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In Vitro Uptake of Salicylate by Human Red Blood Cells

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Abstract □ Distribution and binding properties of sodium salicylate to human red blood cells were studied under various experimental conditions. The effect of tonicity and hemolysis on the steady state level of the drug within the human red blood cells were accounted for in this study. When the washed cells were suspended in normal saline solution, the drug was so rapidly permeated into red cells. Since the pH of the system forces nearly complete ionization of the drug, ionic diffusion through aqueous pores is thought to be the mode of salicylate transport. Human red cell binding capacity and association constant for salicylate were estimated. This work supports the view that the red cells act as an important reservior of salicylate.

Keyphrases□Salicylate-distribution to human red cells; Salicylate-binding to human red cells; Salicylate-fluorescence measurement; Binding-salicylate; Distribution-salicylate; Hemolysis effect to the binding of sodium salicylate; Crenated human cells-salicylate binding.

The red blood cell compartment of the blood is often dismissed as an insignificant consideration in pharmacokinetics.[1,2] Blood and plasma salicylate levels are often merely assumed to be equivalent terms.[1] The possible influence of red blood cells and other cellular components on salicylate binding in blood has been largely neglected. Only recently has binding for salicylate in blood other than plasma received scant attention.[3,4,5,6] The concentration of salicylate within red cells is in excess of the free drug concentration in the plasma over the certain concentration range[3]. The nature of the interaction and accumulation is unknown and probably varies, and the significance of these interactions in the overall pharmacokinetics of the drugs has not been determined. The extent and strength of binding of salicylate in red cells have not been determined. *In vivo*, in the blood circulation, a single event such as red cell uptake is difficult to isolate from the multiplicity of kinetic events occuring. Therefore, to determine the distribution characteristics of salicylate into red cells, and the strength and capacity of salicylate binding, salicylate uptake by human red cell was studied under various experimental conditions *in vitro* in a closed system.

EXPERIMENTAL

Materials

Sodium salicylate (J.T.Baker), potassium bisulfate (J.T.Baker), and spectro grade chloroform(Matheson Coleman & Bell) were obtained from commercial sources. Human red blood cells(HRBC) were obtained from blood bank

stored for 3 weeks at 4°C in acid-citrate-dextrose solution. The whole blood was centrifuged at 1200g for 10 minutes. Plasma and buffy coat were removed. The packed HRBC were washed three times with three volumes of 0.9% normal saline solution. The packed HRBC were collected following a final centrifugation at 1200g for 10 minutes and removal of saline solution.

Uptake of Salicylate by HRBC

The distribution of sodium salicylate into HRBC was determined in plasma, 0.9%, 2.4% saline solution, 4.2% human serum albumin (HSA), and isotonic phosphate buffer with pH 7.4. Five milliliters of washed HRBC was resuspended in 5ml of each suspending medium containing the drug at the concentration of 10, 20, 40, 60, 80, 100, 1200, 400, 600, 800, 1000 μg/ml, respectively. The suspensions were then incubated for 15 minutes at room temperature. After incubation, the samples were immediately centrifuged at 1200g for 10 minutes. Percentage of hemolysis and the concentration of sodium salicylate in the supernatent were measured. The distribution ratio of salicylate between HRBC and suspending medium was calculated. To study the effect of hemolysis and alteration in the nature of the HRBC during the distribution and binding studies, 5 ml of washed HRBC was resuspended in 5 ml of 0.9% saline solution containing various concentrations of sodium salicylate. Each suspension were tumbled up-side down ten times and then incubated for two hours. This procedure was repeated twice. After incubation, the samples were centrifuged at 1200g for 10 minutes. Supernatant was separated and the degree of hemolysis occuring during incubation period was determined. The concentration of salicylate in supernatant was measured with blank.

