The discriminatory effect of different solvents against different bacterial strains suggests the presence of different chemical compounds. The extracts of plants given as a whole might reduce the effect since each plant part has (perhaps) different compounds and their extractability in different solvent varies. Therefore it is necessary to screen the most active compound i.e. the best solvent for a particular compound and then it can be selected for further investigations to determine its therapeutic potential and may be for the synthesis of analogues with improved activity. The results of the present study suggest that the most effective is the compound extracted in 1,4-dioxan. The beneficial action of *P. granatum* might be due to a combination of phytochemicals acting collectively or synergistically.

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Physico-Chemical Aspects of Protein Binding of Nimesulide

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The binding of nimesulide, a cox-2 inhibitor, to bovine serum albumin was investigated by equilibrium dialysis method at different temperatures and pH conditions. The Scatchard plots were prepared based on these drug-protein binding data. The number of binding sites (n), the value of association constant (K) at different conditions and different thermodynamic parameters (i.e., standard free energy change $\triangle G^{\circ}$, standard enthalpy change $\triangle H^{\circ}$ and standard entropy change $\triangle S^{\circ}$) of nimesulide-BSA binding were determined. The result shows that number of binding sites

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is around 2.5 and the value of association constant is decreasing with increasing temperature and pH. It also reveals that the value of $\triangle G^0$ and $\triangle H^0$ were highly negative and $\triangle S^0$ was also negative. The result indicates that the interaction between nimesulide and bovine serum albumin is exothermic and spontaneous in nature. It is postulated that nimesulide–bovine serum albumin interaction may occur due to hydrogen bond formation and ionic interaction.

The binding of a drug to a plasma protein reduces its free fraction in serum, thus reducing its availability for active uptake or diffusion into surrounding tissue and significantly influencing its pharmacokinetics and toxicity[1]. The extent of plasma protein binding is critical to predicting a drug's pharmacokinetic and pharmacodynamic profile. When a small molecule binds to plasma protein, the interaction is typically the result of hydrogen bond formation, van der Waal's[2] attractive force or strong ionic[3] and hydrophobic interactions[4]. Studies have shown that nimesulide is extensively bound to plasma protein[5], but literature regarding a thorough study of physicochemical aspects of protein binding of nimesulide is very meager. The aim of our study was to find out the influence of temperature and pH of the environment on the nimesulide and BSA interaction and also to elucidate a probable mechanism of binding between nimesulide and bovine serum albumin (BSA) through different thermodynamical parameters[6,7]. The present study involves the determination of *in-vitro* binding of the drug, nimesulide, to bovine serum albumin as a function of drug concentration at different temperatures and pH conditions by equilibrium dialysis[8] method.

Nimesulide was obtained from Bengal Chemicals and Pharmaceutical Works Ltd., Kolkata, as a generous gift. BSA was obtained from Merck (I) and dialysis tubing was obtained from Sigma chemical company, St. Louis, USA. BSA solutions were prepared based on average molecular weight of 6 5000. All other chemicals and reagents used were of analytical grade.

Equilibrium dialysis[9,10] experiments were performed under different temperature conditions (20, 30 and 40°) at pH 7.4 and ionic strength 0.1 using dialysis tubing. Various drug concentrations were prepared in the range from 37.7 μM to 125.7 μM. Dialysis sac containing 2 ml of the drug solution in 5 ml of 0.4%w/v BSA solution (61.5 μM) placed inside other compartment which contained 30 ml phosphate buffer solution of specific pH and ionic strength (sodium chloride was used maintain the ionic strength). The incubation was done for 24 h with continuous stirring at very slow speed (10 rpm). Simultaneously a control was run without BSA solution to minimize error due to membrane absorption of nimesulide. The same method was repeated for different pH conditions (pH 7.4, 8.4 and 9.4) using phosphate buffer but at 20° and at ionic strength 0.1. We determined the bound drug concentration indirectly by measuring the concentration of unbound fraction of the drug in the outside sac solution spectrophotometrically at 397 nm using a Thermospectronic UV-1 model (Spectronic, UK) UV/Vis spectrophotometer. Protein binding was expressed in percentage as calculated using the formula, %Bound=100-[(concentration of free drug in outside solution,concentration of free drug in control solution)x100]

The most widely used way to treat the binding data is with Scatchard Plot[11] as per following equation, $r=\frac{nK[D_f]}{1+K[D_f]}$ *Eqn.*1, where r is the molar ration of drug bound (D_b) to portein (P_t), D_f is the molar concentration of free drug, n is the maximum number of binding sites on a particular class of protein site and K is association constant. If independent binding sites with different affinities were involved in drug binding then the number of found drugs to one protein molecule can be expressed as follows: $r=\sum_{i=1}^{m}\frac{n_i K_i[D_f]}{1+K_i[D_f]}$ *Eqn.* 2., where m is the number of binding site classes in an albumin molecule.

