Ion Pair Extraction Technique with Azure A, for Differentiating Biles of Normal Subjects and Patients with Liver Disease from Patients with Crohn’s Disease with Small Bowel Involvement

G. Robert Webb, Ian A. Macdonald,¹ and C. Noel Williams

The ion pair extraction technique with Azure A solution, chloroform/methanol (equal volumes), and dilute sulfuric acid, was used to study bile-rich duodenal aspirates of (a) healthy volunteers and patients with (b) cholestatic liver disease, (c) Crohn’s disease with small bowel involvement, and (d) those with primarily colon involvement. Duplicate aliquots of extracted duodenal aspirates from these four groups were subjected to ion pair extraction, with and without prior acetylation, and the absorbance ratios at 645 nm without acetylation/with acetylation (“acetylation index”) compared; values (±SD) obtained were 0.052 ± 0.0098, 0.038 ± 0.014, 0.136 ± 0.048, and 0.077 ± 0.056, respectively, for the four groups. The acetylation index for group c was significantly (P < 0.001) different from that of groups a and b. All other intergroup comparisons were not significant. The acetylation index correlated positively (r = 0.922) with the ratio of glycine- to taurine-conjugated bile acids, measured independently, implying that it reflects this ratio.

Both ileal and cholestatic liver disease can exert a profound effect on bile-salt metabolism (1, 2). One of the more sensitive variables altered by disease process appears to be the ratio of glycine-conjugated to taurine-conjugated bile salts (G:T ratio) found in duodenal and gallbladder bile. Ileal disease (including Crohn’s disease and ileal fistulae) increases the G:T ratio to values ranging from 5–20 (normal range, 1–5) (3, 4), while liver disease may depress them to values often less than unity (3).

The G:T ratio has been measured by thin-layer chromatography and enzymatic (4) or non-enzymatic estimation (5) of individual bile acids. More recently, it has been measured by enzymatic hydrolysis and estimation of free glycine and taurine (6).

Christie et al. (7) applied acetylation and ion-pair extraction (8, 10), with use of Azure A, to rapidly estimate total taurine-conjugated bile salts and the G:T ratio. This method involves formation of ion pairs (organic sulfates or sulfonates and Azure A) with charge neutralization and extraction of the neutral complex into the organic phase. These authors (7) also imply that various bile salt sulfates and cholesterol sulfates may be estimated by the ion pair extraction technique (Table 1).

This communication describes how their technique may be extended by estimating the ion-pair-extracted Azure A, both with and without the preceding acetylation step, with use of extracts of duodenal aspirates from normal subjects, patients with Crohn’s disease, and patients with liver disease.

Materials and Methods
Reagents
All solvents used were reagent grade, distilled once before use.

Azure A. This solution (Fisher Chemicals, Montreal, Quebec) consisted of 40 mg of Azure A in 5 ml of 2.5 mmol/liter sulfuric acid and 95 ml of distilled water. Solutions were prepared freshly each week and stored in a foil-covered bottle (at room temperature) to minimize exposure to light.

Chloroform/methanol, equal volumes. This mixture was stored at room temperature in a screw-cap reagent bottle.

Standard solution of taurocholate, 2 mmol/liter, in methanol. Solutions were standardized spectrophotometrically by use of 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50; Worthington), 1 g/liter, and complete oxidation as described earlier (7).

Subjects and Patients
Four groups were studied: (a) eight healthy volunteers, who had normal liver-function tests, were asymptomatic, and were taking no drugs; (b) five patients with cholestatic liver disease, comprising four patients with primary biliary cirrhosis and one with

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¹ Address correspondence to this author, at the Clinical Research Centre, 5849 University Ave., Dalhousie University, Halifax, Nova Scotia.

