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# Interaction of Human Serum Albumin with Furosemide Glucuronide: a Role of Albumin in Isomerization, Hydrolysis, Reversible Binding and Irreversible Binding of a 1-*O*-Acyl Glucuronide Metabolite

**Takashi Mizuma*, Leslie Z. Benet and Emil T. Lin**
*Department of Biopharmaceutical Sciences, University of California, San Francisco, CA 94143, USA*

**ABSTRACT:** Furosemide 1-*O*-acyl glucuronide (Fgnd) was reversibly bound to a single class of binding sites on human serum albumin (HSA), and the binding of Fgnd decreased with increasing F concentrations, suggesting that Fgnd binds to the same warfarin binding sites on HSA as F binds. The rate of Fgnd degradation (hydrolysis and acyl migration) decreased in the presence of HSA. Although the formation of acyl migration isomers of Fgnd was slower in the presence of HSA than in its absence, hydrolysis of Fgnd to F was faster in the presence of HSA. Rapid minor irreversible binding of Fgnd to HSA within 30 min was followed by slow major irreversible binding. Slow irreversible binding of Fgnd to HSA was decreased by F, though not significantly. This suggests that major irreversible binding may proceed *via* reversible binding. It has been reported that acyl migration is a prerequisite for irreversible binding. Therefore, these results indicate that HSA decreases irreversible binding of Fgnd to protein by suppressing acyl migration. Furthermore, these results suggest that HSA may prevent irreversible binding of Fgnd to other proteins in the body by decreasing the concentration of reactive Fgnd in the unbound form. HSA eliminates reactive Fgnd by hydrolysis to F. Therefore, it is concluded that HSA works as a scavenger to decrease reactive compounds by reversible binding or eliminates reactive compounds by irreversible binding. 

**Key words:** albumin; 1-*O*-acyl glucuronide; hydrolysis; acyl migration; reversible binding; irreversible binding

## Introduction

Serum (plasma) albumin has a physiological role to preserve oncotic pressure [1]. It is also known that albumin reversibly binds drugs [2]. Since only the free (unbound) form of substrate can be involved in their metabolism and disposition, the reversible binding of drugs and their metabolites by albumin influences their metabolism and disposition [3]. However, the reversible binding of drug glucuronide conjugates (major product by phase II metabolism) to albumin has not been extensively examined. Hepatic uptake of glucuronide conjugates of harmol [4], 4-methylumbelliferone [5,6] and acetaminophen [7] have been reported. Furthermore, deconjugation of glucuronide to parent drug in liver has been observed for 4-methylumbelliferone glucuronide [6]. Therefore, interactions of glucuronide conjugates with albumin, which may affect the disposition of glucuronide conjugates, should be clarified. Furosemide (F), a potent diuretic agent, has been reported to be metabolized to its conjugative metabolite, furosemide 1-*O*-acyl glucuronide (Fgnd), in humans [8–10]. Fgnd was isomerized in aqueous solution by acyl migration [11,12]. However, whether Fgnd undergoes isomerization in the presence of albumin is unclear. Therefore, in this study, the reversible binding of Fgnd to albumin was studied first, then the effect of albumin on acyl migration of Fgnd was studied. Since albumin has been reported to have esterase-like activities on specific sites [13] and nonspecific sites [14], hydrolysis of Fgnd to F by albumin was also studied. Furthermore, Fgnd irreversibly binds to albumin [15]. Since 1-*O*-acyl glucuronide of zomepirac, which was withdrawn from use because of an unexpected high incidence of immunological reactions, has been reported to covalently bind to plasma protein *in vivo* [16] and to albumin *in vitro* [16,17], further analysis of irreversible binding of Fgnd to albumin was also done. Thereby, the physiological role of albumin in metabolism and disposition of the drug and its glucuronide are also discussed.

Abbreviations: F, furosemide; Fgnd, furosemide glucuronide; HSA, human serum albumin.

* Correspondence to: Department of Biopharmaceutics, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan. E-mail: mizuma@ps.toyaku.ac.jp

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## Materials and Methods

### *Materials*

F, indole-3-propionic acid and human serum albumin (HSA) (essentially fatty acid free) were purchased from Sigma (St. Louis, MO). Fgnd was prepared from urine by the method reported previously [18]. Acetonitrile (HPLC grade) was obtained from J.T. Baker Chemical Co. (Milwaukee, WI). All other chemicals were of reagent grade. Due to the rapid photodegradation of Fgnd [12], all experiments with Fgnd were performed under fluorescent lamps covered with translucent plastic frames.

