Methods of Measuring Bile Acids in Bile and Feces

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ABSTRACT

Methods currently available for the determination of bile acids in bile and feces are discussed. While the enzymatic method is suitable for the routine, rapid analysis of total bile acids in bile, specific information on the significance of individual bile acid isomers in metabolic studies can only be gained by a combination of thin-layer and gas-liquid chromatography. Fecal bile acid analysis is complicated and methods are still undergoing substantial modification.

Bile acid excretion is of major interest in studies concerning hyperlipoproteinemia and the effects of hypocholesteremic agents and diet. Bile acids also are of interest in regard to cholestasis, other hepatobiliary disease, and a variety of physiologic functions. Two primary bile acids (cholic and chenodeoxycholic acids) formed from cholesterol in the liver are secreted into the bile as their glycine and taurine conjugates. These bile acids are subjected to bacterial action in the intestine and some of the secondary bile acid thus produced is reabsorbed. Hence, at a given time, bile might contain both primary and secondary bile acids. Since the bile contains a high concentration of bile acids, their measurement is relatively easy. The feces, however, contain a variety of ketonic and 7α-dehydroxylated bile acids and their isomers. Since the bile acids in feces occur along with other lipid classes, purification of them is difficult. In this review, some of the major methods currently available for identifying and measuring bile acids in bile and feces are discussed, including methods that measure the total bile acids and others that give information on a specific bile acid isomer. The choice of method depends on the kind of information needed.

Biliary Bile Acid Determination

A number of methods has been described for the measurement of bile acids. Some of the methods have been specific only for certain component bile acids, while others identify and quantitate all the bile acids. In this review the methods available for the biliary bile acids will be classified on the basis of the specific techniques used to isolate and identify the bile acids.

Differential Extraction-Spectrophotometry

This procedure, described by Levin et al., separates glycine- and taurine-con-
jugated bile acids by simple solvent extraction. The bile samples are deproteinized with an alcohol-zinc sulfate-barium hydroxide mixture, which also removes a considerable amount of biliary pigments. The neutral sterols and other contaminants are extracted with diethyl ether and hexane before acidification. After acidification with dilute hydrochloric acid, the glycine conjugates are extracted with ethyl acetate. After further acidification, the taurine conjugates are extracted with butanol.

The bile acid conjugates are then measured spectrophotometrically. The cholates are measured, after reaction with sulfuric acid and furfural, by their absorbance at 620 nm. The dihydroxycholanates are measured after reaction with a mixture of water, phosphoric acid, and sulfuric acid, 1:4:6 (vol/vol/vol) in the presence of benzaldehyde. The addition of ethyl acetate gives a blue-green color only with dihydroxycholanates.

The authors also noticed that, under certain conditions, the error caused by chenodeoxycholates can be kept low. This procedure does not measure chenodeoxycholate, lithocholate, and other secondary bile acids that normally are found in human bile. Levin et al reported a recovery of 96.8 percent to 104 percent, for the overall procedure, for conjugates added to the bile samples. If specific information is needed on the individual bile acids, one has to use thin-layer chromatography and measure individual bile acids after elution.

Enzymatic-Spectrophotometric Method

This method is based on the concept that an enzyme, β-hydroxysteroid dehydrogenase (NAD-linked), isolated from Pseudomonas testosteronii can oxidize the 3β and 3α groups of any bile acid in the presence of NAD. This method originally was applied to bile acids in blood and now has been applied to bile by Javitt and Emerman.

In this procedure, 10 μl of bile are added to a reaction mixture consisting of 1.0 ml of enzyme solution, 0.5 ml of 1 M hydrazine hydrate, 0.25 ml of 5 mM NADP, and 2.0 ml of 0.1 M potassium phosphate buffer, pH 9.4. The net increase in absorbance at 340 nm (light path = 10 mm), against bile and reagent blanks, is used to calculate the amount of bile acid present:

\[
\text{Bile acid (μg)} = \frac{A \times 3 \times \text{mol wt}}{6.26}
\]

in which A = absorbance of NADH formed; mol wt = molecular weight of the individual bile acid; 6.26 = molar absorbance of NADH; and 3 = volume of the incubation mixture.

