Bile Acid Assays as an Index of Cholestasis

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Four bile acid profiles, bilirubin, alkaline phosphatase, alanine aminotransferase, glutamate dehydrogenase, and isocitrate dehydrogenase were measured in rats in which normal bile flow had been disturbed either by administration of α-naphthylisothiocyanate or by surgical ligation of the bile duct. The most sensitive index to the early onset of cholestasis was an increase in total cholate. This increase was measurable before morphological changes could clearly be identified.

Additional Keyphrases: enzyme activity - early diagnosis - hepatobiliary function - rats - “kit” methods

The use of bile acid assays as an index of hepatobiliary function has been known for many years (1), but until recently the technical difficulties of the assay systems have militated against their widespread use (2, 3).

The introduction of a cholic acid radioimmunoassay procedure in 1973 (4) has been followed by the development of antisera with various specificities. Several kits, covering a wide spectrum of bile acid combinations, are now commercially available and provide rapid and precise assay procedures.

Bile acids, being the endproducts of cholesterol metabolism in the liver, provide a direct measurement of hepatocyte function, and it is not surprising that increased plasma bile acid concentrations have been reported in various hepatobiliary disorders (5–9). The high sensitivity of the assays may be a disadvantage in routine clinical use (10), but represents a considerable advantage in the study of drug-induced hepatotoxic reactions, particularly in the safety evaluation of novel therapeutic agents.

This study was designed to investigate the sensitivity of four commercially available bile acid kits, each designed to assay a different bile acid profile, and to compare these with the more commonly accepted liver enzymes in rats treated with the cholestasis-inducing agent, α-naphthylisothiocyanate (ANIT), or with mechanically occluded bile ducts.

Materials and Methods

Kits and Analytical Procedures

Glycocholic Acid RIA Kit (Nordiclab). The antiserum supplied cross-reacts equally with cholic acid and its glycine and taurine conjugates.

Glychenodeoxycholic Acid RIA Kit (Nordiclab). The antiserum supplied cross-reacts with chenodeoxycholic acid and its glycine and taurine conjugates.

Both of these kits were obtained through Warner Lambert (UK) Ltd., Eastleigh, U.K.

C.G. RIA Kit (Abbott Laboratories, Basingstoke, U.K.). The antiserum was raised against glycocholic acid but also cross-reacts at about 15% affinity with taurocholic acid and glychenodeoxycholic acid.

Conjugated Bile Acid Solid-Phase Radioimmunoassay Kit (Becton Dickinson Immunodiagnostics, Wembley, U.K.). The antiserum supplied binds the primary bile acids with differing affinities. Stated cross reactivities were: taurocholic acid 100%, glycocholic acid 85%, taurochenodeoxycholic acid 210%, glychenodeoxycholic acid 62%.

All assays were performed according to the manufacturers’ protocols, and final radioactivities were counted with a Packard 5221 gamma counter.

Alkaline phosphatase (EC 3.1.3.1): Boehringer Kit 123862, at 30 °C

Alanine aminotransferase (EC 2.6.1.2): Boehringer Kit 124583, at 30 °C

Isocitrate dehydrogenase (EC 1.1.1.42): Boehringer Kit 125989, at 30 °C

All enzyme assays were carried out kinetically with use of the Vitatron AKES.

Bilirubin. Boehringer D.P.D. method, 123951 Vitatron PA800, 37 °C, at 546 nm

All reagents were obtained from BCL, Lewes, U.K.

Procedures

ANIT administration. Forty male Sprague-Dawley CD rats weighing 130–150 g, supplied by Charles River, Manston, U.K., were allocated into four treatment groups in such a way as to give the least difference between group mean body weights.

α-Naphthylisothiocyanate (ANIT) was administered by esophageal gavage at doses of 30, 60, and 120 mg/kg body weight to the three test groups. The fourth group received the vehicle alone (methyl cellulose, 10 g/L of distilled water). The dose volume in each case was 10 mL/kg body weight. Animals were killed 48 h after dosing by exsanguination via the dorsal aorta under pentobarbital anesthesia, blood samples being collected into lithium heparin. Livers were removed for histological examination.

Bile duct ligation. A further 24 rats from the same source were taken, 20 of which underwent bile-duct ligation, the remaining four being subjected to identical surgical treatment but with the ligature left untied. The animals were anesthetized with halothane and a midline abdominal incision was made. The bile duct was ligated near the liver, the wound closed, and the animals were returned to holding cages. Surgical procedures were completed within 10 min; the period of anesthesia averaged 15 min. After 2 h the animals were killed by exsanguination via the dorsal aorta under halothane anesthesia and blood was collected, with lithium heparin as anticoagulant.