### *Reversible Binding of F and Fgnd to HSA*

F (10 mM) in methanol and/or 5.5 mM Fgnd in methanol:acetonitrile (5:3) ($< 27.3$ μL) were added to 1 mL of 50 μM HSA in 0.15 M potassium phosphate buffer (pH 7.4) to make the objective concentrations of F or Fgnd. The resultant solutions were centrifuged with an Amicon centrifree (Amicon Division, WR Grace Co., Denvers, MA) at 1500 rpm for 10 min at room temperature after 80 μL of the solutions were sampled for determination of the total concentrations of F and/or Fgnd. Eighty microliters were sampled from the filtered fractions for determination of free concentrations of F and/or Fgnd, and mixed with 80 μl of acetonitrile (in order to precipitate HSA) containing an internal standard (indole-3-propionic acid). The supernatants obtained after centrifugation (3000 rpm, 10 min) of the samples treated with acetonitrile were injected into an HPLC system.

The HPLC system consisted of a Beckman 110B M pump (Beckman Instruments Inc., Berkeley, CA), a Kratos Spectroflow 783 UV detector (254 nm, Kratos Analytical, Ramsey, NJ), a Shimadzu RF-530 fluorescence detector (Ex 345 nm, Em 425 nm, Shimadzu Corporation, Kyoto, Japan) and an Altex Ultrasphere-ODS column (4.6 mm × 250 mm length, 5 μm particle size, Beckman Instruments Inc.). The flow rate of the mobile phase (30% acetonitrile in water, 0.05% phosphoric acid, pH 3.0) for the determination of F and Fgnd was 1.5 ml/min.

### *Irreversible Binding of Fgnd to HSA*

First, irreversible binding of Fgnd to HSA by 24 h incubation was performed with the samples obtained after reversible binding experiments. Second, the time course of irreversible binding of Fgnd to HSA and the inhibitory effect of F were examined as follows. One milliliter of reaction mixture (50 μM HSA, 100 μM Fgnd) and 0.5 mL of reaction mixture (100 μM HSA, 100 μM Fgnd, and 0 or 500 μM F) were prepared and incubated at room temperature. The reactions were stopped by addition of 3 mL of ice cold acetonitrile:ethanol (2:1) and the mixtures were centrifuged at 3000 rpm for 10 min. After the supernatant was removed, 2 mL of methanol:ether (3:1) was added to the precipitated HSA and it was resuspended. The suspension was centrifuged at 3000 rpm for 20 min and the supernatant was removed. The precipitated HSA was washed five times with 2 mL of methanol:ether (3:1), as described above. The washed HSA was dried and hydrolyzed with 0.25 M KOH (1 mL) at 80°C for 30 min. The solutions of hydrolyzed HSA were acidified with 75 μL of 21.8% phosphoric acid, and the F released from hydrolyzed HSA was extracted with 3 mL of ethyl acetate containing indole-3-propionic acid. After evaporation of ethyl acetate by nitrogen gas, the resultant residue was dissolved in 200 μL of mobile phase and injected into the HPLC system.

### *Acyl Migration and Hydrolysis of Fgnd in the Presence and Absence of HSA*

Fgnd (175 μM) in 0.15 M phosphate buffer (pH 7.4) in the presence of HSA (500 μM) or absence of HSA was incubated at 37°C. The pH of Fgnd solutions was constant. One hundred and fifty microliters of the incubation mixture was mixed with 150 μL of acetonitrile containing indole-3-propionic acid and centrifuged (3000 rpm, 10 min). The resulting supernatant was injected into the HPLC system described above. The flow rate of the mobile phase (20% acetonitrile in water, 0.05% phosphoric acid, pH 3.5) for the acyl migration study was 1.3 ml/min.

### *Data Analysis*

Statistical treatment was performed by a multiple comparison *post hoc* test following ANOVA or Student's *t*-test, as described in the figures. The binding parameters for the reversible binding of Fgnd and F to HSA were calculated by fitting the data to Equation (1), which represents the binding as consisting of a single class binding site using the nonlinear least-square regression computer program (MULTI) [19]. $n$ and $K_d$ represent the number of binding sites and the dissociation constant, respectively; $C_f$ and $C_b$ represent the free concentration and the bound concentration of Fgnd, respectively; $P$ represents the concentration of HSA.

