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ARTICLE

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ACTION OF HIGH DILUTIONS OF DRUGS ON BINDING SITES OF A PROTEIN AND DETERMINATION OF THEIR STANDARD DOSE

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High dilutions (HD) of two drugs, used in homeopathy, have been shown to interact with human serum albumin (HSA) at their binding sites. The objective is to determine the standard dose of HDs showing steady binding interaction. HDs were tested for binding interaction by isothermal titration calorimetry (ITC). The 4 μ1 dose showed uniformity in binding reaction then the 2 μ1 dose tested. So 4 μ1 dose is the standard one for ITC test with HSA.

Introduction

Ultra high dilutions (UHD) of drugs, used in homeopathy, have been found to be effective not only on humans but also on animals, plants, isolated tissue, cells and even in cell-free medium containing enzymes. UHDs are available in liquid form containing water and ethanol and also in sugar globules medicated with a liquid drug. In the medicated globules the crystal water undergoes a change induced by the liquid potency[1-4]. UHDs are usually applied into mouth where they come in contact with a circulating protein albumin. Serum albumin diffuses into oral mucosa. It is a single chain globular protein with its segments folding back on each other. This folding confers structural diversity which helps the protein perform various biological functions[5]. Albumin carries out many functions like transport and binding of endogenous and exogenous ligands[6]. Albumin transports fatty acids, steroids and drugs which are bound to the hydrophobic pockets of the protein. Albumin contains many functional amino and carboxyl groups which help in linking drug molecules to the protein.

Mainstream allopathic drugs are usually designed and developed by utilizing the process of protein-ligand binding. In fact all biological processes involve molecular recognition and binding interaction with high specificity and affinity between proteins and other molecules[7]. If we are to understand the mechanism of action of homeopathic potencies we have to look for binding reaction between a protein and a potentized homeopathic drug. Albumin is, therefore, an ideal candidate for studying binding interaction with a homeopathic potency.

One of the basic principles of pharmacology is dose response. The drug effect is linked to absorption, rise in concentration of drug molecules in plasma and clearance through liver and kidneys. The effect reaches a peak when drug concentration in plasma is maximum. Drugs always show a dose response. While too small a dose is ineffective, too large a dose is toxic. Depending on the chemical nature of the drug the time of clearance varies, but is usually a few hours. For this frequent repetition of drugs is necessary[8]. UHDs, which cross the Avogadro

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number, are devoid of original drug molecules. Do homeopathic potencies vis-à-vis UHDs show any dose response? The present study attempts to explore this phenomenon through protein-ligand binding experiments using the protein human serum albumin (HSA) and three UHDs of two drugs as ligands. We also tested their vehicles ethanol and water. Homeopathic potencies are prepared by serial dilution of a drug in several steps followed by mechanical agitation or succussion in each step. These agitated UHDs are called potencies and are denoted as 6 cH, 30 cH, 200 cH etc according to the number of steps through which they are prepared. The first step in ITC experiments is to establish a proper dose of a UHD so that we can make our results reproducible. In case of material doses of ligands 2 µl/ injection is a standard dose.

In the present study we like to see which of the two doses, 2 µl and 4 µl, would satisfy the basic condition of ITC experiments with UHDs.

Materials and Methods

In the present study we tested three potencies 6 cH, 30 cH and 200 cH of *Calcarea carb*, *Calcarea fluor*, vehicles ethanol and water. The binding interaction of all ligands (potencies) with HSA was tested by isothermal titration calorimetry (ITC 200 GE Health care Bioscience Ltd, Sweden). In this study two doses 2 µl and 4 µl of each ligand were tested on HSA (Sigma: A1887; CAS: 70024-90-7). All the potentized drugs purchased from the local market in Kolkata in sealed vials were products of Dr. Reckeweg, Germany. Ethanol content of all these drugs was 90%. Absolute ethanol (Merck, Germany) was mixed with deionized and distilled (DD) water to make it 90% blank ethanol. All the test potencies and blank 90% ethanol were mixed with DD water 1:1000 (v/v) in order to reduce ethanol content to a negligible amount of 0.09%. At this dilution ethanol does not produce any significant biological effect, but the potencies retain their specific effect 9. Although ethanol itself is used as a homeopathic drug 10, it is used here as an unsuccussed vehicle control. Besides blank ethanol unsuccussed DD water was used as a second control. Optical densities (OD) of all the EtOH containing test samples were measured by a UV-VIS spectrophotometer (Shimadzu, model UV 2401-PC) at 220.5 nm, and found to be same as 0.03.

