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Displacement of Bilirubin From Albumin by Ibuprofen *In Vitro*

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ABSTRACT: Ibuprofen binds to plasma albumin and could interfere with the binding of bilirubin in jaundiced newborn infants. Most clinical studies have not shown increased concentrations of unbound bilirubin (UB) in plasma from infants treated with ibuprofen for a patent ductus arteriosus. However, studies *in vitro* have not been equally conclusive. Plasma were obtained from routine samples from jaundiced newborn infants and pooled. Total and UB were measured with the peroxidase method after addition of ibuprofen or sulfisoxazole as a known bilirubin displacer. Final ibuprofen concentrations varied from 0.43 to 2.6 mM. Bilirubin concentrations were varied from 176 to 708 μM by adding bilirubin to plasma samples. Ibuprofen caused a linear increase in UB up to +54% at a concentration of 1.8 mM, compared with an increase of 87% by sulfisoxazole (1.32 mM). A double reciprocal plot of molar concentrations of bound *versus* UB at bilirubin concentrations ranging from 176 to 708 μM showed a competitive displacement of bilirubin by ibuprofen. The data indicate that ibuprofen is a competitive displacer of bilirubin *in vitro*. Ibuprofen should be used with caution in premature infants with a significant hyperbilirubinemia. **(*Pediatr Res* 67: 614–618, 2010)**

The significance of unconjugated hyperbilirubinemia is the potential for development of bilirubin neurotoxicity and kernicterus. Most healthy term infants with a total serum bilirubin (TB) concentration reaching 425 to 680 μM will, however, escape with no significant damage when treated with phototherapy and/or exchange transfusion according to guidelines (1). However, 8 to 9% of the kernicterus cases occur at a TB concentration <425 μM. Kernicterus has also been reported at TB concentrations <340 μM (2,3). In premature infants, bilirubin toxicity has been observed at even lower TB concentrations (4,5). More recently, high peak TB has been associated with poor long-term prognosis and adverse neurodevelopmental outcome in extremely low birth weight infants (6,7).

The binding site of bilirubin on the albumin molecule is still uncertain, but most likely bilirubin binds to albumin at one high affinity binding site in a 1:1 molar ratio (3,8–10). Clinical and experimental data have also suggested different secondary binding sites. However, these secondary binding sites may not be of any physiologic importance (10). At equilibrium, ~0.005% of bilirubin will be unbound bilirubin (UB). The albumin-bilirubin complex has a high molecular weight and cannot cross capillary walls, whereas UB is available for tissue distribution and elimination (9,11). Bilirubin is not toxic while bound to albumin (BB), and it is thought that the concentration of UB is a better predictor of toxicity than TB. The distribution and elimination of UB causes a shift in the equilibrium, and more bilirubin is released from albumin. The concentration of UB is influenced by several factors like the concentration of TB (2), albumin, and the binding capacity of albumin (5). Of clinical importance is the fact that the binding capacity is influenced by other substances, which also binds to albumin, both endogenous substances such as FFA and hematin (12), as well as several drugs bound to albumin (13,14). These drugs are able to displace bilirubin from its binding sites on albumin, thus increasing the UB concentration and toxicity. This has been demonstrated clinically by increased mortality and kernicterus in newborn infants treated with sulfisoxazole (15) as well as experimentally *in vivo* in Gunn rats given such treatment (16). Drugs that displace bilirubin from albumin generally have a strong binding to albumin (13). Such drugs are therefore routinely not given to jaundiced newborn infants.

A patent ductus arteriosus (PDA) is seen in ~65% of infants born at <28 wk of gestation (17). Infants with PDA are often treated with cyclooxygenase inhibitors to promote ductal closure. This treatment is usually given during the first week of life in which significant jaundice also exists. Historically, indomethacin has been the drug of choice, but recently ibuprofen has become increasingly popular because of a lower renal toxicity (18). Ibuprofen is now the preferred drug for pharmacological treatment of PDA in premature infants. More than 20,000 infants have been treated in Europe since 2001 (www.orphan-europe.com).