Results

Precision of bile acid assays. A sample of normal pooled plasma was included and assayed 20 times within one batch for each diagnostic kit. The same operator was responsible for all assays. Under these conditions the coefficient of variation for the four kits were as follows: total cholate (Nordiclab) 4.84%, glycocholic acid (Abbott) 1.26%, total conjugated bile acids (Becton Dickinson) 6.43%, and total chenodeoxycholic acid 4.21%.

Effect of ANIT administration. In the animals receiving the lowest dose of ANIT (30 mg/kg), the mean value for alanine aminotransferase, bilirubin, and each of the bile acid profiles was higher in the test group than in the control group (Tables...
Table 1. Enzyme Activities in Rat Plasma after ANIT Administration

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>AP</th>
<th>ALT</th>
<th>GDH</th>
<th>ICDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>587.3 (86.8)</td>
<td>31.0 (3.2)</td>
<td>7.9 (3.1)</td>
<td>4.2 (1.5)</td>
</tr>
<tr>
<td>ANIT, 30 mg/kg</td>
<td>576.2 (106.0)</td>
<td>35.1 (3.2)**</td>
<td>8.0 (3.4)</td>
<td>3.5 (2.5)</td>
</tr>
<tr>
<td>ANIT, 60 mg/kg</td>
<td>986.8 (249.6)**</td>
<td>234.5 (129.9)**</td>
<td>248.0 (136.4)**</td>
<td>31.7 (20.2)**</td>
</tr>
<tr>
<td>ANIT, 120 mg/kg</td>
<td>1265.8 (193.8)**</td>
<td>439.9 (129.3)**</td>
<td>397.9 (160.8)**</td>
<td>78.0 (29.4)**</td>
</tr>
</tbody>
</table>

* Arithmetic means (and SD) and statistical significances of difference from mean using Student's t-test are shown: * p < 0.05; ** p < 0.01; *** p < 0.001.

Table 2. Bilirubin and Bile Acid Concentrations in Rat Plasma after ANIT Administration

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Bilirubin</th>
<th>Glycocholate</th>
<th>Cholate</th>
<th>Conjugated primary bile acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.2 (0.9)</td>
<td>1.6 (1.0)</td>
<td>5.0 (2.4)</td>
<td>1.1 (0.7) 3.7 (1.1)</td>
</tr>
<tr>
<td>ANIT, 30 mg/kg</td>
<td>3.4 (0.9)**</td>
<td>2.4 (1.0)*</td>
<td>10.1 (3.6)**</td>
<td>1.8 (0.9)* 4.6 (1.7)</td>
</tr>
<tr>
<td>ANIT, 60 mg/kg</td>
<td>40.7 (35.1)**</td>
<td>50.2 (32.1)**</td>
<td>53.9 (44.0)**</td>
<td>3.0 (1.3)** 118.8 (74.2)**</td>
</tr>
<tr>
<td>ANIT, 120 mg/kg</td>
<td>127.4 (48.7)**</td>
<td>104.5 (31.2)**</td>
<td>102.9 (45.9)**</td>
<td>4.9 (1.3)** 229.5 (72.2)**</td>
</tr>
</tbody>
</table>

* Values and statistical analysis as in Table 1.

Table 3. Enzyme Activities in Rat Plasma after Bile-Duct Ligation for 2 h

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>AP</th>
<th>ALT</th>
<th>GDH</th>
<th>ICDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>410.8 (259.3)</td>
<td>39.3 (9.4)</td>
<td>7.3 (5.7)</td>
<td>0</td>
</tr>
<tr>
<td>Test</td>
<td>346.7 (191.5)</td>
<td>376.5 (222.9)**</td>
<td>593.9 (464.2)**</td>
<td>124.3 (112.5)**</td>
</tr>
</tbody>
</table>

* Values and statistical analysis as in Table 1. * Abbreviations as in Table 1.

Table 4. Bilirubin and Bile Acid Concentrations after Bile Duct Ligation for 2 h

<table>
<thead>
<tr>
<th>Group</th>
<th>Bilirubin</th>
<th>Glycocholate</th>
<th>Cholate</th>
<th>Conjugated primary bile acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.7 (0.4)</td>
<td>0.8 (0.4)</td>
<td>3.4 (2.7)</td>
<td>0.6 (0.7) 3.5 (0.8)</td>
</tr>
<tr>
<td>Test</td>
<td>12.3 (1.3)**</td>
<td>134.3 (53.6)**</td>
<td>526.2 (170.3)**</td>
<td>12.5 (11.1)** 158.1 (35.3)**</td>
</tr>
</tbody>
</table>

* Values and statistical analysis as in Table 1.

1 and 2). This increase was statistically significant for the Abbott C.G. Kit and the Nordiclab glycochenodeoxycholic acid kit (p < 0.05), for bilirubin (p < 0.01), and for the Nordiclab glycochenodeoxycholic acid kit and alanine aminotransferase (p < 0.001).

