Urinary excretion of bile acids in cholestasis: evidence for renal tubular secretion in man

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Summary
1. The apparent renal clearance of intravenously injected $^{14}$C-glycocholate and $^{3}$H-chenodeoxycholate-3-sulphate was estimated in 22 patients with cholestasis. The degree of protein binding of the isotopes in serum from these patients was determined. The effects of pharmacological agents, changes in urine flow rate and pH on renal clearance were studied.

2. The mean renal clearance of $^{14}$C-glycocholate was $1.7 \pm 0.4$ ml/min (mean $\pm$ SEM), and that of $^{3}$H-chenodeoxycholate-3-sulphate was $6.4 \pm 0.9$ ml/min. $^{14}$C-Glycocholate was 80.1% protein bound and $^{3}$H-chenodeoxycholate-3-sulphate 96.5% protein bound.

3. Comparisons of the observed clearance rates with those calculated on the basis of glomerular filtration of the unbound fraction suggest that whereas $^{14}$C-glycocholate is predominantly reabsorbed by the renal tubules, $^{3}$H-chenodeoxycholate-3-sulphate appears in the urine mainly as the result of tubular secretion.

4. Probenecid, ethacrynic acid, frusemide and bendrofluazide decreased the clearance of both bile acids, implying competition for secretion via the proximal tubular organic acid secretory pathway between these compounds and bile acids.

5. Passive non-ionic diffusion does not seem to be an important mechanism in the renal excretion of bile acids as changes in urine flow rate and pH did not influence bile acid clearance.

6. A greater affinity of the proximal tubular organic acid secretory pathway for sulphated than for non-sulphated bile acids may explain the higher observed renal clearance rate of sulphated bile acids.

Key words: bile acids, bile acid sulphates, cholestasis, renal tubular secretion.

Introduction
Studies of the renal clearance of bile acids in patients with cholestasis support the view that non-sulphated bile acids undergo overall re-absorption, the values observed being much less than those for the calculated filtered load [1].

Sulphated bile acids predominate over non-sulphated bile acids in the urine of patients with cholestasis, despite their smaller contribution to total serum bile acid concentrations [2], so that the 'apparent' clearance of sulphated bile acids is 5–100 times greater than that for non-sulphated bile acids [1, 3, 4].

In the isolated rat kidney both sulphated and non-sulphated bile acids are reabsorbed. Competition for reabsorption between these two classes of bile acid has been suggested as an explanation for the difference in their renal clearance rates [5]. In this preparation evidence for tubular secretion was not obtained. However, studies in the dog have demonstrated proximal tubular reabsorption of non-sulphated bile acids [6] and secretion of taurocholate [7].

These studies were designed to define more closely the mechanisms controlling urinary bile acid composition in cholestasis in man. A clearer understanding of these mechanisms might permit interference by pharmacological agents, which could have important therapeutic implications. We have therefore investigated the renal handling
of bile acids in patients with cholestasis after the intravenous injection of $^{[14}C]$glycocholate and $^{[3}H]$chenodeoxycholate-3-sulphate, and studied the effect of various diuretics and drugs known to be secreted by the renal tubule.

**Methods**

**Patients**

Seventeen female and seven male patients with cholestasis were studied (mean age 54.3 years; range 36–80 years). All patients gave informed consent and the study was approved by the Ethical Practices Committee of the Royal Free Hospital. The patients had primary biliary cirrhosis (17), extrahepatic biliary obstruction due to pancreatic neoplasm (four), biliary stricture (one) and sclerosing cholangitis (two). In all patients the diagnosis was established by a compatible history, liver biopsy, mitochondrial antibody and/or cholangiography. No patient had clinical evidence of fluid retention, or was receiving drugs known to influence renal function. The serum bilirubin concentration was elevated in 18 patients (range 30–671 pmol/l). Blood urea, serum creatinine and serum albumin concentrations and creatinine clearance were normal. Total serum bile acid concentrations were raised in all patients (range 27–364 pmol/l).