Using this procedure, Javitt and Emerman found a recovery of greater than 88 percent. For the determination of total bile acids, especially in routine laboratories, this method is fast and accurate. If specific information on the individual bile acids is needed, one has to use thin-layer chromatography and measure individual bile acids after elution.

Paper Chromatography-Spectrophotometric Method

Sjövall's modification of his own original method of measuring bile acids by spectrophotometry after paper chromatography is very elaborate. Various moving phases were used to separate the conjugated and free bile acids on paper chromatography; then the bile acids were eluted with ethanol and the spectra of the bile acids were measured at various wavelengths after reaction with 65 percent sulfuric acid. The time of exposure of the bile acids to the acid was found to be critical. The contribution of other substances to the absorbance was corrected by measuring the spectra of the bile acids in 80 percent ethanol at corresponding wavelengths and subtracting these from the data obtained.

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* Worthington Biochemical Corporation, Freehold, NJ.
Figure 1. Thin-layer chromatography of human biliary bile acids: Silica Gel G (250 μm thick); isooctane-isopropyl ether-acetic acid, 50:25:40 (vol/vol/vol). Plates were stained with 10 percent phosphomolybdic acid in methanol. Bile acids are: 1, cholic; 2, hyodeoxycholic; 3, ursodeoxycholic; 4, chenodeoxycholic; 5, deoxycholic; 6, 3β,12α-dihydroxycholanic; 7, lithocholic; 8, cholesterol, and 9, fatty acids.

The calculation is made from the optical density of reference bile acids.

Using this method, Sjövall calculated the concentration of different bile acids in the bile of rat, rabbit, and man and in human duodenal contents. The recoveries of various added bile acids varied from 90 to 110 percent.

Methods Involving Thin-Layer and Gas-Liquid Chromatography

All methods involving gas-liquid chromatography consist of extraction of bile acids with ethanol and subsequent hydrolysis with alkali or an enzyme, cholylglycyhydrolase. Various concentrations of alkali have been tried; 1 M NaOH at 110° for 6 hours or autoclaving in 1.25 M NaOH for three hours at 15 lb per in² is satisfactory. Enzymatic hydrolysis, described by Nair et al, has the advantage that it is less time-consuming and avoids drastic heating conditions.

After hydrolysis, the saponification mixture is acidified and the bile acids are extracted with ethyl ether and then converted into their methyl esters by reaction with diazomethane. The reaction is rapid and complete methylation occurs very quickly. Bile acids also have been methylated with 5 percent hydrochloric acid in super-dry methanol; however, this does not give complete methylation consistently. The methylated bile acids are then purified by thin-layer chromatography. Various solvent systems have been described for the separation of bile acids. The choice of the solvent system depends on the compound to be separated. An efficient separation of all the biliary bile acids can be obtained in a solvent system consisting of isooctane-isopropyl ether-acetic acid, 50:25:40 (vol/vol/vol) (figure 1).

Other systems described by Hofmann and Eneroth can accomplish similar separation. Thin-layer chromatography is an efficient and fast way of segregating bile acids on the basis of various functional groups. Bile acids from the thin-layer plate can be eluted with methanol or acetone-methanol mixtures and subjected to gas-liquid chromatography after derivatization.

For gas-liquid chromatography, a variety of derivatives and a variety of liquid phases have been used. The best resolution of all the isomers has been obtained by using trifluoroacetates and acetates. Trifluoroacetates also have been found to be excellent for identification of bile acids from various animal sources. In table I are given the retention times of trifluoroacetates of the bile acid methyl esters commonly encountered in human bile and feces. For comparison, the application of the combined thin-layer and gas-liquid chromatographic techniques to identification of biliary bile acids from rats and atherosclerosis-susceptible White Carneau pigeons is also shown in the same table.
TABLE I

