EFFECT OF HEPARIN INJECTION ON PLASMA PROTEIN BINDING OF 1-ANILINO-8-NAPHTHALENESULFONATE AND SALICYLATE IN RATS

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After intravenous injection of heparin, the plasma protein binding of 1-anilino-8-naphthalenesulfonate (ANS) was remarkably decreased in rats. This effect occurred within one min after the injection of 1000 units/kg of heparin and lasted for about 30 min. The change in the binding of ANS was closely related to the plasma concentration of free acids (FFA), which was suggested as one of the heparin-induced inhibitors. The free fatty fraction of salicylate in plasma after the intravenous injection of heparin, had a pronounced variation, and also had a statistically significant correlation with the plasma free fraction of ANS. It was suggested that ANS might be useful for the prediction of the heparin-induced changes in the plasma protein binding of acidic drugs.

Keywords—heparin; salicylate; free fatty acids; 1-anilino-8-naphthalenesulfonate; fluorescence probe; plasma protein binding

Heparin is widely used as an injectable anticoagulant which is administered for the prophylaxis and the treatment of venous thrombembolism. It is also used as a concomitant of the blood exchange transfusions and to facilitate hemodialysis. Previous reports indicated that the systemic administration of heparin caused a decrease of the plasma or serum protein binding of the neutral drugs; digoxin and digitoxin,1 the basic drug; propranolol2 and the acidic drug; salicylate.3, On the other hand, the plasma protein binding of warfarin has been reported to be increased greatly by the injection of heparin.4 It is well known that compounds affecting plasma protein binding may cause significant alterations in drug disposition and pharmacological response. This is especially important with highly bound drugs where small changes in percent of bound drug cause large changes in the free fraction. Previous investigators reported that the effect of heparin injection on plasma protein binding of drugs revealed a good correlation between the change in the protein binding and the concentration of free fatty acid (FFA).1,2 It has been suggested that the effect of heparin was caused by the displacement of drug from the plasma protein binding sites by FFA, of which concentration in plasma was elevated by the increase of lipoprotein lipase activity caused by the administration of heparin.1,2 In the previous report from this laboratory,3 the authors have demonstrated that the plasma protein binding of an anionic fluorescence probe, 1-anilino-8-naphthalenesulfonate (ANS) in rat plasma was greatly influenced by the injection of heparin and suggested that FFA was one of the endogenous inhibitors in plasma protein binding. Also a highly significant correlation was observed between the free fractions of both ANS and phenylbutazone (r = 0.896, p < 0.001). The purpose of the present study was to determine the magnitude and the time course of the effect of heparin on plasma protein binding of ANS.

Adult male wister rats (Nhion Seibutsu Zaityo, Tokyo, Japan), weighing 300—600 g, were used. Under light ether anesthesia, the femoral vein and artery were cannulated with PE-10 and PE-50 polyethylene tubings, respectively. The rats were
given 1000 units/kg of heparin (Japan Upjohn Ltd., Tokyo, Japan) through the femoral vein cannula; blood samples (0.25 ml) were then obtained at 1, 5, 10, 30 and 60 min, in polyethylene centrifuge tubes (Beckman Instruments, Fullerton, CA). Control animals were given a corresponding volume of physiological saline instead of heparin. Plasma was separated by centrifugation for 20 sec in a table-top microfuge (Beckman Instruments, Fullerton, CA). The binding of ANS to plasma was determined at room temperature (19–20°C) by observing the change in ANS fluorescence intensity. The details of the fluorescence method were described previously.\textsuperscript{7,8)} The determination of FFA was carried out using a commercial kits (FFA: NFFA Test, Wako Pure Chemical Industries Ltd., Tokyo, Japan). The extent of plasma protein binding of ANS, expressed as the free fraction, was determined in 6 rats (including 3 rats as the control). For the plasma binding of salicylate, blood samples were collected from the carotid artery from ten seconds to eight minutes after the injection of 1000 units/kg of heparin in 28 rats and the plasma was separated as described above. Two ml of fresh plasma was diluted with 6 ml of 50 mM Tris-HCl buffer (pH 7.4). The bind-