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Table 1. Effect of Acetylation of Sulfates and Sulfonates on Ion Pair Extraction

<table>
<thead>
<tr>
<th>Compound</th>
<th>Without acetic anhydride treatment</th>
<th>With acetic anhydride treatment</th>
<th>Acetylation index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesteryl-3-sulfate</td>
<td>14.5</td>
<td>0.0</td>
<td>infinite</td>
</tr>
<tr>
<td>Lithocholate-3-sulfate</td>
<td>8.0</td>
<td>0.0</td>
<td>infinite</td>
</tr>
<tr>
<td>Glycocholate-3-sulfate</td>
<td>3.2</td>
<td>0.0</td>
<td>infinite</td>
</tr>
<tr>
<td>Taurocholate-3-sulfate</td>
<td>3.0</td>
<td>4.9</td>
<td>0.61</td>
</tr>
<tr>
<td>Taurocholate</td>
<td>0.02</td>
<td>16.2</td>
<td>0.001</td>
</tr>
<tr>
<td>Taurochenodeoxycholate</td>
<td>0.5</td>
<td>16.5</td>
<td>0.03</td>
</tr>
<tr>
<td>Taurodeoxycholate</td>
<td>0.5</td>
<td>16.5</td>
<td>0.03</td>
</tr>
<tr>
<td>Taurocholate</td>
<td>7.0</td>
<td>16.2</td>
<td>0.43</td>
</tr>
<tr>
<td>Sulfatide</td>
<td>12.5</td>
<td>9.5</td>
<td>1.32</td>
</tr>
<tr>
<td>Taurine</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Adapted from Christie et al. (7).

Absorbance of color complexes of 80 nmol compound with Azure A extracted into the chloroform/methanol phase.

Absorbance at 645 nm without acetylation procedure/absorbance at 645 nm with acetylation procedure.

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childhood cirrhosis with paucity of intrahepatic bile ducts; (c) six patients with Crohn’s disease affecting the distal ileum with or without other small bowel involvement (Table 2); and (d) four patients with Crohn’s disease with primary colon involvement. All patients were studied after three days on a diet containing 100 g of fat and 2500 calories, in hospital; the normal subjects ate their usual diet and were studied as outpatient.

Procedure

Collection and extraction of bile-rich duodenal aspirates. Bile-rich duodenal aspirates, 3 to 5 ml, were collected as described previously (11). One milliliter was extracted according to Folch (12) and the upper phase reconstituted into 1 ml of a mixture of methanol and 3% hydrogen peroxide, 5/1 by volume, and stored at 4 °C.

Estimation of total taurine-conjugated bile salts. This was estimated according to Christie et al. (7).

Estimation of the “acetylation index”. We used the ion pair extraction technique described by Christie et al. (7), both with and without the acetylation step previously described. Volumes of 2–100 µl of bile-rich duodenal aspirate were used when the acetylation step was omitted.

The “acetylation index” was defined as:

Absorbance at 645 nm/ unit volume extract without acetylation step

Absorbance at 645 nm/ unit volume extract with acetylation step

Estimations of the acetylation index were done in duplicate in 2–6 (usually 4) serial samples from each subject.

Estimation of the G:T ratio, chromatographically and non-chromatographically. Folch extracts of duodenal aspirates (10–100 µl) were subjected to thin-layer chromatography (solvent system: toluene/acetic acid/water, 10/10/1 by volume) and individual conjugated bile salts were eluted from appropriate sections of the plate and estimated according to Macdonald et al. (13, 14). The G:T ratio was defined by:

Total glycine-conjugated bile salts

Total taurine-conjugated bile salts

Nonchromatographic estimation of the G:T ratio was performed according to Christie et al. (7). This was computed by: (total conjugated bile salts – total taurine

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Table 2. Clinical and Laboratory Data

