

# Bile acid binding in plasma: the importance of lipoproteins

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Bile acid (BA) hydrophobicity, evaluated by the octanol-water partition coefficient, decreases along the series deoxycholic acid – chenodeoxycholic acid – hyodeoxycholic acid – ursodeoxycholic acid – cholic acid (CA) – ursocholic acid (UCA). In vitro experiments carried out using dialysis techniques (to determine the maximum BA binding) and ultrafiltration of plasma pre-incubated with 0.1 mM BA (to assess the distribution of BA between the different lipoprotein fractions) showed that the maximum binding of BA to plasma and lipoproteins follows the same order of hydrophobicity. The fraction not bound to proteins, greater with the hydrophilic BA (UCA and CA), is distributed in the lipoprotein fractions and in particular in high density lipoproteins.

<i>Bile acid</i>	<i>Hydrophobicity</i>	<i>Protein</i>	<i>Lipoprotein binding</i>
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## 1. INTRODUCTION

Physicochemical properties of bile acids (BA) influence their biological activities [1,2] and probably their circulating fraction. In plasma BA are tightly bound to albumin [3,4], but lipoproteins can also influence the unbound BA fraction available to tissues [5].

Surprisingly, in analbuminemia little change in anion binding capacity occurs and no variations of

bile formation have been reported [6,7]. In this condition other plasma proteins and lipoproteins [4] can partially compensate for albumin deficiency [6].

Here we evaluate the in vitro binding of several bile salts, having different physico-chemical properties, to lipoproteins, the latter possessing binding sites for bile salts (BS). In fact surface polar components, such as apoprotein and phospholipids, can interact with BS molecules.

We studied (a) the maximum BA binding capacity of whole plasma and lipoprotein fractions using dialysis techniques, and (b) the distribution of different BS among lipoprotein classes separated by ultra-centrifugation. Since BS binding depends on their physicochemical properties, we also estimated the hydrophobic properties of BS from their octan-1-ol-water distribution.

## 2. MATERIALS AND METHODS

### 2.1. Bile acids

Sodium salts of BA were obtained by neutralizing an alcohol solution with an equivalent amount of NaOH; sodium salts were crystallized after the addition of ether.

**Abbreviations:** VLDL, very low density lipoproteins ( $d < 1.006$  g/ml); LDL, low density lipoproteins ( $d = 1.030$ – $1.063$  g/ml); HDL, high density lipoproteins ( $d = 1.063$ – $1.210$  g/ml);  $d > 1.210$  g/ml, lipoprotein-free fraction; BS, bile salts; BA, bile acids; DCA, deoxycholic acid ( $3\alpha,12\alpha$ -dihydroxy- $5\beta$ -cholanoic acid); CDCA, chenodeoxycholic acid ( $3\alpha,7\alpha$ -dihydroxy- $5\beta$ -cholanoic acid); HDCA, hyodeoxycholic acid ( $3\alpha,6\alpha$ -dihydroxy- $5\beta$ -cholanoic acid); UDCA, ursodeoxycholic acid ( $3\alpha,7\beta$ -dihydroxy- $5\beta$ -cholanoic acid); CA, cholic acid ( $3\alpha,7\alpha,12\alpha$ -trihydroxy- $5\beta$ -cholanoic acid); UCA, ursocholic acid ( $3\alpha,7\beta,12\alpha$ -trihydroxy- $5\beta$ -cholanoic acid); GLC, gas liquid chromatography; TLC, thin layer chromatography; HPLC, high performance liquid chromatography

Tris buffer, pH 7.5, 0.15 M NaCl containing 200 mg/l sodium azide was used in the dialysis experiments.

Sterognost 3 Kit (Bracco-Merck, Milan) was used for BA determination in an LKB Ultralab System 8600 reaction rate analyzer: method sensitivity was 0.5  $\mu$ M/l.

All chemicals were reagent grade. Water was double distilled and decarbonated prior to use.