![Graphs showing the effect of heparin on plasma binding of ANS and FFA](image)

**FIG. 1.** Time Courses of the Effect of Heparin on the Plasma Free Fraction of ANS (● --- ●) and the Plasma Concentration of Free Fatty Acids (FFA) (○ --- ○) in each individual rats. Heparin was injected intravenously at the dose of 1000 units/kg. Panels a, b and c: heparin treated rats; panel d: control rats. See text for details.
ing of salicylate to the diluted plasma was determined by using equilibrium dialysis. The dialysis cell, which has two chambers, was divided with visking membrane (type: 18/32, Visking Co., Chicago, IL). One ml of the diluted plasma was put in one chamber, and 1 ml of 50 mM Tris-HCl buffer containing 0.1 mM salicylate was put in another. After the chambers were gently shaken for 16—18 hr at 25°, the concentration of unbound drug in the buffer solution was determined. The concentration of bound drug was calculated by subtracting that of unbound drug. Under these experimental conditions, the binding of any drug to the Visking membrane was negligible. The free fraction (f) was calculated using the following equation (1).

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f = \frac{C_f}{C_f + C_b}
\]

where \(C_f\) and \(C_b\) are the concentrations of unbound and bound drugs, respectively. The drugs in the buffer solution were determined spectrofluorometrically using a Hitachi MPF-4 fluorospectrometer. After addition of 0.5 ml of 1N HCl to 0.5 ml of the sample, the mixture was extracted with 6 ml of dichlorehthane and then 4 ml of the organic layer was reextracted with 4 ml of 4% Na₂CO₃. The fluorescence of the Na₂CO₃ layer was measured at 410 nm (excited at 300 nm) with a 390 nm cut-off filter in the emission side.

Heparin, 1000 units/kg, caused a remarkable increase of both the free fraction of ANS and the plasma concentration of FFA for 30 and 10 min, respectively, after the injection. At 60 min, both the free fraction of ANS and the plasma concentration of FFA decreased nearly to the normal levels (Fig. la, b and c). The change in the binding of ANS seemed to be closely related to the plasma concentration of FFA. The maximum effect of heparin was observed at one min after the injection, e.g., at the first sampling. In the control animals, the plasma free fraction of ANS and the plasma concentration of FFA, were not altered by saline injection (Fig. 1d). Recently, Bjornsson and Levy reported that the biological half-life of 500 units/kg of heparin in rats, was 69 min.9 Wiegand and Levy reported, however, that the duration of the effect after the same dose of heparin on the plasma protein binding of bilirubin, seemed to be somewhat less than 69 min. In this study, the biological half-lives of the free fraction of ANS and the plasma concentration of FFA, were relatively short (within 10 min) (Fig. la, b and c). These results were in good agreement with those obtained for bilirubin by Wiegand and Levy.3

Based on these findings, it was suggested that there might be a limited amount of precurser of endogenous displacing agent or limited depot of the releasable endogenous displacing agent which is accessible or responsible for the injected heparin, and that the displacing agent itself might

![FIG. 2. Relationship between the Free Fractions of ANS and Salicylate in Plasma of Individual Rats](image-url)
have a very short half-life in plasma. The drug displacement from plasma protein binding sites caused by the heparin injection, has an important pharmacokinetic and toxicological implications as pointed out previously. Heparin, 1000 units/kg i.v. also caused a pronounced variation of the free fraction of salicylate (from 0.38 to 1.0) (Fig. 2), and a highly statistically significant positive correlation between the free fraction of ANS and the free fraction of salicylate \( r = 0.701, p < 0.001 \), was revealed.

In the present study, the authors adopted a fluorescence probe, ANS as a model acidic compound which bound extensively to the plasma protein. It was suggested that ANS might be useful for the prediction of the heparin-induced alterations in the plasma protein binding of many acidic drugs.

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REFERENCES