Each test sample was injected at 2 µl or 4 µl/injection into a measurement cell containing 300 µl of 16 µM HSA solution in DD water every 2 min. Injections were started after thermal equilibrium at a constant temperature of 25 ^{0}C had been established. Ten injections were given for each sample, and binding reaction was measured by the ITC instrument in terms of release of heat (exothermic) or absorption of the same (endothermic). The reference cell containing water only and the sample cell were maintained at a constant temperature of 25 ^{0}C. The instrument was thoroughly rinsed with DD water after each experiment with a sample. ITC data were analysed for binding constant (K), change in enthalpy (ΔH), in entropy (ΔS) and Gibbs free energy (ΔG). ΔG was calculated by the equation $\Delta G = \Delta H - T\Delta S$ where T is the absolute temperature in Kelvin[11]. The ITC instrument contains a software origin[7] for analyzing the data.

Results

Results are presented in figures 1-8 and Table 1. Each figure has two panels A and B. While panel A shows heat change (µcal/sec) due to ligand injection versus time in minutes, B shows heat released per mole of ligand during interaction with HSA in relation to the molar ratio, ligand/ protein in the form of a non-linear regression. Since the ligands are virtually water, the molarity is arbitrarily taken as 100. Each peak in 'A' shows heat change due to an injection of a ligand (drug/control) into the sample cell containing the protein HSA. In case of panel 'B' the best fit parameters are recorded. The parameters include binding constant (K), change in enthalpy (ΔH), in entropy (ΔS),

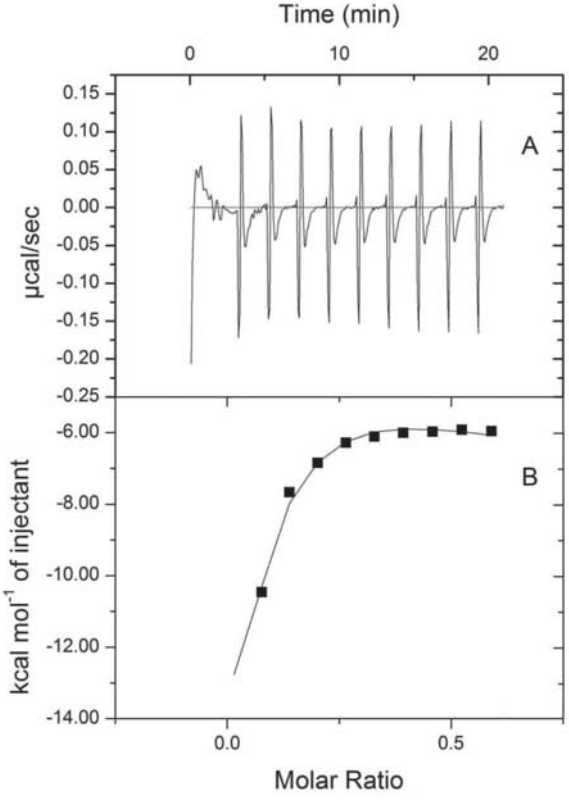

Fig 1x: HSA+ Water, 2µl/injection

Table 1: Thermodynamic parameters of interactions between human serum albumin (HSA) and ligands like Water, Alcohol, *Calcarea carb* and *Calcarea fluor* 6 cH, 30 cH, and 200 cH potencies. All potencies were in 0.09% ethanol. Each ligand was injected 10 times every 2 min at 2 and 4 µl/injection into 16 µm HSA at 25 0C in an isothermal titration calorimetry (ITC) instrument. Control was water.