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Involvement or stage</th>
<th>Medication</th>
<th>Serum AST ml/dl</th>
<th>Serum bilirubin mg/dl</th>
<th>Serum ALP mg/dl</th>
<th>Serum albumin g/dl</th>
<th>G:T ratio</th>
<th>Acetylation index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>51</td>
<td>PBC</td>
<td>IV</td>
<td>n/d</td>
<td>162</td>
<td>1.3</td>
<td>550</td>
<td>3.9</td>
<td>0.5</td>
<td>0.03</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>53</td>
<td>PBC</td>
<td>IV</td>
<td>Prednisone</td>
<td>80</td>
<td>1.2</td>
<td>210</td>
<td>3.8</td>
<td>2.9</td>
<td>0.05</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>48</td>
<td>PBC</td>
<td>III</td>
<td>Imuran</td>
<td>125</td>
<td>3.3</td>
<td>300</td>
<td>4.7</td>
<td>0.3</td>
<td>0.02</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>30</td>
<td>PBC</td>
<td>III</td>
<td>Imuran</td>
<td>92</td>
<td>1.1</td>
<td>585</td>
<td>4.2</td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>11</td>
<td>CPID b</td>
<td>—</td>
<td>n/d</td>
<td>140</td>
<td>2.0</td>
<td>241</td>
<td>—</td>
<td>0.7</td>
<td>0.036</td>
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<tr>
<td>6</td>
<td>M</td>
<td>37</td>
<td>CD</td>
<td>term ileum 10 cm</td>
<td>n/d</td>
<td>35</td>
<td>0.4</td>
<td>70</td>
<td>4.6</td>
<td>2.4</td>
<td>0.08</td>
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<tr>
<td>7</td>
<td>F</td>
<td>16</td>
<td>CD</td>
<td>term ileum 25 cm</td>
<td>n/d</td>
<td>40</td>
<td>0.4</td>
<td>110</td>
<td>3.6</td>
<td>5.1</td>
<td>0.099</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>30</td>
<td>CD</td>
<td>term ileum 25 cm</td>
<td>n/d</td>
<td>25</td>
<td>0.5</td>
<td>85</td>
<td>4.4</td>
<td>15.7</td>
<td>0.155</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>35</td>
<td>CD</td>
<td>term ileum 22 cm</td>
<td>n/d</td>
<td>27</td>
<td>0.9</td>
<td>145</td>
<td>3.5</td>
<td>15.4</td>
<td>0.210</td>
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<tr>
<td>10</td>
<td>M</td>
<td>20</td>
<td>CD</td>
<td>duod-mid jej 25 cm</td>
<td>n/d</td>
<td>37</td>
<td>0.4</td>
<td>70</td>
<td>3.9</td>
<td>7.1</td>
<td>0.164</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>27</td>
<td>CD</td>
<td>jej fistula 17 cm</td>
<td>n/d</td>
<td>40</td>
<td>0.5</td>
<td>60</td>
<td>2.5</td>
<td>13.4</td>
<td>0.110</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>55</td>
<td>CD</td>
<td>total colon</td>
<td>Prednisone</td>
<td>55</td>
<td>0.6</td>
<td>15</td>
<td>3.7</td>
<td>7.3</td>
<td>0.160</td>
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<tr>
<td>13</td>
<td>F</td>
<td>40</td>
<td>CD</td>
<td>total colon</td>
<td>n/d</td>
<td>30</td>
<td>0.6</td>
<td>45</td>
<td>4.0</td>
<td>4.5</td>
<td>0.046</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>35</td>
<td>CD</td>
<td>skip lesions</td>
<td>n/d</td>
<td>30</td>
<td>0.5</td>
<td>82</td>
<td>2.1</td>
<td>3.0</td>
<td>0.063</td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>23</td>
<td>CD</td>
<td>skip lesions</td>
<td>n/d</td>
<td>30</td>
<td>0.5</td>
<td>115</td>
<td>3.3</td>
<td>1.5</td>
<td>0.037</td>
</tr>
</tbody>
</table>

Abbreviations: PBC, Primary biliary cirrhosis; CPID, cirrhosis with paucity of intrahepatic bile ducts; CD, Crohn’s disease; G:T, glycine conjugates/taurine conjugates; duod, duodenum; jej, jejunum; AST, aspartate aminotransferase.

b Patient SH in reference 19.
conjugated bile salts)/total taurine conjugated bile salts, where each estimation is performed in the absence of chromatographic separation of the glycine and taurine conjugates.

