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HEPATIC TRANSPORT OF BILE ACIDS IN THE ISOLATED PERFUSED RAT LIVER

STRUCTURE–KINETIC RELATIONSHIP*

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Abstract—We studied the transport kinetics of a series of bile acids from blood to bile in the isolated perfused rat liver in order to define better the relationship between chemical structure of bile acid molecules and efficiency of the overall hepatic transport process. BA studied were taurocholate (TC), glycocholate (GC), cholate (C), tauroursodeoxycholate (TUDC), ursodeoxycholate (UDC) and hyodeoxycholate (HDC). Estimates of intrinsic hepatic clearance (Cl_{int}), maximal secretory rate (V_{max}) were provided from the analysis of the relationship between bile acid removal rates and sinusoidal concentration under steady-state conditions. TC and TUDC had the highest Cl_{int} (about 5 ml/min/g liver) and V_{max} (about 800 nmol/min/g liver) followed in order by GC (1.71 ml/min/g liver; 442 nmol/min/g liver); C (1.25 ml/min/g liver; 252 nmol/min/g liver); HDC (0.86 ml/min/g liver; 238 nmol/min/g liver); UDC (0.72 ml/min/g liver; 176 nmol/min/g liver). The findings suggest that the efficiency of the overall hepatic transport of bile acids is highly dependent on their molecular structure and that conjugation has a more important effect on both Cl_{int} and V_{max} that the number or position of hydroxyl groups.

Bile acids (BA) are a family of biologically important steroids which are removed from the blood by the liver and secreted into bile. BA present in the blood perfusing the liver differ in their molecular structure, i.e. number and position of hydroxyl groups, conjugation or not with taurine and glycine. Variations in chemical structure strongly influence the hydrophilicity and other physicochemical and metabolic properties of BA [1]. The vectorial transport of these compounds from the sinusoidal blood to bile is a saturable process which includes at least three steps: (a) a sodium dependent cotransport uptake at the sinusoidal membrane, (b) interaction with cytoplasmic proteins and/or intracellular conjugation, and (c) finally secretion into the canaliculus. The relationship between BA structure and uptake kinetics has been studied using isolated perfused rat liver [2] and hepatocyte suspensions [3–5]. In these models, Michaelis–Menten kinetic parameters were found to depend on both the number of hydroxyl groups and conjugation of BA molecules. However, such a kinetic analysis has not been performed to characterize the efficiency of the overall elimination process of BA by the liver.

In the present work, we study the transport kinetics of a series of BA from blood to bile in order to better define the relationship between molecular structure and efficiency of the overall hepatic transport process.

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MATERIALS AND METHODS

Materials. Taurocholate (TC), glycocholate (GC), cholate (C), tauroursodeoxycholate (TUDC), ursodeoxycholate (UDC), hyodeoxycholate (HDC) were purchased from Steraloids and Calbiochem (San Diego, CA), [^{14}C]-TC, -GC and -C from the New England Nuclear, (Boston, MA). [^{14}C]-HDC was prepared as previously described [6]. [^{14}C]-UDC and TUDC were a gift from Roussel Uclaf, France. The purity of BA verified by thin layer chromatography of both the labeled and non-labeled form was better than 95%. Bovine serum albumin was purchased from Calbiochem and 3α-hydroxysteroid dehydrogenase from Worthington Biochemical (Freehold, NJ). All other chemicals were analytical grade and purchased from Prolabo (Paris, France).

Liver perfusion. Inbred male Sprague–Dawley rats (250–350 g) served as liver and blood donors in all experiments. The liver weight varied from 8 to 12 g.

Non fasting rats were anesthetized by IP injection of 60 mg/kg of sodium pentobarbital. After cannulation of the common bile duct, the portal vein was rapidly severed and connected to a reservoir containing oxygenated perfusate; the time elapsed between portal vein cannulation and connection to the perfusate was less than 10 sec.

The liver was then removed and connected to the perfusion apparatus maintained at 37°. The livers were perfused with 140 ml of a recirculating semi-synthetic medium consisting of heparinized rat blood, diluted (1/3, v/v) with Krebs-Ringer bicarbonate buffer (pH 7.4) containing 30 g/l bovine serum albumin and 1.5 g/l glucose, gassed to equilibrium with 95% O_2, 5% CO_2. Hematocrit volume

ranged from 15 to 18%. The livers were perfused under a hydrostatic portal pressure of 13 cm. The setup of the perfusion apparatus was such that the perfusate passing through the liver flowed through a graduated tube before entering the reservoir. This allowed a direct determination of the perfusion rate. The perfusion rate was on the average 1.95 ± 0.32 ml/min/g liver. The routine viability criteria included: (a) stable perfusion rate at constant portal pressure throughout the experiment, (b) O_2 consumption rate higher than 2.5 μmoles/min/g liver, (c) stable pH between 7.3 to 7.4, (d) stable perfusate transaminase concentrations within the reference range.

