Changes in the binding of radioactive conjugated bile salts to serum proteins in cholestatic jaundice

A. CHITRANUKROH AND B. H. BILLING

Academic Department of Medicine, Royal Free Hospital School of Medicine, London

(Received 2 August 1982; accepted 19 January 1983)

Summary

1. A micro-partition centrifugal ultrafiltration technique has been used to monitor the percentage of \([^{14}C]\)glycocholate, \([^{3}H]\)glycochenodeoxycholate and \([^{3}H]\)glycochenodeoxycholate-3-sulphate bound to serum proteins of patients with cholestatic liver disease.

2. In comparison with normal individuals the percentage of binding of \([^{14}C]\)glycocholate and, to a lesser extent, of \([^{3}H]\)glycochenodeoxycholate and \([^{3}H]\)glycochenodeoxycholate-3-sulphate was reduced.

3. The binding of \([^{14}C]\)glycocholate was inversely related to the serum bile salt and bilirubin concentrations. In contrast, the binding of \([^{3}H]\)glycochenodeoxycholate and \([^{3}H]\)glycochenodeoxycholate-3-sulphate were not altered by the severity of the cholestasis.

4. Studies \textit{in vitro} indicated that the reduction in the binding of \([^{14}C]\)glycocholate in cholestatic liver disease was not due to high concentrations of bile salts, unconjugated bilirubin or fatty acids.

Key words: bile salts, cholestasis, protein binding, micro-ultrafiltration technique.

Abbreviations: GC, glycocholate; GCDC, glycochenodeoxycholate; GCDCS, glycochenodeoxycholate-3-sulphate.

Introduction

A large number of compounds, including bile salts, are transported in the blood bound to protein, particularly albumin, and some are known to share common binding sites \([1]\). Since the binding affinities of bile salts are amongst the lowest reported for albumin ligands \([1-3]\) a reduction in the binding of bile salts might be expected as a result of competition for albumin-binding sites by other serum components, which are raised in liver disease.

In order to test this hypothesis a simple micro-partition centrifugal ultrafiltration technique has been used to monitor the binding of \([^{14}C]\)glycocholate, \([^{3}H]\)glycochenodeoxycholate and \([^{3}H]\)glycochenodeoxycholate-3-sulphate in sera from patients with cholestatic liver disease. These bile salts were chosen for the study because glycocholate and glycochenodeoxycholate are the major bile salts of cholestatic serum, whereas glycochenodeoxycholate-3-sulphate is prominent in cholestatic urine and may also be present in the serum \([4, 5]\).

Materials and methods

Radioactive materials

\([^{1-^{14}}C]\)Glycocholate \((^{14}C-GC)\) (specific radioactivity 54 mCi/mmol; \(2 \times 10^{9}\) Bq/mmoll), \(D-^{[1-^{14}}C\) glucose \((50\text{ mCi/mmol; }1.9 \times 10^{9}\text{ Bq/mmoll})\) and \(^{3}H_2O\) (5 Ci/ml; \(18.5 \times 10^{10}\text{ Bq/ml}\)) were purchased from The Radiochemical Centre (Amersham, Bucks, U.K.). \([G-^{3}H]\)Glycochenodeoxycholate \((^{3}H-GCDC)\), specific radioactivity 2.3 Ci/mmoll \((8.5 \times 10^{10}\text{ Bq/mmoll})\), was obtained from New England Nuclear (Boston, MA, U.S.A.). \([G-^{3}H]\)Glycochenodeoxycholate-3-sulphate \((^{3}H-GCDCS)\) (specific radioactivity \(8\text{ mCi/mmoll; }29.6 \times 10^{7}\text{ Bq/mmoll}\)) was synthesized by Dr T. C. Bartholomew \([6]\).

Serum samples

Serial serum samples were obtained from patients with benign recurrent intrahepatic chole-
stasis (one), cholestatic viral hepatitis (one), extra-hepatic cholestasis (one) and chronic active hepatitis (one). They were stored at -20°C until analysed.

**Micro-partition system MPS-1**

The unbound radioactive bile salts were separated from serum proteins by centrifugal ultrafiltration by using a micro-partition system, MPS-1, from Amicon. The device consists of a sample reservoir with cap (to minimize gas exchange), membrane support base, O-ring, YMB membrane, a set of clips which secure the sample reservoir to the support base and a filtrate collection cup. The serum sample is added to the reservoir and during centrifugation the protein-free ultrafiltrate is collected in the cup.