Alkaline phosphatase, glutamate dehydrogenase, and isocitrate dehydrogenase activities were similar in test and control animals. In the intermediate- and high-dose groups (60 and 120 mg/kg) all of the indices studied were significantly increased above control values, the largest percentage mean increases being found for glycochenodeoxycholic acid (Abbott Kit), total conjugated bile acids (Becton Dickinson Kit), and glutamate dehydrogenase.

Examination of liver sections by light microscopy showed no changes in the low-dose group that could be attributed to the administration of ANIT. Liver sections from intermediate- and high-dose animals showed evidence of cholangitis, though these changes were not extensive and only minimal effects on hepatocyte structure were seen.

Bile duct ligation. Bile duct ligation maintained for 2 h produced large increases (p < 0.001) in all of the variables studied except for alkaline phosphatase (Tables 3 and 4). The mean percentage increase in glycochenodeoxycholic acid was by far the greatest, though isocitrate dehydrogenase showed high activities in test animals despite being below the limits of detectability in controls. Because previous work has shown no marked histological effects after 24 h of ligation (11), we did not examine liver sections.

Discussion

The introduction of new drugs is accompanied by trials of therapeutic efficacy and a thorough evaluation of potential hazards that may ensue after their administration. Some drugs now known to have hepatotoxic side effects produce a reaction similar to acute hepatic necrosis, for example, tetracycline (12). Much more common, however, is a reaction resulting in intrahepatic cholestasis, which may be sufficiently severe to halt bile flow and result in visible jaundice.

In the safety evaluation of any new drug it is essential to use the most sensitive indices of early damage, both in humans and in the earlier stages involving animal models. In this latter area, rats are widely used, and alkaline phosphatase is unfortunately a relatively insensitive index to cholestasis in this species.

The experimental induction of intrahepatic cholestasis in the rat with ANIT provides a well-established and predictable model (13, 14). This action of ANIT is via interference with bile acid metabolism, resulting in alteration of the bile acid profile to one in which the concentrations of the less-hydroxylated forms are increased. Morphological damage re-
sulting from prolonged administration of this agent is probably the result of micellar solubilization of cellular structures by the detergent properties of these retained bile acids.

The results of this study indicate that the sensitivity of bile acid measurements as a marker of liver damage depends on the bile acid profile used. We saw only minimal morphological damage in the liver sections of our high- and intermediate-dose groups, yet all indices studied were significantly increased as compared with the control animals. At the low dose, no definite structural changes were visible by light microscopy, but alanine aminotransferase and total cholate were significantly increased \( p < 0.001 \), the percentage mean increase being much greater for total cholate (+101% as compared with +13%)

None of the other enzymes measured was significantly increased at this dose level and, although glycocholic acid and total chenodeoxycholate were significantly \( p < 0.05 \) increased, values for total conjugated bile acids were comparable with controls.

Total conjugated bile acids have previously been measured in manganese sulfate-induced cholestasis in the rat (15), but despite obvious morphological damage and similar enzyme patterns to those seen in the higher-dosed groups in the present study, these workers were unable to demonstrate an increase in total conjugated bile acids of male animals, though an increase was shown in females. This may reflect the varying affinities of the antiserum used for taurine and glycine conjugates. Taurine conjugation occurs much more readily in the rat than does glycine conjugation (16) and the total bile acid as measured by this method would be affected by the relative proportions of each conjugate. Female rats are more susceptible to cytotoxic and cholestatic damage induced by the accumulation of chenodeoxycholate (17), and this probably accounts for the sex-related differences.

These workers also reported increases in enzyme activity and in total conjugated bile acids in the rat after bile-duct ligation for 12 h. Using similar surgical procedures and maintaining the obstruction for only 2 h, we were able to demonstrate increased plasma concentrations of each bile acid profile. The magnitude of the increase in total conjugated bile acids was similar to that previously reported, whilst glycocolate and total cholate showed increases four times greater than that of total conjugated bile acids.

However, we were unable to demonstrate any significant increase in alkaline phosphatase. This may be the result of a more rapid increase in bile acids than in alkaline phosphatase in response to the restricted bile flow. This conclusion would be consistent with the work of Krysiewski et al. (18), who showed that after ligation there was a gradual increase in alkaline phosphatase, reaching a peak at 24 h.

Although a more extensive evaluation of the role of bile acid assays in routine toxicological studies remains to be done, our results indicate that bile acid assays in general provide a sensitive means of assessing cholestasis, and that the assay of total cholate in rats is superior in sensitivity and specificity to other clinical chemical indices. Furthermore, measurable changes in total cholate are apparent before any unequivocal morphological change is visible by light microscopy. On this basis, there is good reason to believe that assays of appropriate bile acids will provide a valuable tool in the assessment of drug-induced cholestasis.

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References