IDENTIFICATION OF BILE ACIDS BY THIN-LAYER (TLC) AND GAS-LIQUID CHROMATOGRAPHY

<table>
<thead>
<tr>
<th>Bile Acid</th>
<th>Type by TLC**</th>
<th>Retention Time on Gas-Liquid Chromatography‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Human Standards</td>
</tr>
<tr>
<td>Lithocholic</td>
<td>Monohydroxy</td>
<td>0.58</td>
</tr>
<tr>
<td>3β,12α-Dihydroxycholanic</td>
<td>Dihydroxy</td>
<td>0.89</td>
</tr>
<tr>
<td>Deoxycholic</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>3α,12β-Dihydroxycholanic</td>
<td></td>
<td>1.12</td>
</tr>
<tr>
<td>Chenodeoxycholic</td>
<td></td>
<td>1.23</td>
</tr>
<tr>
<td>Ursodeoxycholic</td>
<td></td>
<td>1.44</td>
</tr>
<tr>
<td>Hyodeoxycholic</td>
<td></td>
<td>1.45</td>
</tr>
<tr>
<td>Hyocholic</td>
<td>Trihydroxy</td>
<td>1.84</td>
</tr>
<tr>
<td>Cholic</td>
<td></td>
<td>2.10</td>
</tr>
<tr>
<td>α-Muricholic</td>
<td></td>
<td>2.23</td>
</tr>
<tr>
<td>β-Muricholic</td>
<td></td>
<td>2.26</td>
</tr>
<tr>
<td>7-Keto-3α-hydroxycholanic</td>
<td>Monohydroxy,</td>
<td>2.38</td>
</tr>
<tr>
<td>3-Keto-7α-hydroxycholanic</td>
<td>monoketo</td>
<td>2.52</td>
</tr>
<tr>
<td>7-Keto-3α,12α-dihydroxycholanic</td>
<td>Dihydroxy,</td>
<td>3.40</td>
</tr>
<tr>
<td></td>
<td>monoketo</td>
<td></td>
</tr>
</tbody>
</table>

** Based on migration of bile acids as used by Subbiah et al.3,8-40

† Bile acids were chromatographed as their methyl ester trifluoroacetates on 1% QF-1 columns as previously described.3,4 The retention times are relative to deoxycholate.

‡ Due to loss of a trifluoroacetoxyl group in the flash heater.4

For the quantitation of bile acids, an internal standard is used. This internal standard should be structurally similar to bile acids and yet not present in the sample. Subbiah et al.40 used cholesterol acetate as an internal standard for the quantitation of rat bile acids as trifluoroacetates; this also can be used for the analysis of human bile. For the quantitation of bile acids as their trimethylsilyl ethers, 5α-cholestane has been used as an internal standard.11 Recently, Klaassen19 suggested 7-ketolithocholic acid as an internal standard for the determination of bile acid as trifluoroacetates. This acid has a longer retention time than cholic acid. Klaassen also decreased the time of gas-liquid chromatography to four minutes.

When only total bile acid concentrations need to be measured, the enzymatic method is ideal. However, when the specific acids need to be measured, the combination of thin-layer and gas-liquid chromatography is ideal.

Feecal Bile Acid Determination

Determination of bile acids in feces has always been complicated by the presence of multiple bacterial transformation products that are excreted. A number of methods were described before the development of gas-liquid chromatography. These will be discussed only briefly because they are not adequate. The method of Mosbach et al.29 using spectrophotometry, is specific only for dihydroxy- and trihydroxy-bile acids. Since feces contain significant amounts of lithocholic and a variety of keto-bile acids,6,7 this method is not very useful. A method involving ion-exchange chromatography, described by Kuron and Tennent,24 has difficulties in removing other acidic contaminants. The method of Goldsmith et al.10 involving column chromatography and titration, has been found to be inadequate because of the incomplete removal of titratable contaminants.11

Two methods of calculating bile acid excretion by using isotopes deserve discus-
sion. Hellmann et al.\textsuperscript{13} administered labeled cholesterol, determined the radioactivity in the bile acid and sterol fractions of the feces and compared it to the radioactivity in the plasma. The technique is based on the assumption that the specific activity in the plasma is similar to that in the feces when measured simultaneously. However, in this method, a correction factor has to be applied to account for the formation of bile acids from cholesterol and their eventual excretion in the feces. When such correction factors are applied, the method gives bile acid excretion data that are comparable to data obtained by a chromatographic method.\textsuperscript{21} One cannot get information on the bacterial degradation products of bile acids. Another factor to be considered is the delay in equilibration of the administered cholesterol with plasma and the tissue pool.