Ligands injected 2, 4 µl/2min into 16 µM HSA	KM-1 (Binding constant) $\times 10^3$	ΔH cal/mol $\times 10^4$	ΔS Cal/mol /deg $\times 10^3$	ΔG Cal/mol	Binding sites, maximum heat change, Stoichiometry (N)
Water (2µl/injection)	K1:4.35×10 K2:2.40×102 K3:1.18×102	ΔH1:-3.273 ΔH2:5.059 ΔH3:-8.235	ΔS1:-0.0885 ΔS2:0.194 ΔS3:-0.253	ΔG1:-1.0605 ΔG2:0.209 ΔG3:-1.91	Sequential 3, 0.20µcal/s, exothermic, N= 3
Water (4µl/injection)	K:5.71	ΔH:-7.784×10	ΔS:-2.59	ΔG:-13.09	1, 1.4 µcal/s, exothermic, N=1
EtOH (2µl/injection)	K:3.12×102	ΔH:1.255	ΔS:0.0672	ΔG:-0.425	1, 0.45µcal/s, endothermic, N=1
EtOH (4µl/injection)	K1:0.0136	ΔH:-4.349×104	ΔS:-1.46×103	ΔG:-6990	1, 2.25µcal/s, exothermic N=1
Cal c 6cH (2µl/injection)	K1:4.64×10 K2:2.38×102	ΔH1:0.1922 ΔH2:2.507	ΔS1:0.0278 ΔS2:0.109	ΔG1:-0.5028 ΔG2:-0.225	2, 20µcal/s, endothermic, N=2
Cal c 6cH (4µl/injection)	K:0.00112	ΔH:-7.061×105	ΔS:-2.37×104	ΔG:-113,600	1, 10µcal/s, exothermic, N=1
Cal c 30cH (2µl/injection)	K:2.01	ΔH:-8.339×10	ΔS:-2.78	ΔG:-13.89	1, 0.85µcal/s, exothermic, N=1
Cal c 30cH (4µl/injection)	K: 0.00347	ΔH1:-2.69×105	ΔS:-9.02×103	ΔG:-43,600	1, 3.5µcal/s, exothermic, N=1
Cal c 200cH (2µl/injection)	K:8.41	ΔH:-2.246×10	ΔS:-0.735	ΔG:-4.085	1, 0.8µcal/s, exothermic, N=1
Cal c 200cH (4µl/injection)	K:1.55×103	ΔH:-1.171×10	ΔS:-0.364	ΔG:-2.61	1, 3µcal/s, exothermic, N=1
Cal f 6cH (2µl/injection)	K1:2.20×102 K2:3.46×103	ΔH1:3.007×102 ΔH2:-3.195×102	ΔS1:1.01×10 ΔS2:-1.07×10	ΔG1:48.2 ΔG2:-52	2, 20µcal/s, endothermic, N=2
Cal f 6cH (4µl/injection)	K:1.61×10	ΔH:-4.233×10	ΔS:-1.40	ΔG:-7.33	1, 2µcal/s, exothermic, N=1
Cal f 30cH (2µl/injection)	K:3.33×102	ΔH:-5.479	ΔS:-0.158	ΔG:-1.529	1, 1.5µcal/s, exothermic, N=1
Cal f 30cH (4µl/injection)	K:2.36	ΔH:-2.978×102	ΔS:-9.97	ΔG:-48.55	1, 3.5µcal/s, exothermic, N=1
Cal f 200cH (2µl/injection)	K:1.62	ΔH:-3.133×10	ΔS:-1.04	ΔG:-5.33	1, 0.3µcal/s, exothermic, N=1
Cal f 200cH (4µl/injection)	K:3.97×10	ΔH:-3.746×10	ΔS:-1.24	ΔG:-6.46	1, 5µcal/s, exothermic, N=1

free energy (ΔG) and number of binding sites for ligand-protein complex formation. Table1 shows all these parameters.

Fig 1 shows binding interaction between HSA and two doses of each ligand such as DD water at 2 µl (1x) and 4 µl/injection (1 y), 0.09% ethanol at 2 µl, 4 µl (Fig 2 xy), *Calcarea carb* 6 cH 2 µl, 4 µl (Fig 3 xy), *Calcarea carb* 30 cH 2 µl, 4 µl (Fig 4 xy), *Calcarea carb* 200 cH 2 µl, 4 µl (Fig 5 xy), *Calcarea fluor* 6 cH 2 µl, 4 µl (Fig 6 xy), *Calcarea fluor* 30 cH 2 µl, 4 µl (Fig 7 xy) and *Calcarea fluor* 200 cH 2 µl, 4 µl (Fig 8 xy). All the potentized drugs were in 0.09% ethanol. Details of binding parameters are given in Table 1. The common feature in all the samples is that the higher dose (4 µl/injection) shows uniformity in results like exothermic reaction, higher amount of heat change except with *Calcarea carb* 6, visible reduction of heat change with every injection, gradual saturation of binding sites, higher free energy change (ΔG) and single site binding (Fig 1-8, Table 1).

