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Sulphate conjugation enhances reversible binding of drug to human serum albumin

TAKASHI MIZUMA*, MICHIYO KOMORI, MASAHARU UENO†, ISAMU HORIKOSHI, *Department of Hospital Pharmacy, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama, Toyama 760-03, Japan*

Abstract—Reversible binding of model compounds, their conjugated metabolites (sulphates and glucuronides), and also derivatives of the compounds, to human serum albumin (HSA) has been examined using an ultrafiltration method. *p*-Nitrophenol (*p*-NP), α-naphthol (α-NA) and β-naphthol (β-NA) were used as model compounds. Reversible binding of 500 μM *p*-NP sulphate to 4% HSA (96·6 ± 0·35%, mean ± s.d. n = 3) was significantly higher ($P < 0.001$), whereas reversible binding of *p*-NP glucuronide to 4% HSA (33·3 ± 9·82%) was much lower ($P < 0.001$) than that of 500 μM *p*-NP (90·9 ± 0·60%). Reversible binding of 500 μM *p*-NP glucopyranoside to 4% HSA (25·8 ± 2·82%) was comparable with that of the glucuronide, with which it is structurally similar. In contrast, reversible binding of 500 μM *p*-NP phosphate, an anionic compound like *p*-NP sulphate, to 4% HSA (61·4 ± 5·28%) was significantly lower than that of *p*-NP ($P < 0.001$). Similar results were observed in reversible binding of sulphates of α-NA and β-NA. Significant differences of dissociation constants for HSA binding were observed between the parent compound (α- or β-NA) and its sulphate conjugate ($P < 0.005$ for α-NA and α-NA sulphate, $P < 0.001$ for β-NA and β-NA sulphate), but the number of binding sites was the same. These results indicated that sulphate conjugation enhances reversible binding of a parent compound to HSA by increasing the binding affinity of the parent compound to HSA. This enhancement appeared to be advantageous for preventing random distribution of this metabolite to organs in the body.

Although there have been many studies on reversible binding of drugs to human serum albumin (HSA) (see Fehske et al 1981), only few data of reversible binding of conjugates to plasma protein of animals have been reported (Mulder et al 1985; Wang et al 1986). In this report, we describe studies to relate characteristics of the reversible binding of glucuronide and sulphate conjugates with HSA to their disposition in the body. HSA binding of derivatives of the parent compounds were also studied for comparison. *p*-Nitrophenol and α- and β-naphthol were used as model compounds since their structures could be related to those of paracetamol and naproxen, respectively.

Materials and methods

Materials. *p*-Nitrophenol (*p*-NP), *p*-nitrophenyl sulphate (*p*-NPsul), *p*-nitrophenyl glucuronide (*p*-NPglu), *p*-nitrophenyl phosphate (*p*-NPpho), *p*-nitrophenyl glucopyranoside (*p*-NPpyr), α-naphthol (α-NA), α-naphthyl sulphate (α-NAsul), α-naphthyl glucuronide (α-NAglu), α-naphthyl phosphate (α-NApho), β-naphthol (β-NA), β-naphthyl sulphate (β-NAsul), β-naphthyl phosphate (β-NApho), and human serum albumin (HSA, fatty acid free) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Methanol was of HPLC grade (Wako Pure Chemical Industries Ltd, Osaka, Japan) and other reagents were of analytical grade.

Reversible binding experiment. Reversible binding of compounds (*p*-NP, α-NA, β-NA, their conjugates and their derivatives) to HSA was examined using an ultrafiltration method. After mixing 0·6 mL of the compound in 0·1 M sodium phosphate buffer containing 0·9% NaCl (pH 7·4) with 0·6 mL of 8% HSA solution in 0·1 M sodium phosphate buffer containing 0·9% NaCl (pH 7·4), 150 μL of the mixture was sampled for determination of total concentration (C_t) of the compound. The mixture was centrifuged in a microconcentrator, Centricon 30 (Amicon, Denvers, MA, USA), using an angle rotor at 2 500 rev min^{-1} for 9 min. Filtered solution (150 μL) was assayed for determination of free (unbound) concentration (C_f) of the compound. Each solution (150 μL) sampled for determination of C_t and C_f was mixed with 150 μL of 10% perchloric acid containing internal standard for HPLC assay. The internal standards were selected for optimal HPLC assay as follows: for determination of *p*-NP, its conjugates and derivatives, 10 mM *p*-fluorophenol was used; for α-NA, 25 μM β-NA, and its conjugates and derivatives, 10 μM *p*-NP; for conjugates and derivatives of β-NA, 100 μM *p*-fluorophenol. After the mixture had been centrifuged (3000 rev min^{-1}, 10 min), the resultant supernatant was applied to HPLC.