**Procedure**

Serum (600 µl), 5 µl of tracer radioactive bile salt and 5 µl of internal marker were incubated with gentle mixing for 30 min at 37°C. [14C]Glucose was used as the marker for 3H-GCDC and 3H-GCDCS, and 3H2O for 14C-GC. The amounts of radioactivity in the incubation mixture were 54.4 nCi (2 x 103 Bq) for 3H-labelled and 4.5 nCi (1.7 x 102 Bq) for 14C-labelled compounds. The total amount of bile salt added as tracer was less than 1 nmol/ml of total incubation mixture. Duplicate 200 µl portions of the incubation mixture were then centrifuged at 37°C for 30 min at 1500 g. Radioactivity was determined in the total ultrafiltrate and in 100 µl of incubation mixture, after the addition of 10 ml of NE 260 scintillation fluid (Nuclear Enterprises, Edinburgh, U.K.). Samples of ultrafiltrate and incubation mixtures that were icteric were decolourized with hydrogen peroxide (100 µl, 300 g/l) and left overnight at 37°C before the addition of scintillant. The vials were counted for radioactivity for 10 min in a Philips liquid-scintillation spectrometer programmed for double-isotope counting; quenching was corrected by external standardization.

**Calculation**

The use of an internal marker to monitor the movement of unbound serum components obviates the need to measure the ultrafiltrate volume, so that the percentage of bound bile salt was calculated as follows:

\[
\text{Bile salt bound (\%)} = 100 - \left( \frac{\text{Bile salt in ultrafiltrate (\%)}}{\text{Internal marker in ultrafiltrate (\%)}} \right) \times 100
\]

**Serum bile salts**

The total serum 3α-hydroxy bile salt concentration (TSBC) was determined enzymatically [7] after extraction with XAD-7 [8].

**Serum bilirubin**

The total serum bilirubin concentration was determined by the method of Jendrassik & Grof [9].

**Results**

All results were expressed as means ± sd.

**Recovery of isotopes**

The recoveries of the radioactive bile salts and markers when added to phosphate/saline buffer (0.1 mol/l), pH 7.4, or serum were evaluated as described in the Materials and methods section, with centrifugation at 1500 g for 5 min. In the buffer experiments all the solution was transferred to the filtrate cup, which was weighed and the contents were counted for radioactivity. The recoveries for each set of six experiments, expressed as a percentage of the initial value, were 100 ± 2.0% for 14C-GC, 95.3 ± 3.2% for 3H-GCDC and 101.8 ± 2.3% for 3H-GCDCS. The recoveries of the internal markers were 100 ± 0.8% for 3H2O and 101.5 ± 0.8% for [14C]glucose. Mean values for the ratio of the radioactivity of the tracer bile salt in the ultrafiltrate to that of its marker were 1.00 ± 0.02 for 14C-GC, 0.96 ± 0.03 for 3H-GCDC and 1.00 ± 0.02 for 3H-GCDCS.

When the isotopes were added to serum the radioactivity of the bile salt and internal marker was determined in both the total ultrafiltrate and the sample remaining in the reservoir and the sum was expressed as the percentage recovery from 200 µl of incubation mixture. The values for 14C-GC, 3H-GCDC and 3H-GCDCS were 101.3 ± 5.8%, 94.1 ± 2.5% and 104.8 ± 4.0% respectively.

**Time of incubation**

By monitoring the percentage binding of 14C-GC to normal serum (TSBC = 9 µmol/l) at different times of incubation it was shown that the binding of 14C-GC was maximal after 2 min, and was stable over 60 min. For convenience the time for subsequent incubations was chosen as 30 min.

**Effect of ultrafiltrate volume on protein binding**

Different volumes of ultrafiltrate were obtained from 200 µl of serum by varying the time of
centrifugation. From the estimated values of serum bound \(^{14}\)C-GC it was found that the removal of 11–53% of the serum volume as ultrafiltrate did not alter the value for the protein binding of bile salts. The results are shown in Fig. 1, where each point represents the mean of three consecutive experiments. The experiments were performed with normal serum (TSBC = 9 \(\mu\)mol/l; \(^{14}\)C-GC bound = 75.7 ± 0.64%; \(n = 18\)) and with serum from a patient with liver disease (TSBC = 344 \(\mu\)mol/l; \(^{14}\)C-GC bound = 55.1 ± 0.60%; \(n = 18\)).