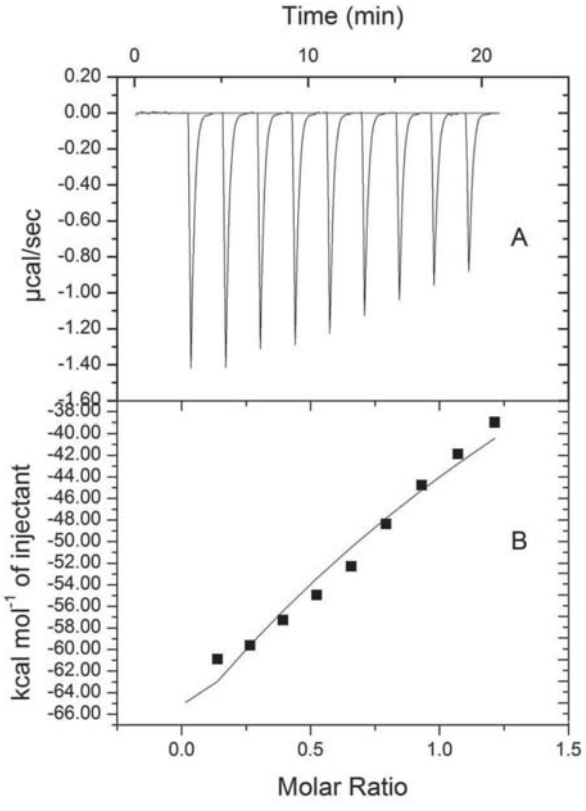

Fig 1y: HSA+ Water, 4µl/injection

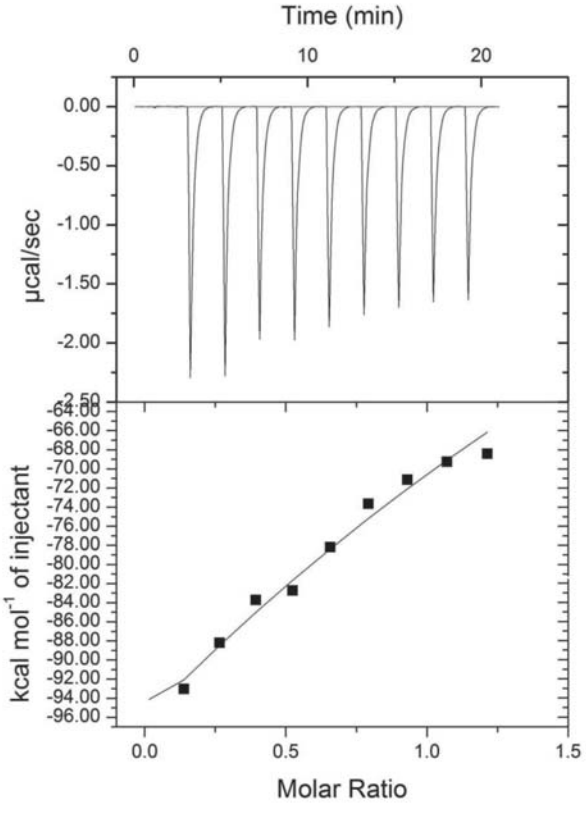

Fig 2y: HSA+ Ethanol, 4 µl/injection

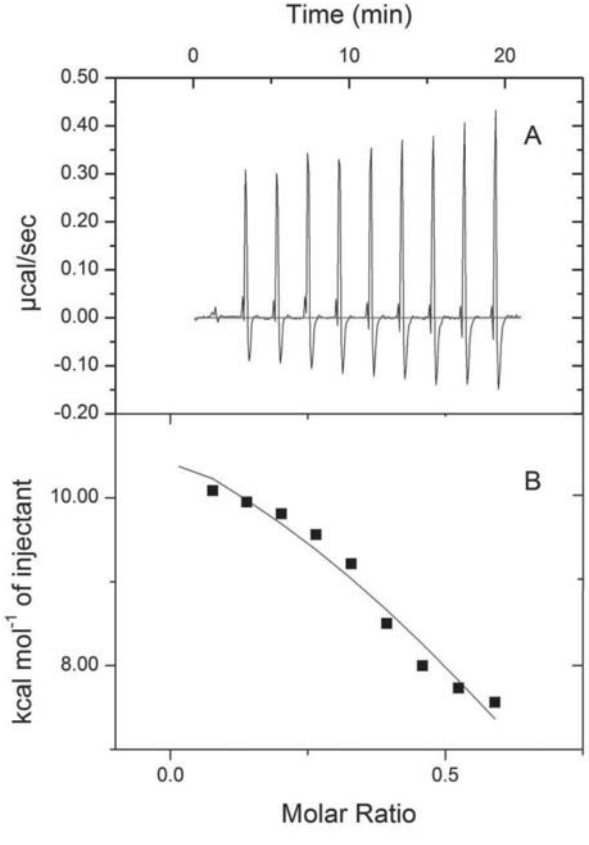

Fig 2x: HSA+ Ethanol, 2µl/injection

