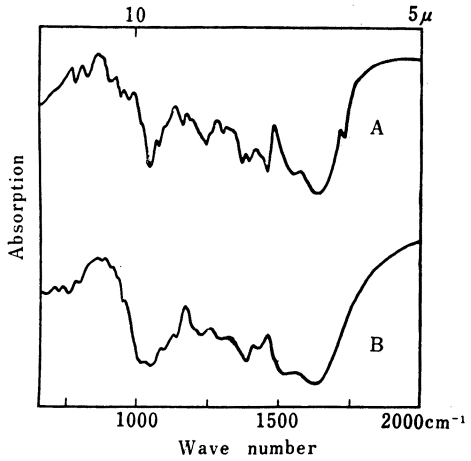

Fig. 5. Infrared Spectra of Abnormal Bile

(A) Bile with large amount of cholesterol (cholelithiasis)

(B) Bile lacking in bile acid (liver cirrhosis)

Fig. 5 (A) is the infrared spectrum of bile with abnormally large quantity of cholesterol, seen in cholelithiasis, and shows a large number of absorptions of cholesterol in the fingerprint region, overlapping normal bile components.

Fig. 5 (B) is the spectrum of bile from liver cirrhosis patient and is characteristic in lacking the absorptions of bile acids and lipids. This spectrum is very like the absorptions of bile mucoic substances described above.

The present series of work has shown that the introduction of infrared spectrum as the new analytical tool for bile acids is very effective in clinical tests.

The authors are grateful to Dr. M. Okudaira and many others for the supply of bile and to Dr. K. Takeda, Prof. K. Yamazaki, and Dr. R. Ohsawa for samples of bile acids. A part of expenses for this work was defrayed by the Asahi Science Fund.

Summary

Infrared spectral measurement was introduced into the analysis of bile and this was proved to be the new, simple, and effective tool for clinical tests.

1) Interpretation of the infrared spectrum of dried whole bile was established and a method was revealed for finding quantitative difference of various components of bile from its absorptions. Some examples of clinical application for the detection of abnormal bile was described,

2) Bile acids, bile pigments, bile lipids, and mucoic substances were separated from bile by a simple procedure and the method was revealed to find quantitative changes in the components constituting each of these fractions from their infrared spectra.

3) This infrared spectral analysis was found to be a new and simple method for finding the kind of bile acids present, which had been difficult by the known methods. It was found that the main component of bile acids in normal human bile is cholic acid and not deoxycholic acid.

(Received October 27, 1961)