HPLC. The HPLC system consisted of a pump (Waters Assoc., 6000A, MA, USA), UV absorbance detector (Shimadzu, SPD-6A, Kyoto, Japan) and an integrator (Shimadzu, Chromatopacc-R2A). Assay conditions for *p*-NP, its conjugates, and derivatives were essentially as reported by Mizuma et al (1982). TSK gel ODS-80TM column (Tosoh, 6·0 mm i.d. × 15 cm length) was used for the *p*-NP, and associated compounds, and an Ultrasphere ODS column (5 μm) (Beckman, 4·6 mm i.d. × 15 cm length) for α- and β-NA, their conjugates and their derivatives. For *p*-NP, and associated compounds, the mobile phase was 38% methanol, 0·3% acetic acid, 16 mg L^{-1} tetrabutyl ammonium bromide and 0·1 g L^{-1} KNO_3 in water and detection was at 300 nm. For α-NA and its associated compounds the mobile phase consisted of 40–45% methanol, 0·3% acetic acid and 20 mg L^{-1} tetrabutyl ammonium bromide in water at 290 nm. For β-NA and its associated compounds, the mobile phase consisted of 35–45% methanol, 0·3% acetic acid, 20 mg L^{-1} tetrabutyl ammonium bromide at 290 nm except for β-NAsul, where the wavelength was set at 280 nm.

Data analysis. Data were analysed by Student's *t*-test. Binding parameters were calculated by fitting data to equation 1, using a non-linear least squares fitting program MULTI (Yamaoka et al 1981).

$$C_b = \frac{n \times P \times C_f}{K_d + C_f} + K_P \times P \times C_f \quad (1)$$

where C_b, C_f and P are bound and free concentrations and HSA concentration, respectively, and n, K_d and K_P are the number of binding sites per HSA molecule, dissociation constant and partition coefficient, respectively.

Results and discussion

Reversible binding of *p*-NP, *p*-NPglu, *p*-NPsul, *p*-NPpyr, and *p*-NPpho (25–500 μM of total concentration) to 4% HSA is shown in Table 1 (mean ± s.d., n = 3). Binding percent of *p*-NPpyr, which is similar in structure to *p*-NPglu, was comparable with

† Present address: Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama, Toyama 760-30, Japan.
* Correspondence and present address: T. Mizuma, Department of Biopharmaceutics, Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan.

Table 1. Binding percent of *p*-nitrophenol, its conjugates and its derivatives to 4% HSA.

Total concn	500 μM mean	s.d.	100 μM mean	s.d.	25 μM mean	s.d.
p-NP	90·9	0·60	94·5	0·52	94·7	0·12
Sul	96·6	0·35	97·9	0·25	97·9*	0·81
Pho	61·4	5·28	60·2	5·34	57·4	5·58
Glu	33·3	9·82	27·2	3·94	23·0	6·65
Pyr	25·8	2·82	20·6	3·75	27·8	7·22

Values were significantly different ($P < 0·001$ except *) from those of parent compound (*p*-NP) at the same total concentration (n = 3). *$P < 0·005$.

Table 2. Binding percent of α-naphthol, its conjugates and its derivative to 4% HSA.