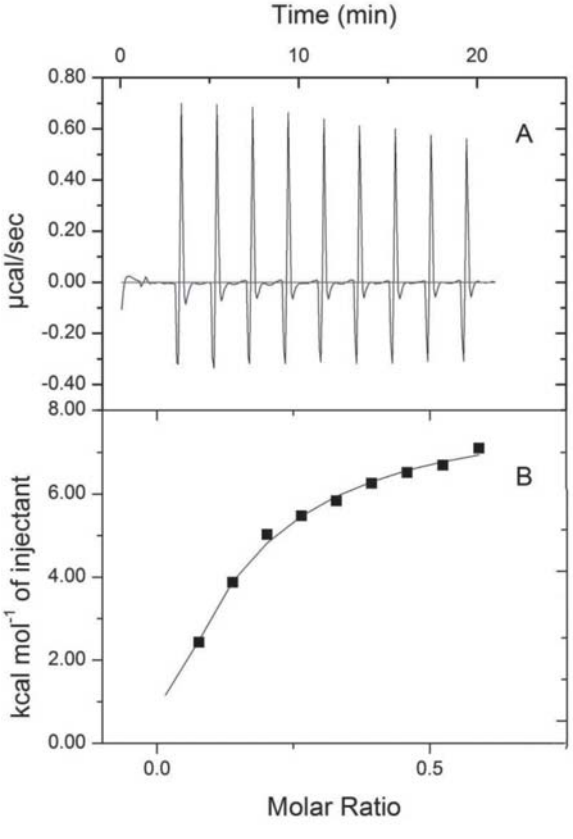

Fig 3x: HSA+ *Calcarea carb* 6 cH, 2 µl/injection

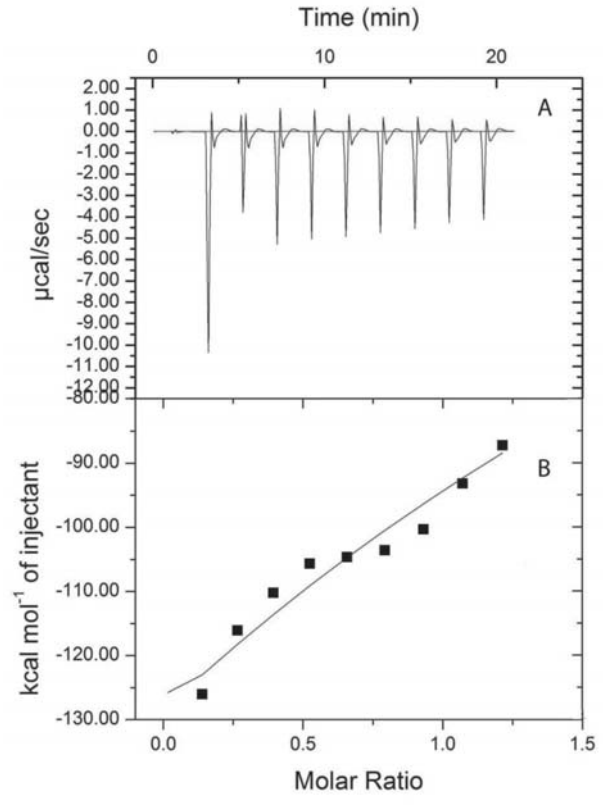

Fig 3y: HSA+ *Calcarea carb* 6 cH, 4 µl/injection

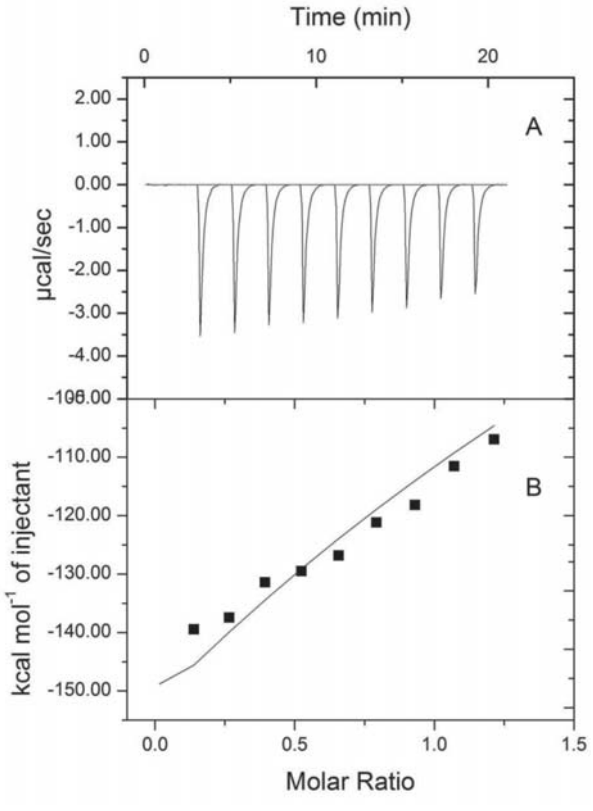