Experimental procedure. The essayed BA was injected into the main reservoir at a constant infusion rate for 60 to 80 min. For each of the BA species 7 to 9 increasing infusion rates (one infusion rate per liver rat perfusion) were studied. Preliminary experiments were performed to determine the time necessary to achieve steady-state conditions and to maintain stability and viability of the recirculating isolated perfused liver system. This preliminary work indicated that the time necessary to achieve and maintain steady-state conditions depends on the nature and on the amount of the BA infused. Therefore the infusion rates used for the study ranged from 50 to 190 nmol/min/g liver for TUDC and TC, from 35 to 130 nmol/min/g liver for GC, from 25 to 130 nmol/min/g liver for C, from 18 to 110 nmol/min/g liver for HDC and from 15 to 75 nmol/min/g liver for UDC. Steady-state conditions as reflected by the constancy of BA concentration in the perfusate and by the constancy of BA excretion in bile were achieved within 40 to 50 min. After this period, four to five samples were taken from the portal (inflow) and the hepatic veins (outflow) perfusate every 5 min. Bile was collected over 5 min periods in preweighed vials and its volume was determined gravimetrically assuming a density of 1.0. BA secretion rates were calculated from the mean values of bile flow and BA concentration of these collection periods.

Analytical methods. The bile acid concentration was measured by using 3α-hydroxysteroid dehydrogenase (Worthington Biochemical, Freehold, NJ). Radioactivity was measured by liquid scintillation counting. The oxygen consumption was monitored using an O_2 electrode.

Kinetic analysis. The relationship between BA secretion rates and BA concentration in perfusate was analysed assuming Michaelis–Menten kinetics. The purpose of doing this is purely descriptive, with no stoichiometric implications. The flow independent sinusoidal (i.e. in the liver sinusoids) substrate concentration was calculated as [7]

$$\hat{C} = \frac{C_{in} - C_{out}}{\mathrm{Ln}(C_{in}/C_{out})}.$$

This term accounts for the decreasing sinusoidal concentration from the inlet to the outlet of the sinusoid created by the transport process. By entering $\hat{C}$ into the Michaelis–Menten relation:

$$V = \frac{V_{max}/\hat{C}}{K_m + \hat{C}}$$

where V_{max} is the maximal elimination rate, K_m the half saturation sinusoidal concentration ($\hat{C}$) and V the biliary elimination rate. V_{max} and apparent K_m were computed non-linearly according to the method of Wilkinson [8]. The robustness of this method has been demonstrated when data are available only in a limited region of the rectangular hyperbolic function [8]. The Wilkinson's approach is therefore appropriate when kinetic parameters of potentially toxic compounds such as BA [10] have to be determined. Intrinsic hepatic clearance Cl_{int}, i.e. the maximal ability of the liver to irreversibly remove BA in the absence of any flow limitations was calculated by two means: (a) as the ratio V_{max}/K_m, V_{max} and K_m being parameter estimates provided by the Wilkinson's method; (b) as $-Q \cdot \ln(1 - E)$ according to the sinusoidal perfusion model of hepatic elimination [7], Q being the liver perfusate flow and E the hepatic extraction ratio of BA calculated as: $\frac{C_{in} - C_{out}}{C_{in}}$. Since Cl_{int} is a first order removal constant, E was calculated for the values of $\hat{C} < 0.5\ K_m$.

Data presentation and statistical methods. Data are presented as mean ± SD. Perfusate BA concentration–time data were statistically examined by least-squares regression analysis of data from individual rat livers to test whether the slope of regression line was different from zero at the 95% confidence limits. Hepatic extraction ratio of BA at each time point by each liver were compared by one way analysis of variance accepting $P < 0.05$ as statistically significant. Comparison of means were made by variance analysis and Student's *t*-test.

RESULTS

Steady-state conditions were achieved in all the experiments. No-time dependent change in BA concentration and hepatic extraction was seen during the experimental period as evaluated by variance analysis. At steady-state the mass of material entering and leaving the liver (via the venous outflow and bile) must be equal; this mass balance condition was verified in each set of perfusion. The differences between the removal rates of BA calculated as $QC_i - QC_o$ and the biliary secretion rates were always less than 5% and did not differ significantly.