Although indomethacin does not seem to affect the binding of bilirubin to albumin (19,20), there is conflicting evidence on the effect of ibuprofen on bilirubin-albumin binding. Cooper-Peel *et al.* (21) found that ibuprofen quadrupled the plasma concentration of UB, and Ahlfors (22) also found that therapeutic concentrations of ibuprofen between 50 and 400 μg/mL (0.22–1.8 mM) significantly increase the UB concentration except at the lowest concentration. However, other studies have not been able to confirm any increase in UB during treatment with ibuprofen (Van Overmeire B *et al.,* Changes in free bilirubin during ibuprofen treatment for patent ductus arteriosus (PDA), 14th European Workshop on Neonatology, August 30 to September 2, Trondheim, Norway,

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Abbreviations: BB, bilirubin bound to albumin; **PDA,** patent ductus arteriosus; **TB,** total bilirubin; **UB,** unbound bilirubin

Abstract 40). Recently, Ambat *et al.* (23) and Aranda *et al.* (Aranda *et al.,* Plasma unbound bilirubin and ibuprofen in preterms. 16th European Workshop on Neonatology, September 4–6, Leuven, Belgium, J Neonatal Perinatal Med 1:262–263) also found that ibuprofen increased UB, but only at high concentrations of ibuprofen and at high bilirubin-albumin molar ratios. However, none of these clinical studies have calculated bilirubin-albumin dissociation constants during treatment with ibuprofen.

The binding potential of albumin as well as total bilirubin concentrations can vary substantially between infants and according to postnatal age (days) during a treatment period with ibuprofen. This study was therefore undertaken to evaluate the possible bilirubin displacing effect of ibuprofen in plasma from newborns during standardized conditions *in vitro* and to calculate dissociation constants of the bilirubin-albumin complex with and without the presence of ibuprofen. This study was approved by the Institutional Review Board.

METHODS

Plasma samples. Excess plasma samples from routine laboratory tests on hyperbilirubinemic newborns were used. Plasma samples were stored for only 2 days in dark at 4°C before pooled and centrifuged at 3000 rpm for 3 min at ambient temperature to precipitate fibrinogens. After centrifugation, the content was divided in aliquots of 250 μL and frozen at −80°C. The plasma albumin concentration was 536 μM.

Bilirubin, drugs, and reagents. A stock solution of bilirubin ($C_{33}H_{36}N_4O_6$, purity ≥99%, lot no: 113K1166 Sigma Chemical Co. Aldrich, Norway) was made by dissolving 13.41 mg bilirubin in 1 mL 0.1M NaOH and was used to adjust plasma bilirubin levels. Bilirubin concentrations in study samples were varied between 176, 223, 347, 546, 616 and 708 μM by addition of bilirubin stock solution to plasma samples. A working solution of ibuprofen ($C_{13}H_{17}O_2Na$, purity ≥99%, CAS no: 31121-93-4 Sigma Chemical Co. Aldrich) was made by dissolving 31 mg ibuprofen sodium salt in 96% ethanol. Plasma ibuprofen concentrations were varied between 0.43, 0.87, 1.3, 1.8, and 2.6 mM by addition of ibuprofen to plasma samples. A working solution of sulfisoxazole ($C_{11}H_{13}N_3O_3S$, purity ≥99%, cas no: 127-69-5 Sigma Chemical Co. Aldrich) was made by dissolving 18 mg sulfisoxazole in 1 mL of 96% ethanol. Sulfisoxazole at plasma study concentrations of 1.32, 2.66, and 3.18 mM was used as a positive control for bilirubin displacement.

Measurement of UB. UB was measured with the peroxidase method (24) using the UB-A1 analyzer (Arrows Co, Osaka, Japan) at the recommended sample dilution 1:42. Enzyme (peroxidase), buffer (0.1 M, glucose-Na_2HPO_4-KH_2PO_4, pH = 7.38), and dilution buffer (buffer without glucose) were provided in reagent kits (lot no: 070516) from Arrows AS, Japan. After addition of 5 μL sulfisoxazole or ibuprofen to plasma samples of 250 μL, the tubes were gently shaken five times and incubated at room temperature (20°C) for 2 min in protection from light. After incubation, the tubes were again gently shaken five times before measurements of TB in measuring cells containing 1 mL buffer and 25 μL plasma. UB was measured in the same cells after addition of 25 μL enzyme solution. All samples were protected from daylight, and measurements were always done in four parallels.

Statistics. Standard paired student *t* tests with a two-tailed significance level of 0.05% were used to compare mean values and percentual increase of UB. One- and two-way ANOVA were used to perform analyses of variance with the post hoc Tukey test for significance using the Microsoft Office Excel 2007 and S-Plus 7.0. Correlation analyses were performed with WINKS 4.80a (TexaSoft, Cedar Hills, TX).

RESULTS

Methodological considerations. No significant ($p > 0.05$) changes in the UB concentration were observed by dilution of plasma samples with drug and bilirubin. Furthermore, the variations in UB measurements within 1 day and between days were both <10%.