The mean volume of ultrafiltrate obtained from 200 \(\mu\)l of serum after centrifugation at 1500 \(g\) for 30 min was 88.6 ± 15.8 \(\mu\)l (\(n = 60\)).

**Intrabatch and interbatch variation**

The reproducibility of the method was assessed by repeated determinations of the percentage binding of \(^{14}\)C-GC, \(^3\)H-GCDC and \(^3\)H-GCDCS to normal serum (TSBC = 9 \(\mu\)mol/l). Because the percentage binding of \(^{14}\)C-GC was found to be markedly reduced in liver disease, the reproducibility of the binding of this bile salt by cholestatic sera was also determined. In all instances the coefficient of variation was less than 2% (Table 1).

**Normal values**

With sera from 12 laboratory staff (eight male, four female) the mean percentages of bound bile salt tracers were 79.9 ± 2.6% for \(^{14}\)C-GC, 95.5 ± 0.9% for \(^3\)H-GCDC and 92.9 ± 1.8% for \(^3\)H-GCDS.

**Patients study**

The results of binding studies in four different cases of cholestatic jaundice, i.e. (a) benign recurrent intrahepatic cholestasis [10], (b) viral hepatitis, (c) extrahepatic obstruction relieved by percutaneous biliary drainage and (d) chronic active hepatitis, are illustrated in Figs. 2(a), 2(b), 2(c) and 2(d) respectively. In all patients the binding of \(^3\)H-GCDC and \(^3\)H-GCDCS remained relatively constant throughout the study but differed for the individuals (Table 2).

In contrast, marked changes in the binding of \(^{14}\)C-GC were observed, the lowest values occurring when TSBC was maximal. In the cases of benign recurrent intrahepatic cholestasis and viral hepatitis the binding values returned to the normal range when the serum bile acid concentration had normalized. However, in the case of extrahepatic obstruction, which was relieved by biliary drainage [11], the percentage binding was still significantly reduced, even when the TSBC had almost returned to normal (25 \(\mu\)mol/l). The TSBC in the patient with chronic hepatitis decreased from 410 to 79 \(\mu\)mol/l, whereas the \(^{14}\)C-GC binding rose from 35 to 58%.

**TABLE 1. Assay variation in percentage binding of bile salts**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Bile salt tracer</th>
<th>Intrabatch ((n = 8))</th>
<th>Interbatch ((n = 8))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Normal</td>
<td>(^{14})C-GC</td>
<td>81.8</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>(^3)H-GCDC</td>
<td>94.9</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>(^3)H-GCDCS</td>
<td>92.3</td>
<td>1.37</td>
</tr>
<tr>
<td>Cholestatic</td>
<td>(^{14})C-GC</td>
<td>59.4*</td>
<td>0.54</td>
</tr>
</tbody>
</table>

* Serum bile salt, 324 \(\mu\)mol/l. † Serum bile salt, 423 \(\mu\)mol/l.
A negative correlation was obtained between the values in the four patients for bound $^{14}$C-GC and those for TSBC ($r = -0.62$, $n = 45$, $P < 0.001$) (Fig. 3). When the patients were considered individually, a higher correlation was obtained ($r = -0.76$, $n = 14$, $P < 0.001$ in benign recurrent intrahepatic cholestasis; $r = -0.93$, $n = 8$, $P < 0.001$ in chronic active hepatitis; $r = -0.93$, $n = 12$, $P < 0.001$ in extrahepatic obstruction; $r = -0.87$, $n = 10$, $P < 0.001$ in viral hepatitis). There was also a negative correlation between the amount of bound $^{14}$C-GC and the serum total bilirubin concentration ($r = -0.78$, $n = 36$, $P < 0.001$) (Fig. 4).
TABLE 2. Changes in radioactive bile salt binding to serum from patients with cholestatic liver disease