**Statistical analysis of data.** We assessed the correlation between the acetylation index and the G:T ratio in all four groups (15). The chromatographic determination of the G:T ratio was similarly compared with its non-chromatographic determination. Intergroup comparison of the acetylation index was assessed by use of the group t-test.

**Results**

Duplicate absorbance estimations for single extracts fell within ±3% for all three groups studied. The coefficients of variation for acetylation indices of 2–6 serial samples were 15%, 17%, 10%, and 30% for groups a to d, respectively. The variation in bile acid concentration of serial samples of the bile rich extracts did not appreciably affect the reproducibility of estimation of either the acetylation index or the G:T ratio. For practical purposes a total bile acid concentration of <3 mmol/liter in a 1.0-ml extract was insufficient for determining the acetylation index.

The mean acetylation index for groups a to d was 0.052 ± 0.0098, 0.038 ± 0.014, 0.136 ± 0.048, and 0.077 ± 0.056, respectively (Figure 1). The acetylation index for the Crohn’s disease groups with ileal involvement, group c, was significantly (P < .001) different from that of the normal group and from the liver disease group (b) (P < .001), whereas the other intergroup differences were not significant (P > .05). Acetylation indices of all patients in group c fell outside our extremes in normal values (0.037 – 0.059). Acetylation indices of two of the five patients in group b fell within the normal range, and these same two individuals had normal G:T ratios. Acetylation indices of three of the four patients in group d fell within this normal range, and these same three individuals had normal G:T ratios.

When the acetylation index was plotted vs. the G:T ratio (Figure 2), a close correlation was observed (r = 0.922; slope = 0.0100; intercept = 0.0289). When the G:T ratios obtained by ion pair extraction (7) were compared with G:T values by thin-layer chromatography (4) the correlation (not shown) was excellent (r = 0.958; slope = 1.01; intercept = –0.30). However, while the acetylation index correlated well with the G:T ratio, it did not correlate with other biochemical data shown in Table 2.

**Discussion**

We have interpreted the acetylation index to simply reflect the amounts of steroid-3-sulfates (Table 1) or other undescribed materials, or both, that will either partly or completely partition into the upper phase during Folch extraction. As mentioned earlier, care and precision during Folch extraction is an essential part of this procedure (7). A “clean” Folch extraction will eliminate unhydrolyzed phospholipid (i.e., cardiolipin) and fat-soluble sulfolipid that may be present in bile and have been described as interfering materials (7, 10). The depressed acetylation index values obtained in our cirrhosis patients was somewhat surprising because bile acid sulfates have been reported in the urine of patients with cholestatic liver disease (16, 17), whereas in Crohn’s disease any bile acid sulfates in urine have yet to be described. We do not, however, yet know the nature of “colorigenic” materials evident in the bile of Crohn’s disease patients but absent in that of patients with cirrhosis. We have not detected any extra bands on thin-layer chromatography of bile-rich extracts from patients with liver disease or normal persons, but are now investigating several extra bands found by thin-layer chromatography of extracts of bile from Crohn’s disease patients. In primary biliary cirrhosis, the relative percentage of the taurocholic acid in bile increases relative to other taurine conjugates (1) and this may partially account for the lowering of the background (Table 1). Although the correlation between the acetylation index and the G:T ratio is good (r = 0.922), the relationship between these parameters is not clear. Elevated G:T ratio and increased amounts of “colorigenic” material in the bile may be related phenomena originating in the liver. The acetylation index may become of value as an adjunct to the G:T ratio, particularly in the diagnosis of ileal disease (18). This work confirms and extends the observations of Christie et al. (7) and thus the acetylation index may represent an empirical estimation of the G:T ratio.
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References