The excretion of bile acids also can be calculated by the isotope turnover method described by Lindstedt.\textsuperscript{26} In this method, labeled cholic and chenodeoxycholic acids are administered and duodenal samples are obtained (by intubation) over a period of nine days. On the basis of the specific activity of the cholic and chenodeoxycholic acids in the bile, the pool size and turnover of the primary bile acids can be calculated. Such methods have been used by Kottke\textsuperscript{20} to calculate the bile acid excretion in patients with type II and type IV hyperlipoproteinemia. It is not possible by this method to get data on the bacterial degradation of bile acids.

The only satisfactory techniques for identifying and measuring individual bile acids in the feces are those involving thin-layer and gas-liquid chromatography. Grundy et al.\textsuperscript{11} described such a method. After a mild saponification of the feces with 20 ml of 1 N NaOH in 90 percent ethanol, the neutral sterols are extracted with petroleum ether. To the lower aqueous phase containing the bile acids, 2 ml of 10 N NaOH are added and the mixture is saponified at two atmospheres for three hours in a pressure cooker. After acidification, the bile acids are extracted with chloroform-methanol (2:1) and chloroform. The bile acids are then methylated with 5 percent hydrochloric acid in super-dry methanol, and the methyl esters are separated from fatty acids by thin-layer chromatography with benzene and isooctane-isopropanol-acetic acid, 120:40:1 (vol/vol/vol). After elution with methanol, the bile acids can be converted into their trimethylsilyl ethers\textsuperscript{27} and chromatographed on DC-500 or SE-30 columns with 5α-cholestane as an internal standard. (Because it elutes close to the solvent front on gas-liquid chromatography, 5α-cholestane is not ideal.) However, Grundy et al.\textsuperscript{11} measured total bile acid areas and the concentrations of individual bile acids were not measured. This method has been used to study the excretion of bile acids by patients with various types of hyperlipoproteinemias.\textsuperscript{13}

Ali et al.\textsuperscript{1} described a method in which, after extraction, the bile acids are subjected to column chromatography to remove fatty acids and then measured by gas-liquid chromatography; the bile acid methyl esters are chromatographed as their trifluoroacetates with chenodeoxycholic acid as an internal standard. Since chenodeoxycholic acid can be present in fecal samples, especially in patients treated with antibiotics, samples are subjected to chromatography with and without the internal standard. In their method, Ali et al did not use standards to account for procedural losses. Evrard and Janssen\textsuperscript{8} were the first to use an internal standard (23-nordeoxycholate) that could be added at the beginning of the extraction and followed through the entire procedure of purification and quantitation by gas-liquid chromatography. However, these authors oxidized the bile acids and measured the resulting keto
bile acids. Hence, not much data could be obtained regarding the bacterial degradation products within a given class.

This suggested the necessity of using an internal standard that could be added at the beginning of extraction in a procedure in which the individual bile acids could be measured by gas-liquid chromatography. After experimenting with a number of bile acids in our laboratory, it was found that hyocholic acid could serve as an ideal standard for the quantitation of human fecal bile acids for the following reasons: (1) it does not occur in human feces, (2) it accompanies the bile acids during the extraction and purification procedure, (3) it is eluted at a reasonably good position when the bile acids are chromatographed as their methyl ester trifluoroacetates on QF-1 columns and (4) it is completely resolved from all the other bile acids that occur in normal feces. The recovery of hyocholate and other labeled bile acids added to the samples was good.

In our procedure, the sterols are removed from fecal samples as described by Miettinen et al. To the aqueous phase, two ml of 10 N NaOH are added and the mixture is saponified for three to five hours at 110°. The bile acids are then extracted with chloroform-methanol (2:1), ethyl acetate, or chloroform. The bile acids are methylated with diazomethane. The methyl esters are purified by thin-layer chromatography as described by Grundy et al. For complete recovery of the bile acid methyl esters from the silica gel, elution is performed twice with 25 ml of methanol and once with 25 ml of acetone-methanol (1:9). The bile acids are converted into their trifluoroacetates and analyzed on 1 percent QF-1 columns. All the bile acids are completely resolved, and the amount of each bile acid can be calculated from the concentration of hyocholate added. With this procedure, the concentration of bile acids in six stool specimens (from the same day's collection) was 2.69 ± 0.25 mg per g.

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