Analytical Method

One ml of each samples was acidified with 1 ml of 10% potassium bisulfate solution in 15 ml glass-stoppered centrifuge tubes. Five ml of chloroform were added to each tubes and these were then agitated on a mechanical shaker (Thermolyne Sybron Co.) for two minutes and situated for minimum 30 minutes. Aqueous and buffy layer were pipetted off. The concentration of salicylate in chloroform war measured by spectrophotofluorometer (Perkin-Elmer model 204). The fluorescence of salicylate was determined by activating at a wave-

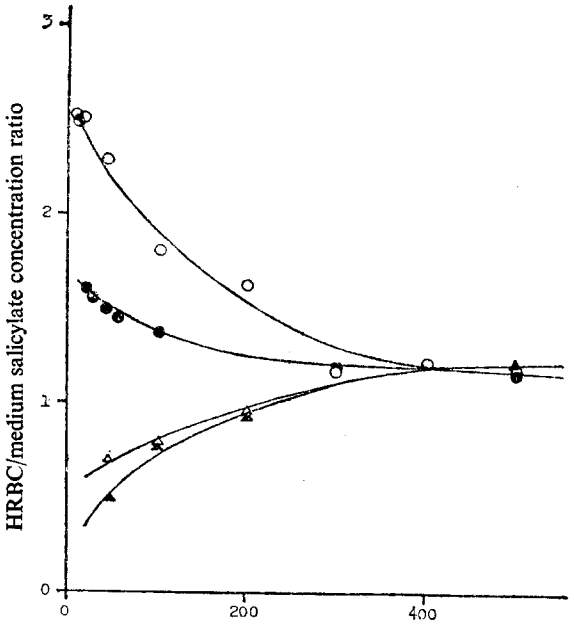

Fig. 1: Distribution of salicylate in HRBC suspending medium. ●=0.9% Saline Solution ○=2.4 % Saline Solution ▲=Plasma △=4.2% HSA Solution

length of 313nm and reading the emission at 445nm. The method used to determine the degree of hemolysis is based on the fact that the quantity of oxyhemoglobin exuded from the HRBC of a blood samples is a direct function of the proportion of HRBC hemolyzed. A photoelectric colorimeter (Klett MCG Co.) with a No. 54 green filter was adjusted to read zero absorbance with the tube containing the last portion of 0.9% saline solution after the packed HRBC were washed three times. Each absorbance reading was compared with a total hemolysis reading obtained by lyzed HRBC in distilled water. The degree of hemolysis occuring in each test solution was calculated as a percent of total hemolysis. Each of supernatant was read for its absorbance and present hemolysis was determined.

RESULTS AND DISCUSSION

After addition of sodium salicylate to the washed HRBC suspension in normal saline solution *in vitro,* salicylate was freely permeable throughthered cell membrane and rapidly equilibeated between the extra and intracellular fluid. Vesell[7] summarized the plasma levels of various drugs under different conditions. In the case of salicylate, 50~100 μg/ml were considered to be in the range required for analgesia, while levels greater than 250 μg/ml were required for the treatment of rheumatic fever. In the treatment of arthritis, plasma levels of 350~500 μg/ml were cited. These values were taken as general guideline in this study. Figure 1 shows that the distribution ratio of salicylate in HRBC to plasma or 4.2% HSA solution are increased initially in a nonlinear fashion with increasing blood salicylate concentration, and linear fashion beyond a blood concentration of 500 μg/ml. Concentration of salicylate within red cells obtained is in excess of the free drug concentration in the plasma over the concentration range reported[3]. The distribution of salicylate in HRBC suspension was studied to determine the binding parameters. It is found that the ratio of the red blood cell to saline medium salicylate concentration is decreased initially in a nonlinear fashion with increasing medium salicylate concentration up to 250 μg/ml as shown in Figure 1. It was reported that red blood cell uptake is a sum of two processes[3,4]: one is the physical dissolution of drug in intracellular fluid and the other