Results are means ± SD, or ranges where indicated.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Percentage binding of radioisotope</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^3$H-GC&lt;sub&gt;D&lt;/sub&gt;C</td>
</tr>
<tr>
<td>Normal</td>
<td>95.5 ± 0.9</td>
</tr>
<tr>
<td>Benign recurrent intrahepatic cholestasis</td>
<td>91.0 ± 1.9</td>
</tr>
<tr>
<td>Viral hepatitis</td>
<td>91.3 ± 1.7</td>
</tr>
<tr>
<td>Extrahepatic obstruction</td>
<td>89.5 ± 1.7</td>
</tr>
<tr>
<td>Chronic active hepatitis</td>
<td>85.8 ± 1.9</td>
</tr>
</tbody>
</table>

FIG. 3. Relationship between percentage $^{14}$C-GC bound and total $3\alpha$-hydroxy bile salt concentration in sera from patients with benign recurrent intrahepatic cholestasis (○), chronic active hepatitis (△), extrahepatic obstruction relieved by biliary drainage (●) and viral hepatitis (●) ($r = -0.62$, $n = 45$, $P < 0.001$).

Effect of albumin concentrations on $^{14}$C-GC binding

The percentage of $^{14}$C-GC bound was determined in increasing concentrations (400–1250 μmol/l) of human serum albumin (Sigma Chemical Co., London) dissolved in sodium phosphate buffer (20 μmol/l), containing NaCl (100 mmol/l), pH 7.4. When the binding of $^{14}$C-GC was plotted against the concentration of albumin, a hyperbolic saturation curve was attained. The results are shown in Fig. 5, where the normal ranges of the bound $^{14}$C-GC (75–85%) and the concentration of albumin (520–750 μmol/l) in serum are also indicated. Within the normal serum albumin range the $^{14}$C-GC binding only changed from 73 to 78%. The serum albumin concentration of the cholestatic patients fell within this range and did not change significantly during the period of study.

Bile salts. In order to see if the reduction in $^{14}$C-GC binding was due to the high concentrations of intercurrent organic anions, the percentage of $^{14}$C-GC bound was determined in increasing concentrations of total serum bilirubin in sera from patients with benign recurrent intrahepatic cholestasis (○), chronic active hepatitis (△), extrahepatic obstruction relieved by biliary drainage (●) and viral hepatitis (●) ($r = 0.78$, $n = 36$, $P < 0.001$).

FIG. 4. Relationship between percentage $^{14}$C-GC bound and total bilirubin concentration in sera from patients with benign recurrent intrahepatic cholestasis (○), chronic active hepatitis (△), extrahepatic obstruction relieved by biliary drainage (●) and viral hepatitis (●) ($r = 0.78$, $n = 36$, $P < 0.001$).

FIG. 5. Effect of albumin concentration on the percentage binding of $^{14}$C-GC. The stippled areas indicate the range of values in normal serum.
of conjugated primary bile salts in cholestatic serum, increasing concentrations of glycocholate, glycochenodeoxycholate, taurocholate and taurochenodeoxycholate were added to normal serum and the percentage of bound $^{14}$C-GC was estimated. An experiment was also performed with added glycochenodeoxycholate-3-sulphate. The reductions observed were similar for all the bile salts tested (Fig. 6). The maximum reduction in $^{14}$C-GC binding observed at the highest concentration (500 µmol/l) of added bile salt amounted to only 5%.

**Bilirubin.** The addition of unconjugated bilirubin in concentrations up to 200 µmol/l to normal serum did not cause any reduction in the $^{14}$C-GC binding.

**Fatty acids.** Oleic acid and palmitic acid are typical examples of saturated and unsaturated fatty acids which are known to be raised in liver disease [12]. When added in concentrations of up to 800 µmol/l, which is greatly in excess of that present in cholestatic serum, neither fatty acid had any effect on $^{14}$C-GC binding. Lauric acid, which has been shown to displace tryptophan bound to albumin [13], also had no effect on the binding of $^{14}$C-GC.

**Discussion**

The binding of bile salts to serum has been examined by using equilibrium dialysis [2, 14] and steady-state gel filtration [3]. Both techniques require relatively large samples of serum and are time-consuming. The percentage of bound bile salts in serum has also been determined directly by ultrafiltration [15] with Centriflo membrane cones (Aminco Corp.). However, this technique requires 3 ml of serum for duplicate determinations and problems have recently been encountered with some batches of cones, which were found to exhibit non-specific binding for bile salts. It is for this reason that the results in this paper differ quantitatively, but not qualitatively, from those presented previously [16].