We computed the equilibrium dialysis study data for the binding of nimesulide to BSA at different temperatures and analyzed those using graph pad prism 3-version software to perform a nonlinear regression. A Scatchard plot was obtained as shown in fig.1.We observed a sharp bend near the abscissa that indicates existence of more than one type of binding site in the BSA molecule for nimesulide. The prism analyzed data depicted two K_d (dissociation constant) values but the second type of binding site is not considered for further studies because the binding affinity of the second type is significantly smaller than that of first type. To obtain a best-fit straight line and the equation, the linear regression analysis for first type of binding sites at different temperature conditions were performed by Windows MSExcel were as follows: At 20°, r/Df=17.032-7.0553r (correlationcoefficient=0.9941)...Eqn. 3, at 30°, r/D_f=12.774-4.8043r (correlation coefficient=0.9980)...Eqn. 4, and at 40°,

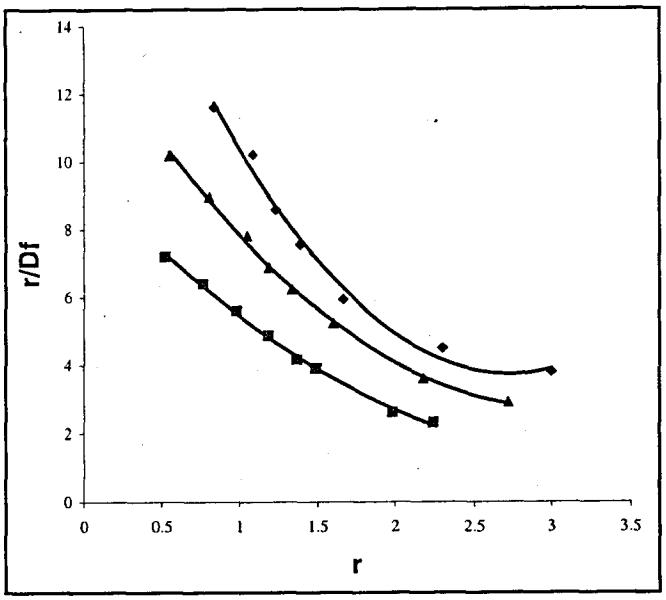

Fig. 1: Non linear Scatchard plot of nimesulide binding to BSA.

A non linear Scatchard plot showing the binding of nimesulide to BSA at 20° (-♦-), 30° (-▲-) and 40°(-□-) at constant pH 7.4 and ionic strength 0.1. r is the molar ratio of drug bound to total protein, D_f is the molar concentration of free drug

4.8043r (correlation coefficient=0.9980)...Eqn. 4, and at 40°, r/D_f=9.0125-3.4753r (correlation coefficient=0.9991)...Eqn. 5. The values of n for the first type of binding site (2.5 at 20°, 2.65 at 30° and 2.59 at 40°) were rounded off to their nearest integer 3. K value obtained from the slope were 70 553, 48 043 and 34 753 mol^{-1} at 20°, 30° and 40°, respectively.

We calculated the thermodynamic data such as the standard free energy of drug-protein binding and the standard enthalpy change using the following equations; ΔG^0= -2.303 RT logK...Eqn. 6. The standard enthalpy change ΔH^0 can also be obtained from the following relationship, Log K=-ΔH^0/2.303RT+constant...Eqn. 7. The constant term in Eqn. 7 is actually equals to ΔS^0/2.303RT, where ΔS^0 represents the standard entropy change.

The value of ΔG^0 at different temperatures were determined and recorded in Table 1. A best-fit linear plot of logK vs. 1/T (T= temperature in °+273) was made (not shown in the figure) and the regression equation obtained as follows, LogK=1435.31/T-0.0435,correlation coefficient=0.9976) ... Eqn. 8. The value of ΔH^0 and ΔS^0 were obtained from the slope and intercept of the above equation respectively. The value of ΔH^0 and ΔS^0 were obtained – 6.5680 Kcal and – 0.199 x 10^{-3} kcl/°k, respectively.