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

## Results and Discussion

### *Reversible Binding of Fgnd and F to HSA*

A Rosenthal plot of the reversible binding of Fgnd to HSA (Figure 1) shows that the reversible binding consists of a single class of binding sites ($n = 1.80$, $K_d = 99.8$ μM). The $n$ and $K_d$ for reversible binding

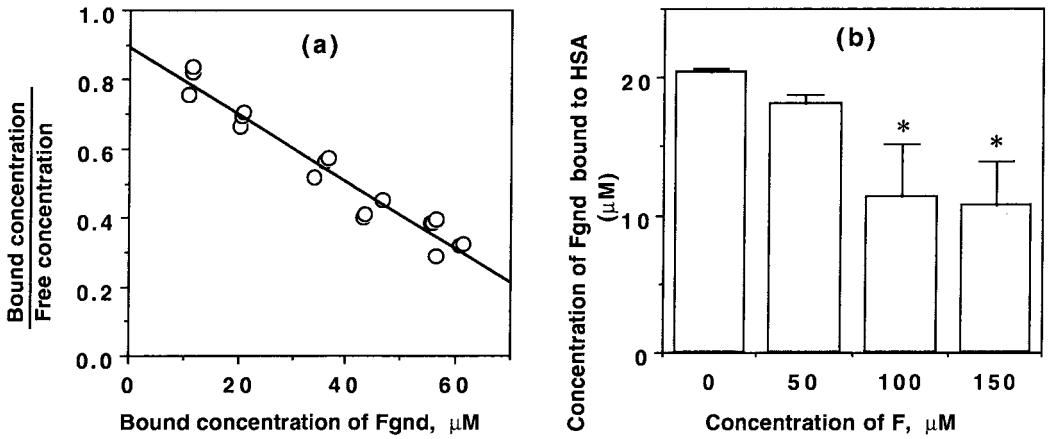



Figure 1. Reversible binding of furosemide glucuronide to HSA (50 μM). (a) Rosenthal plot of reversible binding of furosemide glucuronide to HSA. (b) Effect of furosemide on the reversible binding of furosemide glucuronide to HSA. Furosemide glucuronide (50 μM). Data represent mean ± S.E. ($n = 3$). An asterisk represents a significant value compared with the value in the absence of F (Dunnett's multiple comparison *post hoc* test, $p < 0.01$)

of F to HSA were 6.66 and 39.4 μM, respectively (data not shown). The bound concentration of Fgnd decreased with increasing F concentrations (Figure 1(b)). Since F binds to the warfarin binding site on HSA [20], this decrease by the presence of F suggests that Fgnd binds to the warfarin binding site of HSA. The $K_d$ (99.8 μM) for the reversible binding of Fgnd to HSA was larger than that for F (39.4 μM), indicating that the glucuronic acid moiety affects the affinity of Fgnd for HSA. The number of binding sites ($n = 1.80$) of Fgnd on HSA was less than that of F (6.66), indicating that conjugation of the glucuronic acid moiety to F decreases the number of binding sites, probably because of the increase in molecular size. It has also been reported that reversible binding of glucuronides of acetaminophen [21], oxazepam [22], harmol [23], *p*-nitrophenol [24] and α-naphthol [24] to HSA are lower than that of the respective parent drugs. Therefore, decreasing of binding affinity and the number of binding sites by conjugation of glucuronic acid moiety seems to be a general phenomenon, although sulfate conjugation (one of phase II metabolism) enhances reversible binding to HSA [24]. Even though Fgnd reversibly binds to HSA with less affinity than F, it is predicted that approximately 90% of Fgnd is still preserved in bound form in plasma after a 80 mg oral dose of F. Because the plasma concentration of F after a 80 mg oral dose of F was less than 3 μM [9], and thereby even if the Fgnd concentration in plasma is 50 times higher (e.g. 150 μM) than that of F (3 μM), Fgnd is still around 90% based on Equation (1) and estimated binding parameters.