## 2.2. Lipoprotein fraction preparation

Fasting blood samples were collected in EDTA (1 mg/ml) from healthy subjects. Lipoproteins (VLDL, LDL, HDL) and the fraction with  $d > 1.210$  g/ml (lipoprotein-free fraction) were obtained by sequential ultracentrifugation in an L5-65 Beckman ultracentrifuge at 6°C using solid potassium bromide to obtain the desired densities. Plasma was first diluted with 10 mM Tris buffer to final pH 7.5. Lipoprotein fractions were dialyzed for 24 h at 4°C against saline containing 200 mg% EDTA prior to the experiments.

## 2.3. Binding of different bile acids to lipoproteins

Before evaluating binding, lipoprotein fractions or plasma were diluted with Tris buffer, pH 7.5. BS solutions in Tris buffer (pH 7.5), 0.15 M NaCl were added to obtain a final concentration of 0.1 mM. After sonication they were incubated for 24 h at 37°C prior to dialysis.

Lipoprotein binding was determined by equilibrium dialysis at 37°C [9,10]. Experiments were performed in a cellulose sac (Visking dialysis tubing 8/32, 6 mm diameter) having a minimum molecular mass retention of about 12 kDa. Aliquots of 1.0 ml BS solution were loaded into the dialysis tubes, previously washed with 10 mM Tris buffer in saline, pH 7.5, at 37°C. 2 ml BS solution were dialyzed against 5 ml of the same buffer. The system was incubated for 36 h in a shaking bath at 37°C.

At the end of incubation, BA were determined in triplicate on the dialysate and retentate; the volume shift from buffer to plasma during equilibrium dialysis was also evaluated. Dialysis equilibrium for each BS was also determined to check that BS were completely dialyzed in the absence of proteins and lipoproteins. The percentage of BS bound to proteins or lipoproteins can be found from the BS concentration of the dialyzed

solution (free fraction) and retentate (bound + free fraction).

Values are expressed as percentage of BS bound to proteins or lipoprotein fractions.

## 2.4. Distribution of bile salts between lipoprotein classes

BS distribution between lipoprotein classes was studied by incubating plasma containing 4 g% protein and 0.1 mM BS. Sequential runs were performed at 20°C to obtain VLDL, LDL, HDL and the fraction of  $d > 1.210$  g/ml. BA content of each fraction was determined by enzymatic methods.

## 2.5. Hydrophobic properties of bile acids

The hydrophobic character of BA was studied by evaluating the partition coefficient ( $P$ ) between octan-1-ol and water [11].

Octan-1-ol RP (Carlo Erba, Milan) was saturated with Tris buffer, pH 7.5, in saline prior to partitioning. BS at 0.1 mM were prepared in 10 mM Tris buffer, pH 7.5, and saturated with octan-1-ol. 3 ml BS solutions were added to 3 ml octanol and shaken. After 6 h, the tubes were centrifuged for 30 min at 6000 rpm and the aqueous phase analyzed for BA content.

# 3. RESULTS

## 3.1. Maximum binding of bile salts to lipoproteins

Table 1 shows the composition of the plasma and lipoprotein fractions used for determining maximum BS binding. Table 2 reports the percentages of bound BS, as evaluated by experimental

Table 1  
Composition of pooled plasma and lipoprotein fractions used in dialysis experiments

	Protein <sup>a</sup>	Phospho- lipids	Cho- lesterol	Trigly- cerides
Plasma	7.0	182	229	154
VLDL	0.035	15	39	110
LDL	0.12	42	150	48
HDL	0.30	107	40	16
$d > 1.210$ g/ml	3.5	18	—	—