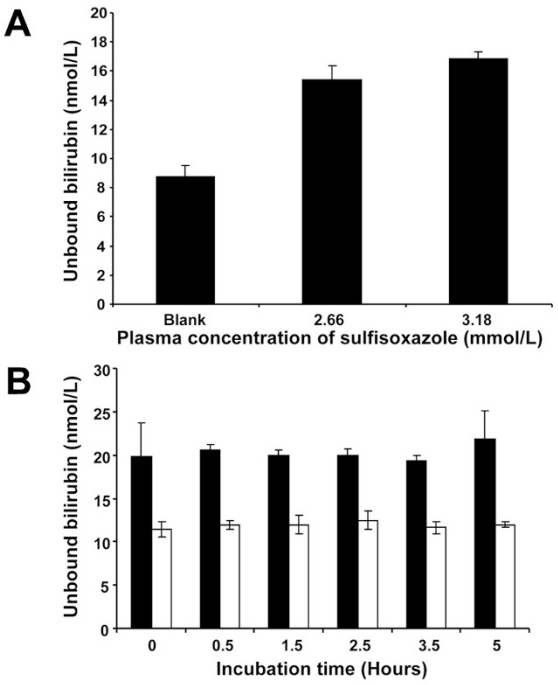

Figure 1. Effect of sulfisoxazole and incubation time on UB levels. *A*, Effect of sulfisoxazole (2.66 and 3.18 mM) on UB levels in pooled plasma samples from jaundiced newborn infants. Total bilirubin concentration was 176 μM, and albumin concentration was 536 μM. *B*, Effect of incubation time on UB levels in pooled plasma samples from jaundiced newborn infants after addition of sulfisoxazole, ■ (1.32 μM) compared with □. Total bilirubin concentration was 176 μM, and albumin concentration was 536 μM.

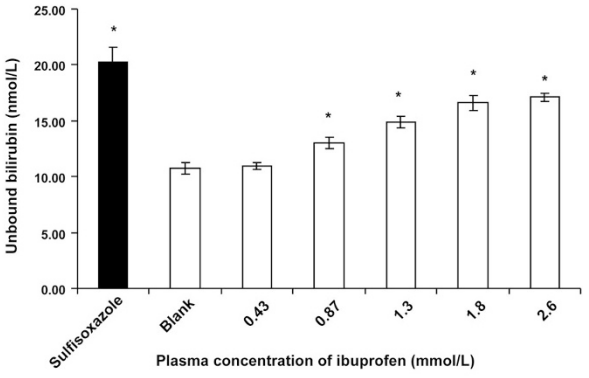

Figure 2. Effect of sulfisoxazole and ibuprofen on UB levels. Effect of sulfisoxazole (1.32 mM, ■) and ibuprofen (□) in concentrations from 0.43 to 2.6 mM on UB levels in pooled plasma samples from jaundiced newborn infants. Total bilirubin concentration was 176 μM, and albumin concentration was 536 μM. * indicates a significant ($p < 0.05$) increase in UB from blank values.

Effect of sulfisoxazole on UB in plasma samples. As shown in Figure 1*A*, UB concentrations increased by 80 and 90% after addition of sulfisoxazole to give plasma concentrations of 2.66 and 3.18 mM, respectively. This effect was not potentiated by different incubation times (0, 0.5, 1.5, 2.5, 3.5, and 5 h) as seen in Figure 1*B*.

Effect of ibuprofen on UB in plasma samples. As shown in Figure 2, ibuprofen at four different concentrations (0.43, 0.87, 1.3, and 1.8 mM) increased the UB linearly (y =

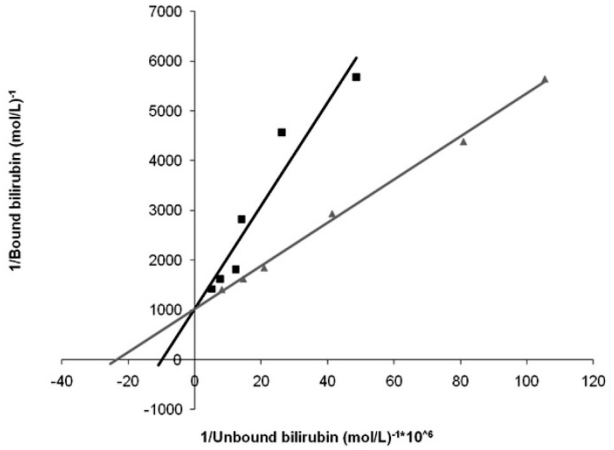

Figure 3. Binding characteristics of bilirubin to albumin. Double reciprocal plot of molar concentrations of BB (1/BB) *vs* UB (1/UB) at plasma bilirubin values from 176 to 708 μM at albumin concentration of 536 μM. Data shown with (■, $r^2 = 0.9198$) and without (▲, $r^2 = 0.9969$) the presence of ibuprofen 2.6 mM.