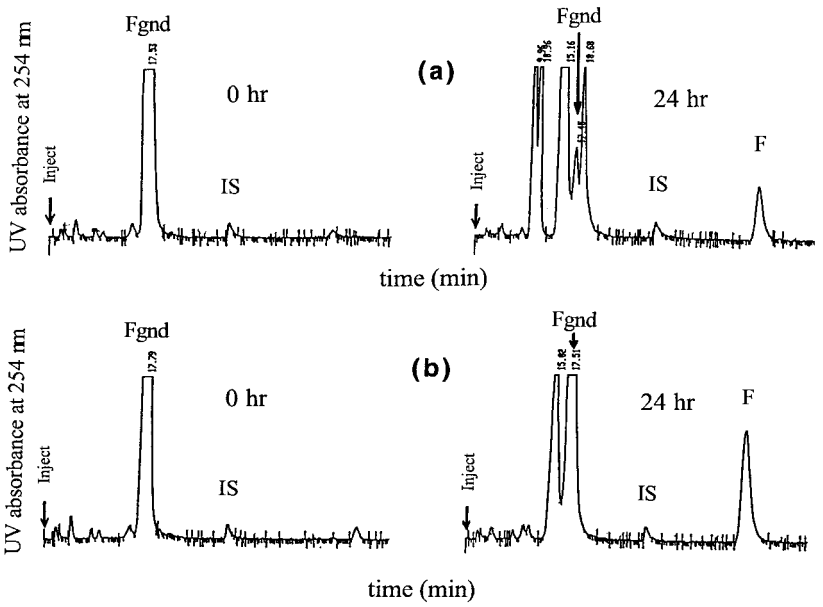
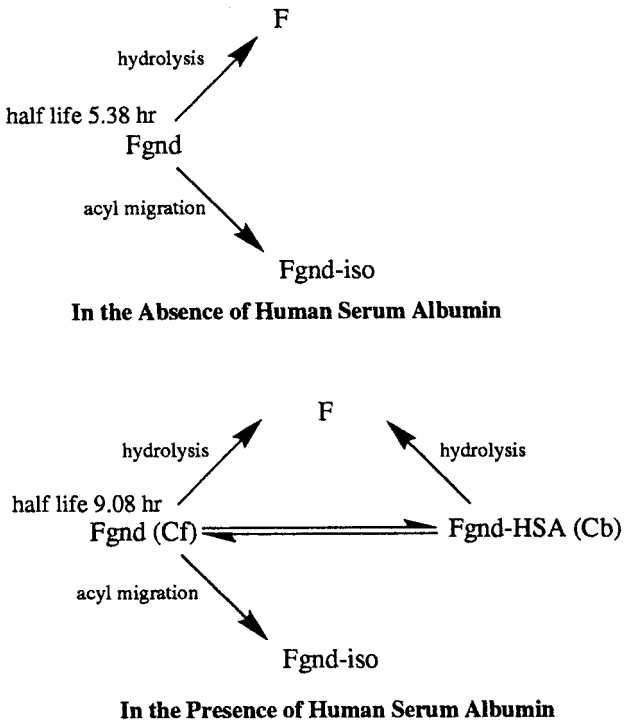



Figure 2. HPLC chromatograms of furosemide glucuronide in pH 7.4 buffer 0 or 24 h after incubation at 37°C in the absence (a) or presence (b) of HSA. Furosemide glucuronide, 175 μM; HSA, 500 μM. Data represent mean ± S.E. ($n = 3$). Fgnd, furosemide glucuronide; IS, internal standard; F, furosemide

### *Effect of HSA on Acyl Migration and Hydrolysis of Fgnd*

Figure 2(a) and (b) show the HPLC chromatograms of Fgnd, which were incubated in pH 7.4 buffer in the absence or presence of HSA, respectively, for 24 h at 37°C. Seven peaks of Fgnd acyl migration products and one peak of F were observed in the HPLC chromatogram of the Fgnd solution after incubation for 24 h in the absence of HSA, as observed in the authors' previous study [12]. On the other hand, only one peak of an acyl migration product other than that of Fgnd was observed in the HPLC chromatogram of the Fgnd solution after incubation for 24 h in the presence of HSA (Figure 2(b)). These results indicate that the formation rate of acyl migration product of Fgnd decreased in the presence of HSA. Since approximately 90% of Fgnd in HSA solution was bound to HSA, HSA reversible binding may prevent acyl migration of Fgnd. Furthermore, since acyl migration is a prerequisite for irreversible binding to protein [15], this reversible binding may prevent irreversible binding to endogenous compounds such as protein.