Fig 4y: HSA+ *Calcarea carb* 30 cH, 4 µl/injection

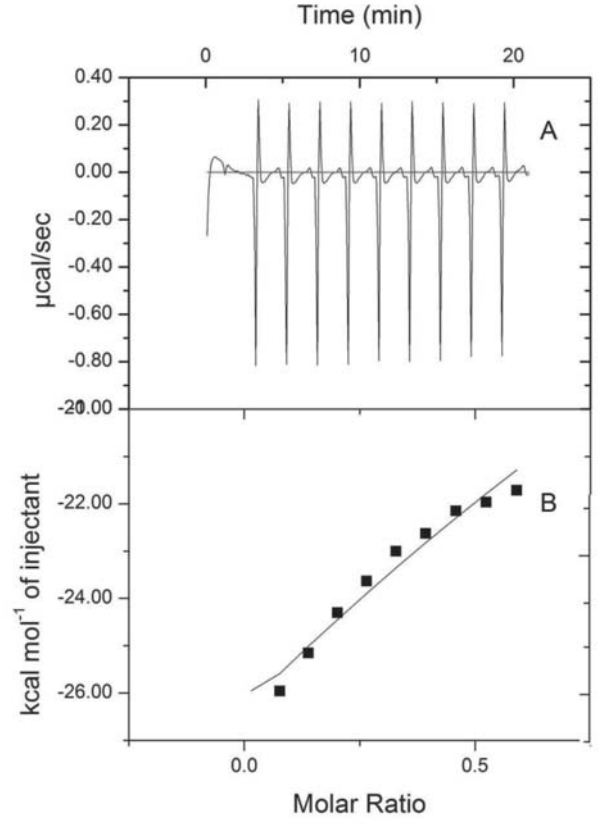

Fig 4x: HSA+ *Calcarea carb* 30 cH, 2 µl/injection

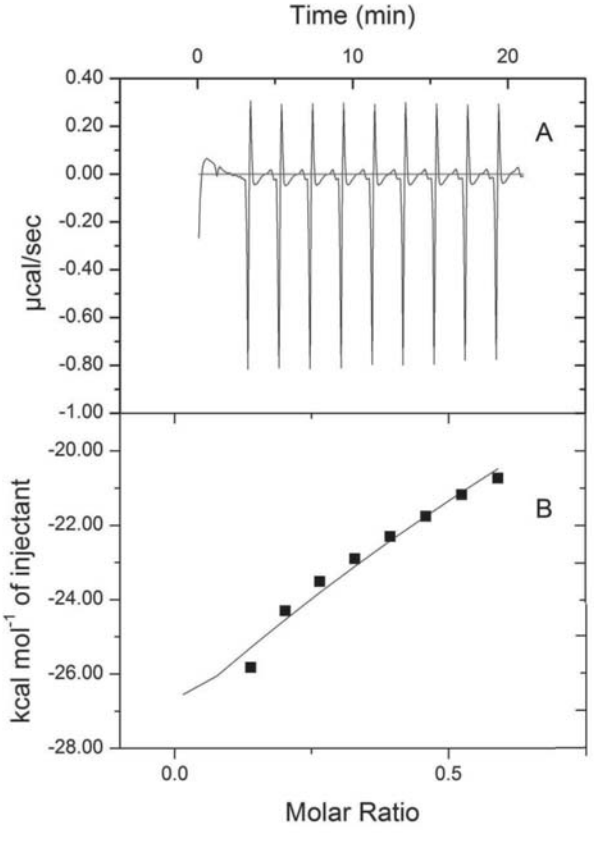