**Radioactive bile acids**

$^{[1-14}C]$Glycocholate, specific radioactivity 54 mCi/mmol (2 X 10$^9$ Bq/mmol), $^{[11,12(n)-3}H]$chenodeoxycholate, specific radioactivity 37 Ci/mmol (1.4 X 10$^{12}$ Bq/mmol) and $^{[carboxyl-14}C]$chenodeoxycholate, specific radioactivity 60 mCi/mmol (2.2 X 10$^{12}$ Bq/mmol), were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). $^{[3}H(G)]$Glycochenodeoxycholate, specific radioactivity 2.3 Ci/mmol (8.5 X 10$^9$ Bq/mmol), was purchased from New England Nuclear (Boston, Mass., U.S.A.). Chenodeoxycholate-3-sulphate and glycochenodeoxycholate-3-sulphate were synthesized by the method of Parmentier & Eysen [8]. The isotopes were purified by elution from DEAP-Sephadex LH-20 by the method of Aimé et al. [9] and were dissolved in sodium chloride solution (150 mmol/l). Fifteen patients received two isotopes, 10 $\mu$Ci (3.7 X 10$^5$ Bq) of $^{[14}C]$glycocholate and 25 $\mu$Ci (9.3 X 10$^5$ Bq) of $^{[3}H]$chenodeoxycholate-3-sulphate, simultaneously. Two received only $^{[3}H]$chenodeoxycholate-3-sulphate and five $^{[14}C]$glycocholate. Two patients received simultaneously 9 $\mu$Ci (3.3 X 10$^5$ Bq) of $^{[14}C]$chenodeoxycholate-3-sulphate and 14 $\mu$Ci (5.2 X 10$^5$ Bq) of $^{[3}H]$glycochenodeoxycholate-3-sulphate.

**Radioactivity measurement**

Serum (0.2 ml) and urine (1.0 ml) samples were counted for radioactivity in duplicate after incubation at 45°C for 1 h with NCS solubilizer (Amersham/Searle Corporation, Arlington Heights, Illinois, U.S.A.), decolorization with hydrogen peroxide (100 $\mu$l, 300 g/l), and the addition of 15 ml of NE 260 scintillation fluid (Nuclear Enterprises, Edinburgh, Scotland, U.K.) in a Phillips liquid scintillation spectrometer programmed for double isotope counting. Samples were counted for 10 min and quench was corrected by external standardization.

**Experimental**

Patients were studied 1–2 h after a standard hospital breakfast. Oral fluid intake was encouraged to maintain an adequate urine output (mean urine flow rate before drug administration 3.89 ml/min; SEM 0.42; range 2.1–7.0; n = 22). Timed urine collections ($V$) were made at approximately 1 h intervals for up to 8 h and blood samples were taken from an indwelling venous catheter at the midpoint of each urine collection in most cases. When urine was not passed at the required time then an appropriate midpoint blood level was read from the plasma disappearance curve. Clearance calculations were based on serum ($S$) and urine ($U$) radioactivity measurements, but were not made in the first hour after isotope injection because of the rapid decline in serum radioactivity during this time.

The effect of a variety of drugs on bile acid clearance was investigated in 16 patients. The drugs used were probenecid (three), ethacrynic acid (two), frusemid (two), fusidic acid (two), tienilic acid (two), hydrocortisone (two), bendrofluazide (one), methyl testosterone (one) and norethandrolone (one). Drugs were given after two or three control clearance measurements had been made. In addition, the effects of water diuresis (three) and urinary alkalinization (two) were studied.

**Chemical determinations**

Total serum bile acid concentrations were determined by the method of Murphy et al. [10] (normal range <16 $\mu$mol/l). Serum and urine concentrations of creatinine were measured in samples from each clearance period after dialysis to remove bilirubin [11]. In some studies serum
and urine concentrations of urate were also measured [12].

**Bile acid protein binding**

Serum samples (3 ml) from eight patients undergoing clearance studies were incubated with 0.1 μCi (3.7 × 10³ Bq) of [14C]glycocholate and 0.2 μCi (7.4 × 10³ Bq) of [3H]chenodeoxycholate-3-sulphate in 50 μl with continuous mixing for 30 min at 37°C. Aliquots (0.2 ml) were counted for radioactivity in duplicate and 1 ml was centrifuged in an ultrafiltration cone (Centriflow Membrane Cones, Amicon, Woking, Surrey, U.K.) with a molecular-weight exclusion of 50 000 daltons, for 15 min at 800 g [13]. The ultrafiltrate (0.2 ml) was counted and the protein binding (expressed as a percentage) calculated.