Further evidence of absence of hepatocellular toxicity of the infused BA was the stability of portal pressure, O_2 consumption and transaminase activity in the perfusate. For each BA species, there was a linear relationship between bile flow and BA output in bile ($r = 0.83$ to 0.97) and no systematic deviation from the regression line was observed at the highest infusion rates. The choleretic response was in the order HDC (16 nl/nmol), C (15 nl/nmol), UDC (15 nl/nmol), TC (9 nl/nmol), GC (6 nl/nmol), TUDC (2 nl/nmol).

Figure 1 shows for each BA the relationships between BA output in bile (v) and the logarithmic average sinusoidal concentration ($\hat{C}$) at steady-state.

Estimates of V_{max}, K_m as well as Cl_{int} are given in Table 1. Cl_{int} calculated as $-Q \cdot \ln(1 - E)$ and the ratio V_{max}/K_m were highly correlated ($r = 0.996$, $P < 0.001$) (Fig. 2). Statistical analysis revealed that

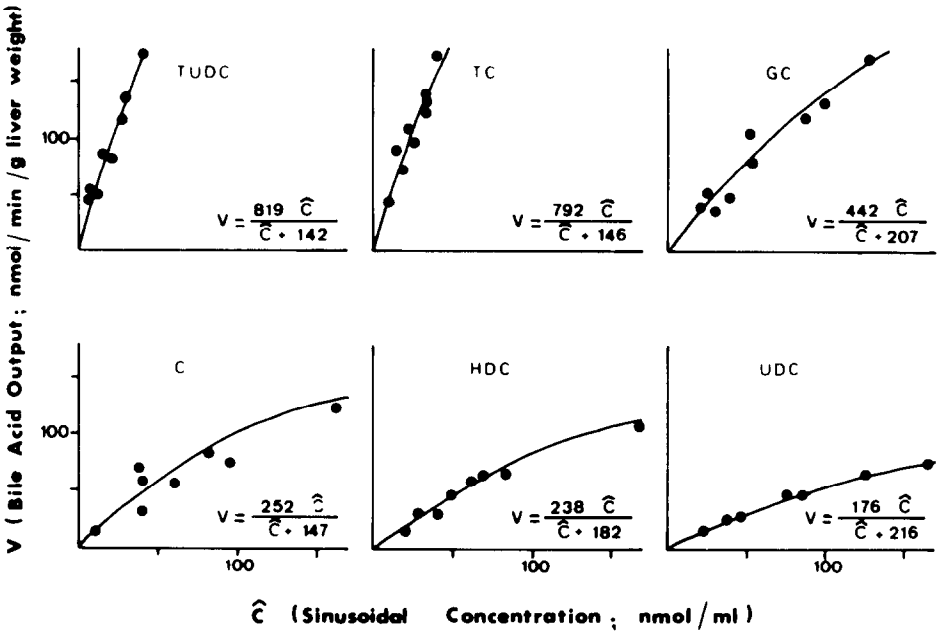

Fig. 1. Relation between bile acid secretion rates (v) and sinusoidal concentration ($\hat{C} = C_{in} - C_{out}/\ln (C_{in}/C_{out})$. The data are fitted (solid line) according a rectangular hyperbola obeying the Michaelis–Menten equation.

Table 1. Bile acid kinetic data (means ± SD)

Bile acids	V_{max}* (nmol/min/g liver)	K_m† (nmol/ml)	V_{max}/K_m (ml/min/gL)	Cl_{int}‡ (ml/min/g liver)
TUDC§	819 ± 354	142 ± 62	5.77	4.99 ± 0.71
TC	792 ± 260	146 ± 52	5.42	4.33 ± 0.79
GC	442 ± 106	207 ± 48	2.14	1.71 ± 0.56
C	252 ± 63	147 ± 48	1.71	1.25 ± 0.66
HDC	238 ± 97	182 ± 81	1.31	0.86 ± 0.08
UDC	176 ± 97	216 ± 45	0.81	0.72 ± 0.07

* V_{max}, bile acid maximal secretion rate in bile.
† K_m, sinusoidal bile acid concentration corresponding to $V_{max}/2$.
‡ Cl_{int}, intrinsic hepatic clearance of bile acid.
§ Abbreviations as in the text.