The decrease in association constant of BSA-nimesulide interaction with increasing temperature clearly explains that it is a temperature dependant phenomenon and also is an exothermic reaction and the negative sign for ΔG^0 indicates that the binding process is spontaneous. The nature of drug-protein interaction could be predicted from the thermodynamic parameters for the binding[12]. For electrostatic interactions $\Delta H^0$is very small, nearly zero[13-14] and thus electrostatic interactions in this case are not present. The high negative ΔH^0 value suggests that donor-acceptor interactions between nimesulide and BSA[15]. The negative enthalpy and entropy changes also dictates the possibility of drug binding due to ionic interactions and hydrogen bond formation[7]. The selectivity of the binding sites is explained by a large contribution of enthalpy to free energy. So finally it can be concluded that both ionic interaction and hydrogen bond interaction played major role in nimesulide BSA binding.

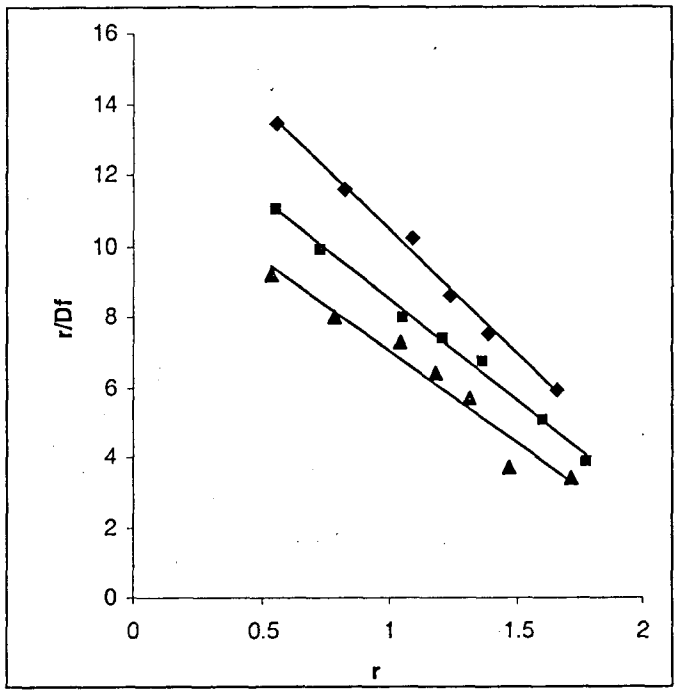

Fig. 2: Scatchard plot showing nimesulide binding with BSA at different pH

A Scatchard plot showing nimesulide binding with BSA at pH 7.4 (-♦-), pH 8.4 (-□-) and pH 9.4 (-▲-) but at 20° and ionic strength 0.1 . r is the molar ratio of drug bound to total protein, D_f is the molar concentration of free drug

TABLE 1: DIFFERENT PARAMETERS FOR NIMESULIDE-BSA BINDING AT DIFFERENT TEMPERATURES AND PH CONDITIONS

Temperature (°)	pH	K (in mol^{-1})	$\triangle G^0$ (in calories)	n
20°	7.4	69628	-6493.4	2.5
30°	7.4	48043	-6488.7	2.65
40°	7.4	34753	-6502.9	2.59
20°	8.4	56915	-6375.7	2.49
20°	9.4	52248	-6326.0	2.35

K= association constant; n=number of binding sites; $\triangle G^0$ standard free energy change

To examine the effects of charge on the drug-protein interaction[16], the affinity of nimesulide for the BSA at different pH was determined. The data obtained from nimesulide-BSA binding at constant temperature (20°) and ionic strength 0.1 (μ=0.1) and at different pH (pH 7.4, pH 8.4 and pH 9.4) were also analyzed and plotted as Scatchard plot (fig. 2) Here the nimesulide concentration was restricted at lower side to obtain the first type of binding site only. The regression equations obtained according to previous method were as follows, At pH 7.4, r/D_f=17.032-7.0553 r ...Eqn. 9, at pH 8.4, r/D_f =14.163-5.6915 r...Eqn. 10, at pH 9.4, r/D_f=12.2530-5.2248 r.....Eqn. 11. The value of number of binding sites (n) and intrinsic association constants were computed from the slope and intercept. The values of $\triangle G^0$ were calculated from equation number 6. All these values were recorded in Table 1.

From these studies it is evident that values of n were around 3, standard free energy change ($\triangle G^0$) were highly negative and the value of intrinsic association constants were decreasing with increasing pH. At all those pH conditions nimesulide and BSA will be ionized and fraction ionized will be increasing with increasing pH this suggests that more binding occurs when the drug is unionized. So the formation of hydrogen bond and ionic interaction between BSA and nimesulide is confirmed.

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