<sup>a</sup> g/dl

Values expressed as mg/dl

Table 2  
Binding of different bile acids to plasma and lipoprotein fractions

	VLDL	LDL	HDL	$d > 1.210$	Plasma
DCA	$15.4 \pm 2.2$	$20.6 \pm 3.4$	$49.5 \pm 5.9$	$95.3 \pm 8.5$	$97.6 \pm 9.6$
CDCA	$16.2 \pm 1.7$	$21.7 \pm 3.7$	$50.2 \pm 5.1$	$94.6 \pm 9.1$	$96.2 \pm 8.3$
HDCA	$9.6 \pm 1.4$	$11.7 \pm 1.2$	$28.4 \pm 2.8$	$74.3 \pm 7.6$	$81.2 \pm 9.3$
UDCA	$10.8 \pm 1.0$	$16.1 \pm 1.7$	$27.6 \pm 3.1$	$71.7 \pm 6.2$	$76.4 \pm 7.7$
CA	$7.3 \pm 0.3$	$9.5 \pm 1.2$	$19.7 \pm 1.2$	$48.3 \pm 4.5$	$50.2 \pm 4.9$
UCA	$7.1 \pm 0.4$	$9.8 \pm 0.8$	$16.6 \pm 2.1$	$28.2 \pm 3.0$	$29.6 \pm 3.1$

Values are expressed as percentage of initial bile salt concentration (0.1 mM) bound to the studied fraction. Mean values  $\pm$  SD of 5 experiments

Table 3  
Distribution of bile salts between the lipoprotein fractions

	VLDL	LDL	HDL	$d > 1.210$
DCA	—	$1.8 \pm 0.4$	$6.1 \pm 1.7$	$92.0 \pm 3.1$
CDCA	—	$2.2 \pm 0.3$	$6.7 \pm 1.7$	$91.1 \pm 5.2$
HDCA	$0.9 \pm 0.3$	$7.6 \pm 2.2$	$12.1 \pm 1.8$	$79.1 \pm 3.8$
UDCA	$0.6 \pm 0.2$	$4.7 \pm 0.9$	$10.6 \pm 1.4$	$84.2 \pm 4.2$
CA	$1.4 \pm 0.8$	$10.2 \pm 1.8$	$18.3 \pm 2.1$	$70.1 \pm 5.4$
UCA	$2.1 \pm 0.6$	$18.2 \pm 2.3$	$29.2 \pm 2.4$	$50.5 \pm 6.8$

Values (mean of 5 experiments  $\pm$  SD) indicate the bile acid content of each fraction (as percentage of the initial amount)

dialysis. Binding to plasma and other lipoprotein fractions decreases along the series UCA – CA – UDCA – HDCA – DCA – CDCA. Binding to VLDL and LDL is very low for all BS studied. HDL are bound by some dihydroxy BA, in particular DCA and CDCA. In contrast, maximum HDL binding capacity for HDCA and UDCA is very low. The fraction with  $d > 1.210$  g/ml and total plasma show the highest binding capacity, markedly greater than the lipoprotein fractions.

### 3.2. Distribution of bile salts among lipoprotein fractions

Table 3 gives the values, expressed as percent of initial content, of the distribution of each BS between the different lipoprotein fractions. BS distribution seems to depend on their molecular hydrophobicity. In fact, the most hydrophilic BA (UCA, CA) are less bound to the protein-rich and lipoprotein-free fraction ( $d > 1.210$  g/ml). This

'unbound' BS fraction is that found in lipoproteins, HDL in particular.

CA behaves similarly to UCA, whereas DCA and CDCA are tightly bound to the fraction with  $d > 1.210$  g/ml. UDCA and HDCA have intermediate binding to HDL and LDL.

### 3.3. Determination of octanol-water partition of bile acids

Table 4 gives the values expressed as percent initial BA concentration remaining in the aqueous phase after shaking with octan-1-ol. Molecular lipophilicity reduces the amounts of BA remaining in the aqueous phase. Hydrophobicity decreases along the series DCA – CDCA – HDCA – UDCA – CA – UCA. The behavior of  $3\alpha,7\beta$ - and  $3\alpha,6\alpha$ -dihydroxy- $5\beta$ -cholanoic acids is different from that expected from reversed phase HPLC data [10].