**Calculations**

The 'apparent' renal clearance of bile acids was calculated as \((U \cdot V)/S\), where \(U\) and \(S\) were the urine and serum radioactivity concentrations in d.p.m./ml, and \(V\) was the urine flow rate in ml/min. Plasma disappearance data were fitted by computer to a two-exponential curve of the form \(y = Ae^{-at} + Be^{-bt}\) with the method of least mean squares.

Results were expressed as the mean and SEM and statistical comparisons were made with Student's t-test.

**Results**

**Plasma disappearance (Fig. 1)**

In all instances [3H]chenodeoxycholate-3-sulphate was cleared more slowly from the plasma than [14C]glycocholate. Calculated half-lives for the first and second exponentials of the plasma disappearance curves in five patients who did not receive an agent which altered urinary bile acid clearance were 6.1 ± 2.1 and 807 ± 126 min respectively for [14C]glycocholate and 23.5 ± 2.5 and 479.8 ± 41 min respectively for [3H]chenodeoxycholate-3-sulphate. These values did not differ significantly from those for the whole group of patients studied, including those who received agents which altered urinary bile acid clearance.

**Protein binding**

Protein binding of [3H]chenodeoxycholate-3-sulphate (96.5 ± 0.5%; \(n = 8\)) was significantly greater than that of [14C]glycocholate (80.1 ± 2.1%; \(n = 8\); \(P < 0.001\)). Protein binding was independent of the serum bilirubin and serum bile acid concentrations.

**Clearance studies**

The reproducibility of bile acid-clearance measurements was assessed in nine patients. In three consecutive 1 h collections the mean [14C]glycocholate clearance was 92.8 ± 4.1 and 94.2 ± 6.4% of the initial value, and the mean [3H]chenodeoxycholate-3-sulphate clearance was 92.0 ± 6.5 and 95.7 ± 6.2%. These variations were not significant.

The renal clearance of [3H]chenodeoxycholate-3-sulphate (6.4 ± 0.9 ml/min; \(n = 17\)) was significantly greater \((P < 0.001)\) than that of [14C]glycocholate (1.7 ± 0.4 ml/min; \(n = 20\)). The mean creatinine clearance in these patients was 95.7 ± 5.6 ml/min \((n = 22)\).

In two studies the renal clearance of [14C]-chenodeoxycholate-3-sulphate was found to be similar to that of [3H]glycochenodeoxycholate-3-sulphate, the values being 3.3 and 3.8 ml/min respectively in the first study and 6.8 and 5.5 ml/min respectively in the second.
Effect of water diuresis and urinary alkalinization (Fig. 2)

The effect of changes in urine flow rate, induced by drinking tap water, on bile acid clearance was assessed in three patients. In two patients given \(^{14}\text{C}\)glycocholate the maximum variations in bile acid clearance were from 2.0 to 2.5 and 2.4 to 2.9 ml/min, whilst urine flow rates increased from 0.5 to 5.5 and 0.4 to 9.0 ml/min respectively. One patient received both isotopes and the maximum variation in \(^{14}\text{C}\)glycocholate clearance was from 0.7 to 0.9 ml/min, and that for \(^{3}\text{H}\)chenodeoxycholate-3-sulphate was from 3.6 to 4.2 ml/min, the urine flow rate increasing from 0.7 to 5.6 ml/min.

Two patients received 500 ml of sodium bicarbonate solution (27.4 g/l; 0.33 mol/l) intravenously over 2 h to raise urinary pH to above 7.5. The clearance of \(^{14}\text{C}\)glycocholate was unchanged. Typical examples of the effect of water diuresis and urinary alkalinization are shown in Fig. 2.

Effect of probenecid

Three patients were given probenecid (500 mg, orally) 3 h after isotope injection (Fig. 3). The bile acid clearance was reduced in 4–6 h to 57, 42 and 44% of the initial value for \(^{14}\text{C}\)glycocholate in three patients, and to 37 and 33% for \(^{3}\text{H}\)chenodeoxycholate-3-sulphate in two patients. Creatinine clearance was unaltered, but urate clearance increased. Although the percentage changes in clearance were similar for both isotopes, the absolute reductions in clearance for \(^{14}\text{C}\)glycocholate (1.3, 1.7 and 0.5 ml/min) were much smaller than those for \(^{3}\text{H}\)chenodeoxycholate-3-sulphate (3.7 and 3.9 ml/min).