Table 1. *Binding parameters for bilirubin in plasma from newborns with and without the presence of ibuprofen*

	n	N	K_D (M)	K_D/n (slope)
With ibuprofen	9.8×10^{-4}	1.8	9.9×10^{-8}	1×10^{-4}
Without ibuprofen	9.8×10^{-4}	1.8	3.9×10^{-8}	4×10^{-5}

Total number of binding sites (per litre) available for bilirubin in plasma from newborns (n), available binding sites for bilirubin per albumin molecule (N), and dissociation constant of the bilirubin-albumin complex (K_D), with and without the presence of ibuprofen. Data calculated from Figure 3.

$38.376x - 13.666$) ($R^2 = 0.999$) from 10.8 to 16.1 nmol/L (corresponding to an increase of 54% $p < 0.05$), before it leveled off at 17.1 nmol/L (59%) at an ibuprofen concentration of 2.6 mM. In comparison, sulfisoxazole (1.32 mM) as a positive displacer, increased the UB concentrations to 20.1 nmol/L corresponding to 87%.

Double reciprocal plot of UB and BB with and without ibuprofen. Figure 3 shows a linear relationship in a double reciprocal plot of inverse molar concentrations of UB *versus* BB, in the presence ($y = 0.0001 \times + 1015.2$) and absence ($y = 4 \times 10^{-5} \times + 1015.9$) of 2.6 mM ibuprofen. R^2 values were 0.9198 and 0.9969, respectively. Different displacement parameters calculated from Figure 3 are presented in Table 1. The addition of ibuprofen did not change the total number of binding sites (n) for bilirubin in plasma or the binding sites available for binding (N) per albumin molecule. The dissociation constant (K_D) increased from 3.9×10^{-8} to 9.9×10^{-8} M after addition of ibuprofen. The results thus indicate a competitive displacement of bilirubin from albumin in plasma from newborns by ibuprofen.

DISCUSSION

The concentrations of ibuprofen used in this study were comparable to, as well as higher, than ibuprofen concentrations measured after a single i.v. dose of 10 mg/kg (25,26). However, neonates are often treated with several doses with 12-h intervals and may reach even higher plasma concentrations of ibuprofen. The long half-life of the drug may also cause an accumulation and increase the ibuprofen concentrations in the first days of life when TB concentrations are also high. The concentrations of bilirubin in study samples were comparable to levels usually found in newborn premature infants.

Although clinical *in vivo* studies on the binding of bilirubin to albumin have found no or insignificant effects of ibuprofen on UB in infants during treatment, both the present as well as earlier *in vitro* studies have clearly shown that ibuprofen increases UB in hyperbilirubinemic plasma (21,22). Therefore, the question is how to explain the seemingly different results from *in vivo* and *in vitro* studies. In the *in vivo* situation, there exists equilibrium between BB and UB in plasma, and UB in plasma is in equilibrium with UB diffused into different tissues causing toxicity. With a reduced binding of bilirubin to albumin, the immediate increase in UB will *in vivo* cause more UB to diffuse into tissues and establish a new equilibrium between BB, UB-plasma, and UB-tissue. Because the distribution space for UB-tissue probably is greater than UB-plasma, the net increase in UB-plasma at the new equilibrium will probably be much less than the acute increase seen immediately after the introduction of the displacing drug. It is thus likely that any small differences in UB in clinical studies will not be detectable with standard methods, also due to differences and variations in binding capacity and TB levels both in the same infant as well as between infants. *In vivo* experiments of bilirubin displacement have also shown that this shift of UB into tissue compartments may actually cause a decrease in TB concentrations in plasma (16). With regard to this, it is interesting to note that in the study by Aranda *et al.* (Aranda *et al.,* Plasma unbound bilirubin and ibuprofen in preterms. 16th European Workshop on Neonatology, September 4–6, Leuven, Belgium, J Neonatal Perinatal Med 1:262–263), TB levels were lower in infants treated with ibuprofen than in infants not treated. Furthermore, TB levels also tended to fall on consecutive postnatal days when TB levels would have been expected to rise as a result of the normal development of the physiologic neonatal hyperbilirubinemia (27), indicating that more bilirubin could have been shifted from plasma into the tissue space during treatment with ibuprofen. On the other hand, in two recent clinical studies, ibuprofen has been shown to slightly but significantly increase TB in infants with PDA (28,29). However, UB concentrations were not measured in these studies. In the study by Rheinlaender *et al.* (28), infants treated with ibuprofen were compared with infants treated with indomethacin. Although neurodevelopmental outcome at 2 y of age did not differ between the groups, single case analysis identified four cases of adverse neurodevelopmental outcome in the ibuprofen group despite inconspicuous clinical course. The authors speculate that the increase in TB concentrations could be caused by inhibition of hepatic glucuronidation of bilirubin by ibuprofen. Differences in results between these studies might be explained by differences in clinical parameters not accounted for.