On the other hand, the peak of F formed by hydrolysis of Fgnd in the presence of HSA was greater than that in its absence (Figure 2(a) and (b)). This indicates that the rate of hydrolysis of Fgnd to F increased in the presence of HSA (Figures 2(b) and 3). The hydrolysis of oxaprozin acyl glucuronide to oxaprozin by HSA had also been reported [25]. Kurono *et al*. [13,14,26] reported that HSA has several kinds of esterase-like activities. Therefore, hydrolysis of Fgnd to F may be due to the esterase-like activity of HSA.




Scheme 1. Proposed pathway of furosemide glucuronide degradation in the absence and presence of HSA

The half-life of Fgnd in pH 7.4 buffer in the presence of HSA was longer than in its absence ($9.08 \pm 0.20$ h ($n = 3$) *versus* $5.38 \pm 0.28$ h ($n = 3$), mean $\pm$ S.E.) (Scheme 1). The half-life of Fgnd in pH 7.4 buffer (5.38 h) is in good accordance with the value (5.29 h) reported by Rachmel *et al*. [11]. This indicates that the larger contribution of acyl migration to Fgnd degradation than hydrolysis results in longer half-life in the presence of HSA. Watt and

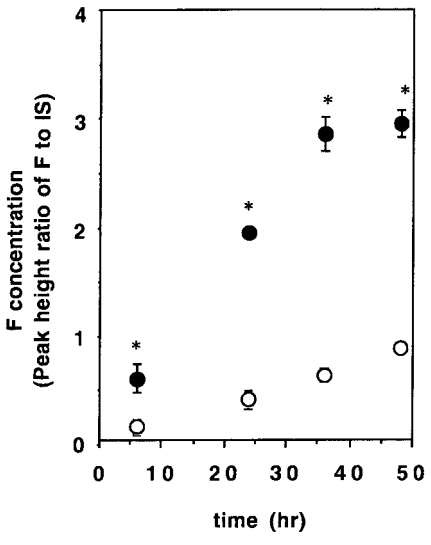



Figure 3. Time course of hydrolysis of furosemide glucuronide to furosemide in the absence (○) and presence (●) of HSA in pH 7.4 buffer at 37°C. Conditions were the same as in Figure 2. An asterisk represents a significant value compared with the value in the absence of HSA (Student's *t*-test, $p < 0.05$)

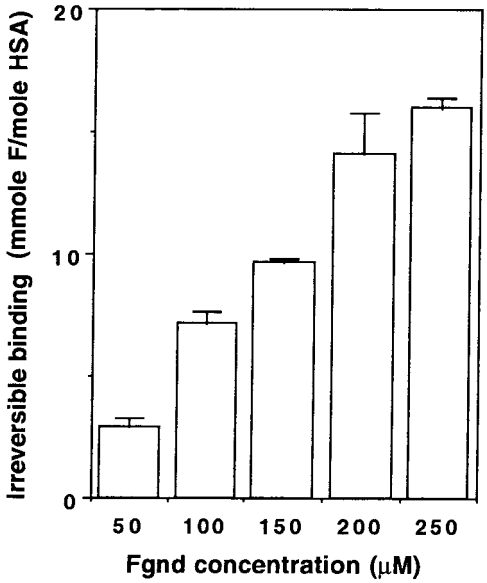



Figure 4. Irreversible binding of furosemide glucuronide to HSA. Conditions: HSA, 50 μM; furosemide glucuronide concentrations, 50–250 μM; incubation time, 24 h. Data represent mean $\pm$ S.E. ($n = 3$)