Effect of diuretics

Ethacrynic acid (20 mg) and frusemide (20 mg) were each given intravenously to two patients (Fig. 4). Isotope clearance was decreased in all cases, the maximum change being 41–72% for \(^{14}\text{C}\)glycocholate and 33–63% for \(^{3}\text{H}\)chenodeoxycholate-3-sulphate. With ethacrynic
acid the decreases in bile acid clearance were greater than could be accounted for by changes in creatinine clearance alone (Table 1), and persisted after the creatinine clearance had returned to control levels. With frusemide one patient demonstrated decreases in bile acid clearance greater than those of creatinine clearance, whereas in the other patient changes
TABLE 1. Percentage change in the bile acid clearance/creatinine clearance ratio 4 h after administration of ethacrynic acid or frusemide

<table>
<thead>
<tr>
<th>Drug</th>
<th>Patient no.</th>
<th>Clearance ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethacrynic acid</td>
<td>1</td>
<td>-50</td>
</tr>
<tr>
<td>Ethacrynic acid</td>
<td>2</td>
<td>-50</td>
</tr>
<tr>
<td>Frusemide</td>
<td>3</td>
<td>-28</td>
</tr>
<tr>
<td>Frusemide</td>
<td>4</td>
<td>-10</td>
</tr>
</tbody>
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paralleled more closely those of creatinine clearance. In both these patients the changes in bile acid clearance were of shorter duration than those seen with ethacrynic acid.

Bendrofluazide (10 mg orally) produced a maximum decrease in clearance of 61% for \([^{14}C]\)glycocholate and 27% for \([^{3}H]\)chenodeoxycholate-3-sulphate in one patient. Tienilic acid (250 mg orally) produced a maximum decrease in clearance of about 28% for both \([^{14}C]\)chenodeoxycholate-3-sulphate and \([^{3}H]\)glycoxeno-

Other pharmacological agents

Norethandrolone (10 mg) and methyl testosterone (10 mg), both known to alleviate pruritus in cholestasis in man [14], had no effect on bile acid clearance. Fusidic acid (250 mg), a steroid antibiotic which has some structural similarities to bile acids, and is excreted in bile [15], and hydrocortisone (100 mg), also did not alter bile acid clearance.

Discussion

In this investigation in cholestatic patients glycocholate was selected as the non-sulphated bile acid for study since cholate conjugates are not extensively sulphated and for the most part appear unaltered in the urine. In contrast, glycochenodeoxycholate is mainly excreted in the urine of cholestatic patients as glycochenodeoxycholate sulphate [2]. It would, therefore, have been impossible to interpret the clearance data if radioactive glycochenodeoxycholate had been administered since it would have been partly sulphated during the experimental period. The rate of disappearance of radioactivity in the plasma from labelled glyco- and tauro-cholate has been shown to be only slightly greater than that from labelled glyco- and tauro-chenodeoxy-

cholate [13]. Sulphate esters of both primary bile acids have similar biological half-lives and are excreted significantly more rapidly than non-sulphated bile acids [16]. In the present study it was shown that the radioactive isotopes of glycochenosulphate and chenosulphate are cleared at similar rates, so that as use of the latter isotope was more economical it was employed in this investigation. Present evidence therefore indicates that the renal clearance of primary bile acids in man is regulated by the presence or absence of sulphate rather than their basic structure or type of conjugation. Data obtained with \([^{14}C]\)glycocholate and \([^{3}H]\)chenodeoxycholate-3-sulphate can therefore be taken as representative of non-sulphated and sulphated bile acids.

The ratios of the clearance values of these two bile acids (expressed in terms of their isotope labelling) ranged from 0.9 to 10.6, and are somewhat lower than those reported in previous studies using direct chemical determinations [3, 17]. The reason for this difference in 'apparent' clearance rates is unclear. Palmer [18] has suggested that the higher renal clearance of sulphated bile acids is related to their greater water solubility. Summerfield et al. [2] postulated that either renal synthesis of bile acid sulphates occurs or that tubular reabsorption of sulphated bile acids is inhibited by non-sulphated bile acids. If one takes into account that sulphated bile acids are significantly more tightly bound to plasma proteins than non-sulphated bile acids (mean 96.5% vs 80.1%) then it is clear that the filtered load (glomerular filtration rate \(\times\) 'unbound' bile acid) will be less for \([^{3}H]\)chenodeoxycholate-3-sulphate than for \([^{14}C]\)glycocholate despite the fact that the urinary excretion is greater. The mean calculated values for overall renal clearance with 'unbound' bile acid concentrations amount to 9.3 ml/min for \([^{14}C]\)glycocholate and 147 ml/min for \([^{3}H]\)chenodeoxycholate-3-sulphate. All our patients had values for creatinine clearance within the normal range (95.7 ± 5.6 ml/min) so that it appears that \([^{14}C]\)glycocholate is largely reabsorbed whereas \([^{3}H]\)chenodeoxycholate-3-sulphate is secreted. Our data do not show whether \([^{3}H]\)chenodeoxycholate-3-sulphate is also reabsorbed.