In an *in vitro* situation, UB cannot diffuse out of the plasma compartment. Any reduction in the binding capacity of albumin for bilirubin will therefore result in a more profound

increase in plasma UB compared with an *in vivo* situation. The *in vitro* situation must therefore be considered more sensitive to changes in the binding affinity for bilirubin to albumin. This can probably explain the different results from *in vivo* and *in vitro* studies. Ibuprofen probably also diffuses out of the plasma compartment and thus might compete with bilirubin on tissue binding sites as well and possibly influence the tissue toxicity of UB.

The total number of binding sites (n) for bilirubin on albumin in plasma was approximately the same with and without addition of ibuprofen, whereas the slope (K_D/n) was different (Table 1 and Fig. 3) indicating a competitive displacement of bilirubin from albumin by ibuprofen (30). A competitive inhibition involves a competition between two ligands (bilirubin and ibuprofen) for the same binding site. At any given inhibitor concentration, the inhibition can be terminated by increasing the ligand concentration (31). The concentrations of UB will thus vary in relation to both the concentrations of ibuprofen and bilirubin. Different concentration ratios of these substances can result in similar concentrations of UB. This might explain some of the different results in clinical as well as *in vitro* and *in vivo* studies.

The number of binding sites for bilirubin on albumin in this study was 1.8 (Table 1). However, as pointed out by Siam *et al.* (10), secondary binding sites may be physiologically irrelevant because they only are detected at bilirubin:albumin molar ratios >1:1, in which supersaturation will occur due to the low solubility of bilirubin at physiologic pH. This situation leads to temporary binding of bilirubin onto the bilirubin-albumin 1:1 molar complex before bilirubin precipitates (10). The findings of 1.8 binding sites in this study could be related to the inherent dilutions of the samples in the peroxidase method, which has been shown to increase the binding affinity (32,33). It has therefore been argued that the high dilution of the samples when using the UB-analyzer substantially reduces the ability to detect an increase in UB from a weak displacer, which could have been detected in the undiluted sample (22). However, the dissociation constant K_D of 3.9×10^{-8} M (Table 1) in this study is in the same range (10^{-8} M) as calculated by others (14) for the high affinity bilirubin binding site on albumin. This supports our conclusion that ibuprofen displaces bilirubin from its high affinity binding site on the albumin molecule. Ghuman *et al.* (34) have reported the precise architecture of two primary drug binding sites on the albumin molecule, including the binding site for ibuprofen (drug binding site 2). Because this study showed a competitive displacement of bilirubin by ibuprofen, this is probably also the binding site for bilirubin. Interestingly, indomethacin was found to bind to a different binding site (binding site 1).

Bilirubin has been shown to induce apoptosis and necrosis in human NT2-N neurons (35). Recently, Berns *et al.* (36) also reported that ibuprofen alone increased apoptosis and necrosis in cultures of rat cortical neurons and also augmented the effect of bilirubin. They concluded that ibuprofen in therapeutic concentrations is toxic to embryonic neuronal cells, and that combined exposure of ibuprofen and bilirubin is more toxic than exposure to either substance alone. In their experiments, they used a bilirubin:albumin molar ratio of 3.0, which is a higher bilirubin:albumin molar ratios than found in plasma from newborns, probably resulting in high UB bilirubin levels in study as well as in control cell cultures. However, UB was not measured, and it is therefore not possible to conclude that the increased toxicity of the combined exposure was caused by bilirubin displacement.

The displacing effect of ibuprofen seems to be significant but quantitatively small. However, because the present data indicate a competitive and dose-related linear relationship between ibuprofen concentrations and bilirubin displacement, this effect might be of clinical significance in some infants. Therefore, ibuprofen should be used with caution in hyperbilirubinemic premature infants.

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