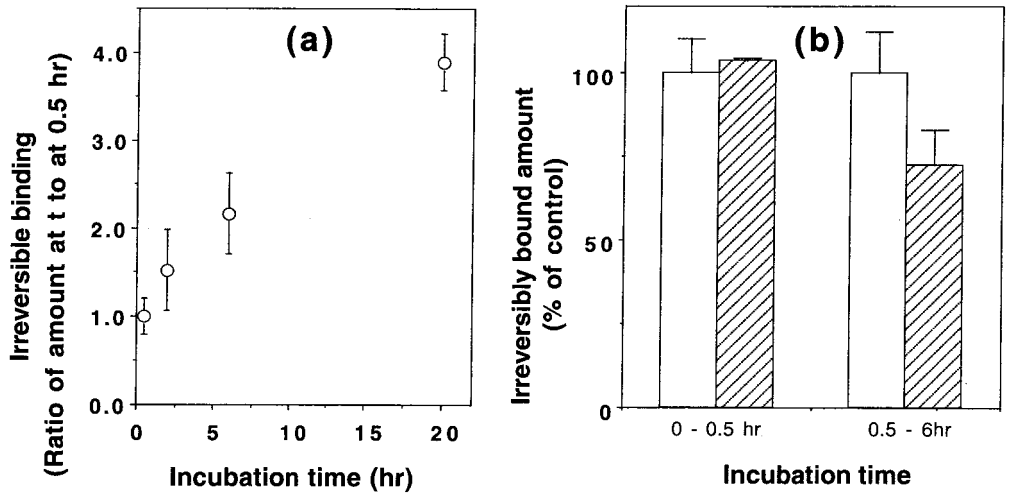



Figure 5. Time dependency of irreversible binding of furosemide glucuronide and inhibitory effect of furosemide on it. (a) Time course of irreversible binding of furosemide glucuronide to HSA. Concentrations: HSA, 50 μM; furosemide glucuronide, 100 μM. Data represent mean ± S.E. ($n = 3$). (b) Effect of furosemide on the irreversible binding of furosemide glucuronide. Control, open column; 100 μM furosemide, slushed column. Concentrations: HSA, 100 μM; furosemide glucuronide, 100 μM. Data represent mean ± S.E. ($n = 3$)

Dickinson [27] also reported that degradation of diflunisal glucuronide in HSA solutions (95 min half-life) was retarded compared with in buffer alone (35 min half-life). Therefore, these strongly suggest that the HSA reversible binding plays a role in stabilizing Fgnd.

### *Irreversible Binding of Fgnd to HSA*

Irreversible binding of Fgnd (50–250 μM) to HSA (50 μM) was observed after incubation of the mixture for 24 h. The irreversible binding was dependent on the Fgnd concentration (Figure 4). Figure 5(a) shows the time course of irreversible binding of Fgnd to HSA. The irreversible binding time profile indicates a biphasic reaction, which consists of a rapid reaction within 30 min and a slow reaction after 30 min. Contribution of the slow reaction after 30 min to total irreversible binding was major compared with the rapid reaction. Van Breemen and Fenselau [17] also reported irreversible binding of flufenamic 1-*O*-acyl glucuronide, which consisted of rapid binding within 30 min and then slow binding. The amount of Fgnd irreversibly bound to HSA at 30 min of incubation was the same in the presence and absence of F (Figure 5(b)). However, the amount of Fgnd irreversibly bound to HSA after 30 min–6 h in the presence of F was lower than in the absence of F, though not significantly (Figure 5(b)). This suggests that the slow irreversible binding site of Fgnd is also the reversible binding site. The small extent of the decrease in the irreversible binding of Fgnd is reflected by the reversible binding percent of Fgnd (56% in the absence of F *versus* 50% in the presence of F under this condition). Therefore, these results suggest the irreversible binding of Fgnd to HSA may occur in part via reversible binding. Ruelius [25] also reported the importance of reversible binding as the first step in the irreversible binding of oxaprozin acyl glucuronide to HSA.

This study indicated the following: (1) HSA reversibly binds reactive Fgnd to decrease free concentration of reactive Fgnd and to suppress acyl migration, and this is the initial step in the slow irreversible binding reaction. (2) HSA hydrolyzes reactive Fgnd to decrease reactive Fgnd concentrations. (3) HSA irreversibly binds reactive Fgnd to decrease concentrations of reactive Fgnd, possibly indicating its role as a scavenger of reactive Fgnd in blood to prevent irreversible binding of Fgnd to other proteins.

In summary, Fgnd reversibly binds to the warfarin binding site on HSA. Fgnd irreversibly binds to HSA by two kinds of reactions. Time-dependent major irreversible binding of Fgnd occurs via reversible binding. Furthermore, HSA suppresses acyl migration of Fgnd, but enhances the hydrolysis of Fgnd. HSA decreases the free concentration of reactive Fgnd, preventing irreversible binding of Fgnd to other proteins.

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