The micro-partition system used in this study has proved to be a simple and reliable method for the direct determination of bound bile salts in undiluted serum. It is suitable for multiple determinations and can be used with small sample volumes (i.e. 200-300 µl). The recoveries of the tracer bile salts from aqueous solutions were greater than 95% in all instances, thereby indicating that non-specific binding was low and indeed for glycocholate and glycochenodeoxycholate-3-sulphate was almost negligible. The use of internal markers, as advocated by Hammond et al. [17], eliminated the need to measure the ultrafiltrate volume. Since the values for the ratios of the recoveries for bile salt tracers to those of the internal markers in a protein-free solution approximated to one, it is apparent that the unbound bile salts pass freely through the membrane. The precision of the estimates for the percentage of bound bile salt was not influenced either by the time of incubation or the ultrafiltrate volume. This technique therefore has considerable potential for physiological studies involving hepatic, renal and intestinal transport in which the relative importance of free and bound bile acids is still debatable.

The mean results obtained in control subjects for the binding of the two main primary bile salts $^{14}$C-GC (79.9 ± 2.6%) and $^{3}$H-GCDC (95.5 ± 0.9%) were of the same order as those previously reported from ultrafiltration with cones [15, 18]. There has been no previous report on conjugated chenodeoxycholate sulphates; the mean value of 92.9 ± 1.8% for $^{3}$H-GCDCS was, however, similar to that reported for chenodeoxycholate sulphate [18].

In liver disease serial determinations showed that the binding of $^{3}$H-GCDC and $^{3}$H-GCDCS was relatively unchanged during the course of the illness, although the absolute values tended to be somewhat lower than those given by the control subjects and reductions of up to 10% for $^{3}$H-GCDC and 5% for $^{3}$H-GCDCS were noted; these changes were not related to the albumin concentration of the serum. The reductions in the binding of $^{14}$C-GC were very much greater (Fig. 2) and were shown to be inversely related to the total 3α-hydroxy bile salt concentration (Fig. 3). However, when a high concentration (500 µmol/l) of glyco-
cholate, glycochenodeoxycholate or glycochenodeoxycholate-3-sulphate was added to normal serum the reduction in 14C-GC binding amounted to only 5% of the initial value (Fig. 6), which was markedly less than that seen in the patients. Since these bile salts would account for 90% or more of the total bile salts in cholestatic serum [5], it is unlikely that the reduction in 14C-GC binding is due to the raised concentration of serum total bile salts, which is merely an index of the degree of cholestasis.

The marked changes in the percentages of 14C-GC bound, when compared with the relatively constant binding of 3H-GCDC and 3H-GCDCS, in cholestatic liver disease could be explained by the lower binding affinity of glycocholate for albumin [19]. The finding that 14C-GC binding was reduced to a similar extent by added concentrations of glycocholate, glycochenodeoxycholate or glycochenodeoxycholate-3-sulphate (Fig. 6) suggests, however, that glycocholate has binding sites on the albumin molecule which are not shared by glycochenodeoxycholate or glycochenodeoxycholate-3-sulphate.

An inverse correlation (Fig. 4) was also observed between the 14C-GC binding and serum total bilirubin. The serum pigments in the patients studied were predominantly bilirubin conjugates, as judged with liver disease [12, 21] is responsible for the reduction in 14C-GC binding. It therefore seems likely that the reduction in the binding of 14C-GC is due to competition for albumin binding sites by compounds other than bile salts, unconjugated bilirubin or fatty acids. The variable extent of the reduction in the 14C-GC binding seen could be taken as an index of the amounts of such compounds in cholestatic sera. Further investigations are therefore necessary to determine their identity. The effect of changes in the conformation of the albumin molecule [22] on the binding of 14C-GC in cholestatic serum cannot, however, be excluded.

Acknowledgments
We are grateful to Dr T. C. Bartholomew for preparing the glycochenodeoxycholate-3-sulphate, and to Dr J. S. Dooley and Dr J. A. Summerfield for providing the serum samples. We also thank Professor Dame Sheila Sherlock and the Medical Research Council for their support.

References