Total concn	500 μM mean	s.d.	100 μM mean	s.d.	25 μM mean	s.d.
α-NA	98·0	0·16	99·5	0·22	ND	ND
Sul	98·9	0·02	$99·6^{NS}$	0·03	ND	ND
Pho	94·2	0·74	95·8	0·35	96·6	0·26
Glu	69·0	1·24	75·5	0·71	76·8	0·53

Values were significantly different ($P < 0·001$), except noted, from those of parent compound (α-NA) at the same total concentration (n = 3). NS, not significant ($P > 0·1$); ND, not detected.

that of *p*-NPglu. In contrast, binding percent of *p*-NPpho, which is an anionic compound like *p*-NPsul, was significantly lower than that of *p*-NP ($P < 0·001$). These results indicated that only *p*-NPsul bound to HSA to a greater extent than did the parent compound.

Reversible binding results for α-NA, its conjugates and derivatives (25–500 μM of total concentration) to 4% HSA are shown in Table 2 (mean ± s.d., n = 3).

Reversible binding of α-NA and α-NAsul at 25 μM to 4% HSA was so great that free concentrations could not be detected. At 500 μM, α-NA binding was 98·0 ± 0·16%, α-NAglu 69·0 ± 1·24% (significantly lower, $P < 0·001$) and α-NAsul was 98·9 ± 0·02%. The binding of α-NApho was 94·2 ± 0·74% (significantly lower than α-NA $P < 0·001$). Other results were similar to those of *p*-NP, and its compounds. There was no significant difference between α-NA and α-NAsul 100 μM ($P > 0·1$).

Results similar to those of *p*-NPsul and α-NAsul were observed with β-NAsul (data not shown). The physiological concentration of binding protein (4% HSA) examined here, resulted in failure to show a significant difference of reversible binding between sulphate conjugates and its parent compound, α- or β-NA, at low concentrations (25 and 100 μM). Therefore, reversible binding to 0·33% HSA was studied, and binding parameters were obtained, using Scatchard plots (Figs 1, 2).

The curved plot indicated that reversible binding of sulphate conjugates and their parent compounds consisted of one specific binding site, with non-specific binding shown by the shallow part. Binding parameters were calculated by fitting data to equation 1 and are shown in Table 3.

When binding parameters of α-NA were compared with those of α-NAsul, there was a significant difference only in K_d ($P < 0·005$). There was also a significant difference only in K_d when binding parameters for β-NA were compared with that for β-NAsul ($P < 0·001$). These results indicate that differences of HSA binding between a parent compound and its sulphate resulted from the change of binding affinity (K_d) of compound to HSA. Furthermore, a 7-fold difference of K_d between α-NAsul and β-NAsul was observed ($P < 0·001$), whereas there was no significant difference of K_d between α-NA and β-NA. This result indicates that sulphate conjugation enhances the difference in characteristics between the isomers in terms of HSA binding.

Results similar to ours were reported for 4-methylumbelliferone (Mulder et al 1985) and paracetamol (Wang et al 1986). In those reports, binding percent of sulphate conjugate to rat or pregnant sheep plasma protein was higher and of glucuronide conjugate was lower than that of the parent compound. Since values of the binding percent of phosphates were lower than those of parent compounds, unlike sulphate conjugates, an addition of a negative charge to the drug does not always seem to enhance reversible binding of drugs to HSA. Therefore, the higher binding of sulphate conjugate to HSA compared with its parent compound, was considered to be a specific characteristic of the sulphate conjugate.