Proximal tubular secretion of taurocholate has been demonstrated in stop-flow studies in the dog [7] and indeed a large number of organic acids are known to compete for secretion in the proximal tubule [19]. One of these compounds, probenecid, has been widely used to demonstrate competition between different substances for this organic acid secretory pathway [19]. The
reduction in the clearance of bile acids observed with probenecid therefore suggests that both sulphated and non-sulphated bile acids are secreted by this pathway in cholestasis in man. The decrease in clearance with diuretics may be due to the same effect, as thiazides [20], ethacrynic acid [21] and frusemide [22] are all secreted by this pathway. The lack of effect of changes in urinary flow rate and pH on bile acid clearance indicates that passive non-ionic reabsorption, which occurs with compounds such as probenecid and salicylates which are secreted by the organic acid pathway [19], is not an important mechanism controlling the renal excretion of bile acids.

These findings suggest that glycocholate is filtered by the glomeruli and undergoes both tubular secretion and reabsorption, with the latter predominating. Studies in the dog [6] and the isolated rat kidney [5] suggest that reabsorption occurs in the proximal tubule. Chenodeoxycholate-3-sulphate is filtered to a lesser extent, and tubular secretion may be the major factor in its urinary excretion. However, bidirectional transport of sulphated bile acids in the proximal tubule cannot be excluded.

In the isolated rat kidney sulphated bile acids are reabsorbed, and apparently compete for reabsorption with non-sulphated bile acids [5], a situation analogous to that in the human ileum, where non-sulphated bile acids inhibit the reabsorption of sulphated bile acids [23]. The linear relationship between sulphated bile acid clearance and total urinary bile acid output in cholestatic patients described by Summerfield et al. [2] is compatible with this hypothesis. Our findings do not exclude such mechanisms, but the magnitude of the effect of probenecid on bile acid clearance suggests that, in cholestasis in man, tubular secretion is a more important determinant of urinary bile acid excretion than competition for reabsorption.

Some of the findings in the rat kidney studies, such as the evidence for a component of passive non-ionic reabsorption, and the failure of p-aminohippuric acid to inhibit urinary bile acid excretion [5], are difficult to reconcile with our data. Species differences may be important and it is possible that the kidney preparation in vitro behaves differently in some respects from the kidney in the intact animal. Such discrepancies have been noted for other actively transporting tissues, such as small intestinal mucosa [24].

Abnormalities in renal tubular function have been reported in primary biliary cirrhosis, and in other forms of chronic liver disease [25]. Their relationship to the findings in our studies is unclear, but would seem unlikely to affect our conclusions with respect to cholestasis.

Tubular secretion of bile acids may offer an explanation for the difference in observed clearance rates between sulphated and non-sulphated bile acids. The greater absolute reduction in clearance of chenodeoxycholate-3-sulphate than of glycocholate by probenecid may imply that the secretory pathway transports a greater load of sulphated than non-sulphated bile acids. Since the serum concentrations of sulphates are very low compared with those of non-sulphates in cholestasis, this suggests a higher affinity of the pathway for sulphates.

Sulphated bile acids are efficiently excreted by the kidney in cholestasis in man, up to 30% of the injected dose of chenodeoxycholate-3-sulphate appearing in the urine within 9 h in the present studies. Since this appears to be accomplished in major part by tubular secretion, it seems unlikely that their rate of excretion can be increased by pharmacological agents. Injected glycocholate is excreted slowly in the urine, only 5% appearing in the urine within 9 h in these studies, probably because of efficient tubular reabsorption since biliary excretion was minimal. None of the agents studied produced any increase in glycocholate or chenodeoxycholate-3-sulphate clearance but it is possible that bile acid analogues may be found which would interfere with glycocholate reabsorption and hence increase its urinary excretion.

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