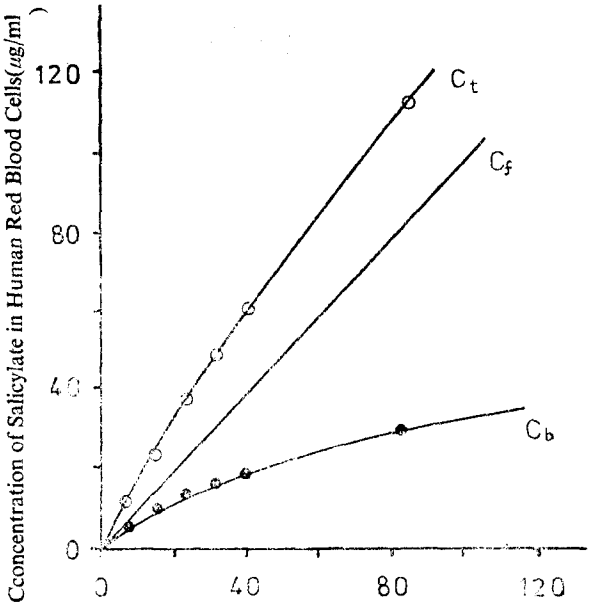

Fig. 2: Plot of total unbound and bound concentration of salicylate as a function of unbound concentration in HRBC

is a non-linear saturable drug binding process. By preparing human red cell suspension standards over a concentration range of 10–500 μg/ml, centrifuging to separate medium, and measuring total, total red cells and medium salicylate concentrations, it would be able to compare the proportions of the salicylate within HRBC which are either bound to cell consti tuents or free in intracellular fluid as shown on Figure 2. It was found that as salicylate concentration is increased the cell components binding sites become saturated and then progressively more salicylate is present in unbound form in the intracellular fluid. McArther *et al.*[4] found that bound drug by red cells in normal saline solution was linearly increased up to 3,000 μg/ml. Our findings sho wn in Figure 1 and 2 indicate that the ratio of bound to unbound salicylate concentration is widely variable over the therapeutic salicylate concentration range, and that the ratio to be constant in conjunction with the salicylate concentration beyond a blood concentration of 500/μgm*l*. The extent of binding

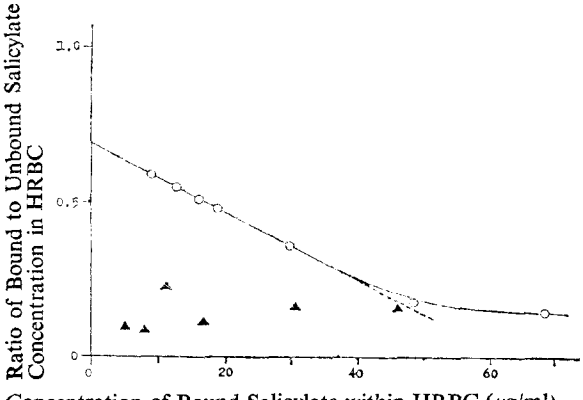

Fig. 3: Rosenthal plot of the bound salicylate to HRBC in 0.9% saline solution. ○=intact HRBC; ▲=fragile HRBC.

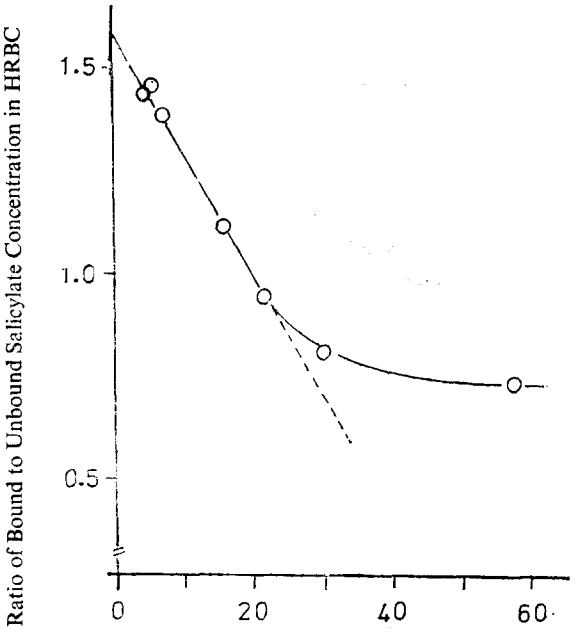

Fig. 4: Rosenthal plot of the binding of salicylate to HRBC in 2.4% saline solution

in red cells can be calculated on the basis of the following equations.