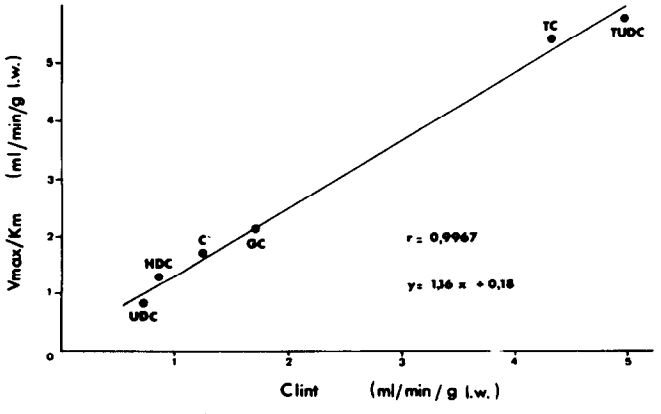

Fig. 2. Relation between the ratio V_{max}/K_m and intrinsic hepatic clearance (Cl_{int}) of bile acids, V_{max} is the apparent maximal elimination rate; K_m is the sinusoidal concentration corresponding to $V_{max}/2$.

Cl_{int} of each BA differed significantly from each other ($P < 0.05$ or lower) except for TUDC vs TC, CA vs HDCA, HDCA vs UDC.

DISCUSSION

The aim of the present study was to define the relation between chemical structure of BA and their removal rates from blood to bile. Relationships between BA removal rates and their sinusoidal concentrations were analysed at steady-state in an isolated perfused rat liver preparation with a recirculating system. Achievement of steady-state was obtained as reflected by the absence of change in perfusate concentration with time and by mass balance condition. The removal of BA calculated as the hepatic flow rate multiplied by inlet–outlet concentration difference and the amount of bile acid secreted in bile during the same period did not differ significantly.

The relationship between elimination rates and sinusoidal concentrations were analysed assuming saturation kinetics. This relationship was, however, studied in a limited region of the hyperbolic function describing Michaelis–Menten kinetics to avoid any toxicity of high concentration of bile acids to the liver [10]. Indeed, it is well known that the maximal biliary secretion of BA is a compromise between their hepatocellular toxicity and the capacity of the transport system [10]. Over the range of BA infusion rates used (15 to 190 nmol/min/g liver) satisfactory steady-state conditions and viability of the liver preparation were demonstrated.

The Wilkinson's method used to analyse the relation between elimination rates and sinusoidal concentration has been shown to provide accurate parameter estimation even if data are limited within the lower half of the curve [9]. In the present study, total BA (bound + free) concentration in the perfusate were measured; binding of BA to albumin (30 g/l in the perfusate) was not determined. Cl_{int} was therefore the total intrinsic hepatic clearance, i.e. the intrinsic hepatic clearance of both bound and unbound BA present in the perfusate.

An important finding of the present study was that the efficiency of the overall hepatic transport of BA as assessed by V_{max} and Cl_{int} was highly dependent on the chemical differences in the structure of BA. Firstly conjugation appears as the major determinant of the transport efficiency. TCA the predominant bile acid in rat bile and TUDC the taurine conjugate of a bile acid not found in rat bile had the highest V_{max} and Cl_{int}; V_{max} and Cl_{int} of TC were 3 to 4 times higher than that of CA; V_{max} and Cl_{int} of TUDC were 5 times higher than that of UDCA; conjugation with taurine appeared more effective than conjugation with glycine since V_{max} and Cl_{int} of TC were about 2 times higher than that of GC. Secondly, the number and position of hydroxyl groups influence to a lesser degree the transport efficiency since the values of V_{max} and Cl_{int} for unconjugated CA or unconjugated di-OH BA, (UDC or HDC) were very close. The findings that conjugation has a more important effect on hepatic transport that number or position of OH well agree with previous work using a different methodology [11–13]. In contrast, such results are at variance with these of Reichen and Paumgartner [2] who have studied the relationship between bile acid structure and uptake. Their data showed that the maximal uptake velocity was mainly influenced by the number of OH group rather than conjugation. These difference could probably be explained by the fact that different mechanisms operate at each step of the transport through the hepatocyte. Values of V_{max} obtained in the present study are lower than those reported for uptake of BA [2]. For CA, V_{max} is five times lower than that reported for uptake. However for TCA, V_{max} is only two times lower than that reported for uptake. Thus, the disproportion between uptake and secretory capacity seems highly dependent of the BA species.

Conjugated BA are secreted unchanged into bile. Unconjugated BA are secreted into bile, after being metabolized as tauro- or glycoconjugated or for a minor fraction unchanged. Since it is generally assumed that uptake is not the limiting step of the overall hepatic transport [12], the differences in the efficiency of BA transport observed in the present study might theoretically be explained by the degree of hydrophilicity of unchanged BA or conjugated metabolites secreted into bile or by the rate of formation of conjugated BA.

Whatever the mechanisms our results show that: (a) the efficiency of the overall hepatic transport of BA is highly dependent on their molecular structure; (b) taurine and glycine increase Cl_{int} and V_{max} of BA; however taurine appeared to be more effective than glycine.

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