Sulphate conjugates are well known to be excreted in urine for

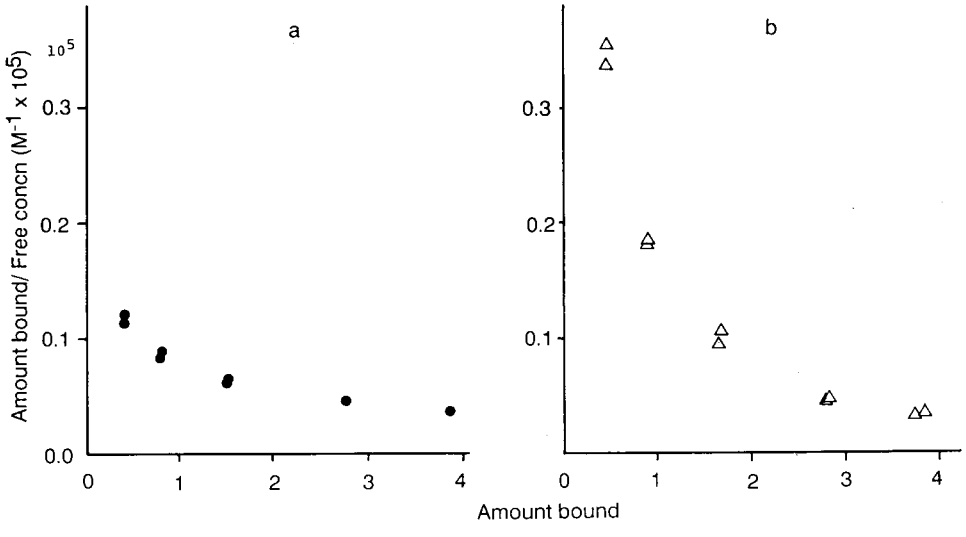

FIG. 1. Scatchard plot of reversible binding of (a) α-naphthol and (b) α-naphthol sulphate to 0·33% human serum albumin.

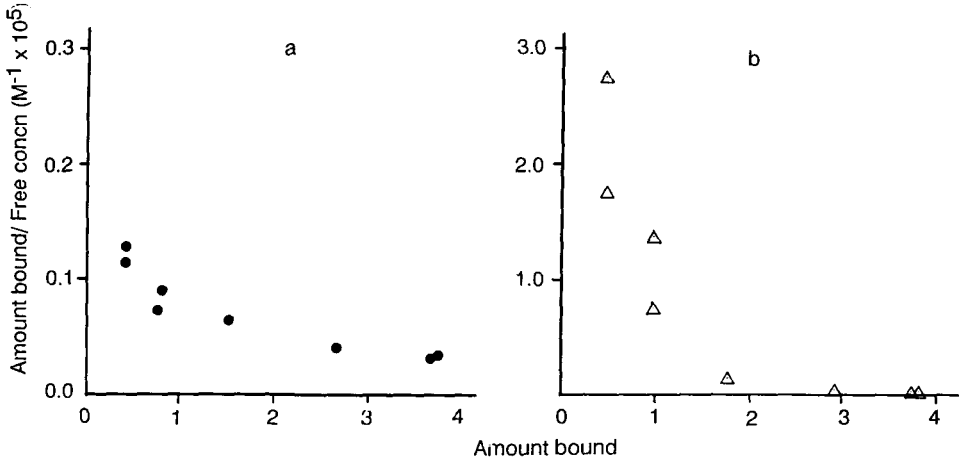

FIG. 2. Scatchard plot of reversible binding of (a) β-naphthol and (b) β-naphthol sulphate to 0·33% human serum albumin.

Table 3. Parameters for reversible binding of α- and β-naphthol and their sulphates to human serum albumin.

	α-NA		α-NAsul		β-NA		β-NAsul	
	mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.
n	1·23	0·18	1·44	0·13	1·24	0·28	1·49	0·15
K_d (μM^{-1})	10·41	2·07	3·27	0·53	9·71	3·11	0·476	0·102
K_p (μM^{-1})	0·0270	0·0021	0·0231	0·0021	0·0236	0·0031	0·0226	0·0031

Parameters were calculated from data shown in Figs 1 and 2.

which purpose they must be released from the metabolizing organ (e.g. liver) into the blood. However, reverse transport of sulphates of harmol and paracetamol from blood to liver has been reported (Sundheimer & Brendel 1983; Iida et al 1989). Therefore, high affinity binding of sulphate conjugate to HSA is considered to be advantageous in preventing a return of sulphate conjugate to the metabolizing organ or for preventing distribution of sulphate conjugate to other non-excretory organs.

Even if drugs are metabolized by both types of conjugation (sulphate, glucuronide), sulphate conjugation is a primary metabolic pathway for drug concentrations below the Michaelis constant (K_m) for glucuronide conjugation. For many drugs, such as paracetamol (Mizuma et al 1985), the Michaelis constant for sulphate conjugation is lower than that for glucuronide conjugation. Further studies on the binding site for sulphate conjugate, by which an interaction between drug and its sulphate conjugate in the body can be predicted in terms of HSA binding, is required.

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