Overall concentration of salicylate in HRBC (C_t)=Concentration of free salicylate in HRBC(C_f)+Concentration of bound salicylate in HRBC(C_b) .....(1)

At steady state-equilibrium the diffusible free salicylate concentration(C_f) in the red cells is assumed to be equal to the concentration of free salicylate in extracellular fluid(C_{ef}).

$$C_f = C_{ef} \quad \text{.....(2)}$$

The concentration of salicylate bound by red cells at equilibrium steady-state can be obtained from EQ(1) and EQ(2)

$$C_b = C_t - C_{ef} \quad \text{.....(3)}$$

Table I: Binding of Salicylate to Human Red Blood Cell at Room Temperature

No. of Experiment	Distribution System	Ka(ml/μg)	nM(μg/ml)	ϕ*	% Hemolysis
1	HRBC in 0.9% Saline Sol'n	1×10^{-2}	68	0.5	8.6×10^{-3}
2	HRBC in pH 7.4 Isotonic Phosphate Buffer	0.85×10^{-2}	86.95	0.5	3.6×10^{-2}
3	HRBC in 0.9% Saline Sol'n	1.2×10^{-2}	58	0.5	6×10^{-3}
4	HRBC in 0.9% Saline Sol'n	1.68×10^{-3}	92.35	0.5	2.5~3.5
5	HRBC in 2.4% Saline Sol'n	3×10^{-2}	53	0.35	

* ϕ is the hematocrite

The concentration of salicylate bound by red cells obtained from EQ(3) was linearized as follows[8].

$$\frac{C_b}{C_{ef}} = nMK_a - K_aC_b \quad \cdots\cdots\cdots\cdots (4)$$

This equation can be applied by plotting C_b/C_{ef} as a function of C_b as shown in Figure 3 and 4. Thus two coordinates are independent of the concentration of macromolecule. EQ(4) is a straight line with a slope of $-K_a$, and the product(nM) of the number of binding sites(n) and the concentration of macromolecule(M) can be obtained. Binding data were linearized according to EQ(4), and the binding parameters were calculated by least squares linear regression analysis, which is presented in Table I. The concentration of macromolecules is unknown and therefore only product(nM) is a useful parameter, since it represent the maximum binding capacity of red cells to salicylate, which have a range 50~90 μg/ml. Blood samples varied slightly in their maximum binding capacity and association constant for salicylate. Zaroslinski *et al.*[9] reported that salicylate binding to protein was not affected by electrostatic repulsive forces. Thus possible explanation for these result could be the presence of some agent competing with binding sites of red cell components for salicylate. One source of a potential competitor could be a material released from the red cells upon hemolysis. The ratio of bound salicylate to unbound salicylate in fragile human red cells was plotted in order to compare the experimental data for intact red cells. Figure 3 clearly illustrated that variation of binding data could be attributed to an alteration in the nature of the human red cell during the distribution and binding studies. Hemolysis may result in marked shifts in the evaluation of binding parameters. For this reason extracellular fluid samples employed in drug binding studies should be obtained from nonhemolyzed blood specimens.

The present results show that salicylate is freely permeable and that it binds to HRBC. The amount of free and bound salicylate in HRBC varies according to the concentration of drug. At lower therapeutic levels, the binding sites in HRBC would be involved in binding salicylate. At higher levels, binding sites would approach saturation. The determination of salicylate binding to separated human plasma would yield misleading information about the ratio of bound to un-

bound salicylate in the circulation. The fraction of unbound salicylate is available at any one time interval to reach the target sites, and initiate drug actions. The determination of this fraction is of obvious importance in relating *in vitro* effects to *in vivo* actions in the assessment of the efficiency of a drug in different formulations and dosage forms. The interaction of salicylate with HRBC should be determined in addition to the plasma-salicylate interaction for the pharmacokinetic studies. The present work supports the view that HRBC act as an important reservior of salicylate.

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