Table 4

Partition of the studied bile acids in an octanol-water system

	Percent in water	$P^a$
DCA	3.2 ± 1.4	30.2
CDCA	4.1 ± 1.3	23.3
HDCA	6.2 ± 1.5	23.3
UDCA	16.7 ± 2.7	4.9
CA	71.2 ± 8.4	3.7
UCA	93.2 ± 10.1	0.1

<sup>a</sup>  $P$  (partition coefficient) evaluated from the relationship:

$$P = \frac{V_{aq}}{V_{or}} \left[ \frac{C_i}{C_f} - 1 \right]$$

where:  $V_{aq}$  = volume of aqueous phase,  $V_{or}$  = volume of organic phase,  $C_i$  = bile acid concentration in aqueous phase,  $C_f$  = bile acid concentration in aqueous phase after extraction with octanol

#### 4. DISCUSSION

Our findings show that BS bind to different plasma components. The most hydrophobic BA (according to the HPLC definition of Armstrong and Carey [1]) are most tightly bound to proteins contained in the fraction with  $d > 1.210$  g/ml.

However, some poorly hydrophobic BA are strongly bound to proteins: such is the case for UDCA and HCA, which show greater binding to proteins and lipoproteins than CA, in spite of their shorter retention times on reverse-phase liquid chromatography [1]. Therefore these results and other data gleaned from the literature [4,12] do not fit HPLC retention time values.

The partition coefficient in the octan-1-ol-water system is widely utilized for describing the hydrophobic properties of drugs, and the correlation between biological activity of molecules and octanol-water partition is frequently addressed [2]. The presence of the  $7\beta$ - or  $6\alpha$ -hydroxy groups on the non-polar side of the molecule reduces both hydrophobicity and protein binding, and increases either the affinity for lipoproteins and the solubility in the aqueous phase of the water-octanol system, or both. An inverse correlation was found between the percentage of BA binding to protein and the water-octanol partition (fig.1).

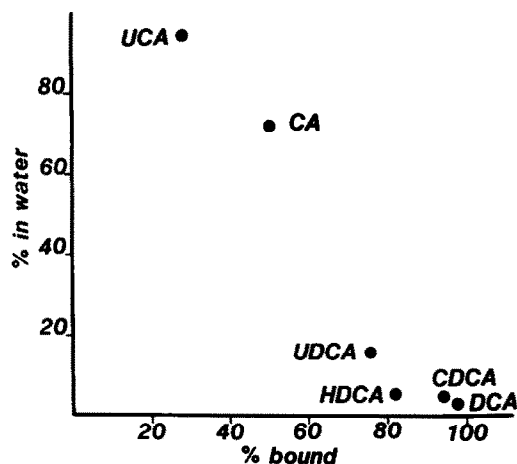


Fig.1. Correlation between percent of BA binding to plasma and percent in water in an octanol-water system of different bile salts.

The molar relationship of binding between plasma component and BA is low (about 12) for apoproteins/BA, intermediate (about 57) for albumin/BA, and high (about 320) for phospholipid/BA. Therefore circulating phospholipids are the most represented components which can pick up BA. Although BA binding to albumin varies in nature and strength, when BA are not extensively bound to proteins, lipoproteins become more important and transport increasing quantities of BA (UCA > CA > HDCA > UDCA > CDCA > DCA).

In particular, the fractions richer in phospholipids and proteins are the most active. Using photo-affinity labeling techniques, Kramer et al. [13] demonstrated that about 30% of taurocholate is present in HDL and 15% in LDL fraction.

Other circulating membranes, such as erythrocytes, can transport BA, but very detergent BA are tightly bound to proteins and so their possible damaging effects on the membranes are prevented.

In conclusion, lipoproteins seem to represent a reserve transport system for BA in serum, especially for hydrophilic BA less bound to proteins. The hydrophobic-hydrophilic balance of BA molecules does not correlate with their maximum binding to proteins and lipoproteins, or with the distribution into different lipoprotein classes. On the contrary, the binding pattern shows a good correlation with octanol-water partition. This test is easy to per-

the binding pattern shows a good correlation with octanol-water partition. This test is easy to perform and can be used to estimate some chemico-physical properties of bile acids.

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