Fig 5x: HSA+ *Calcarea carb* 200 cH, 2 µl/injection

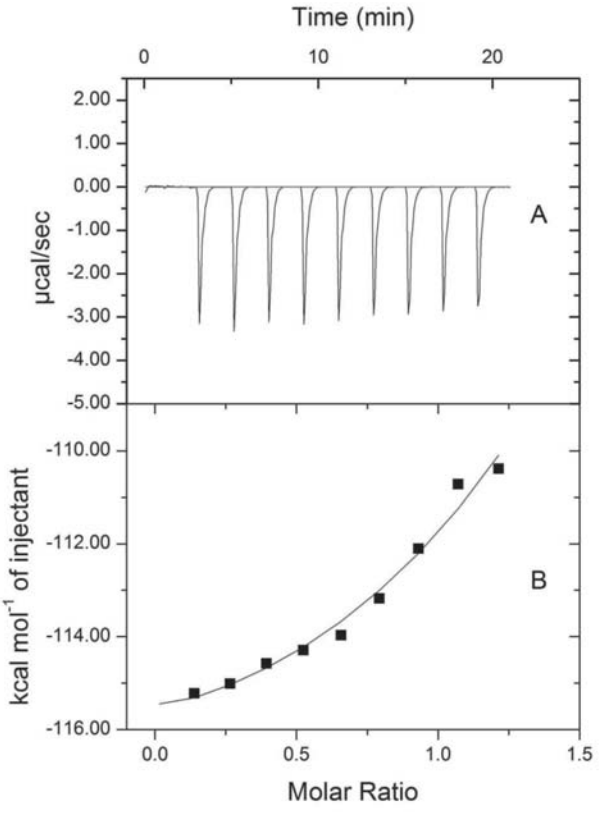

Fig 5y: HSA+ *Calcarea carb* 200 cH, 4 µl/injection

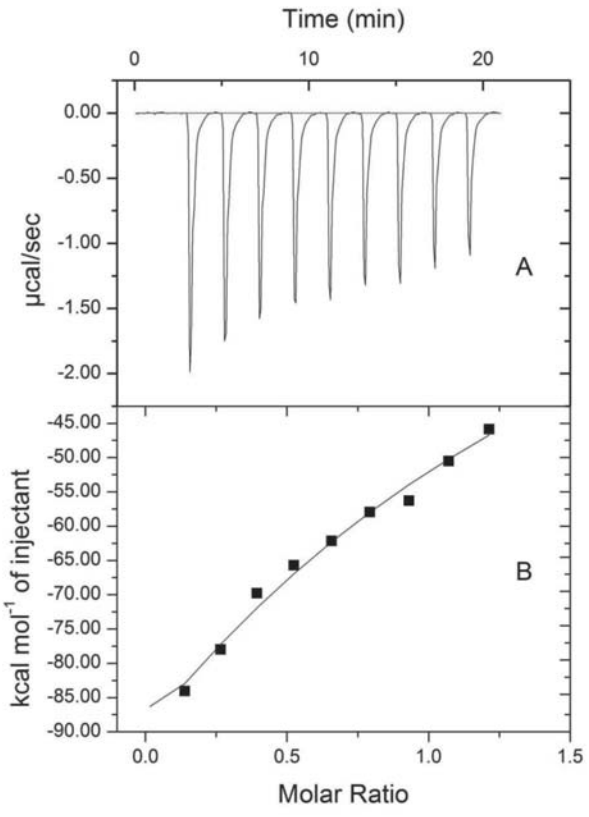

Fig 6y: HSA+ *Calcarea fluor* 6cH, 4 µl/injection

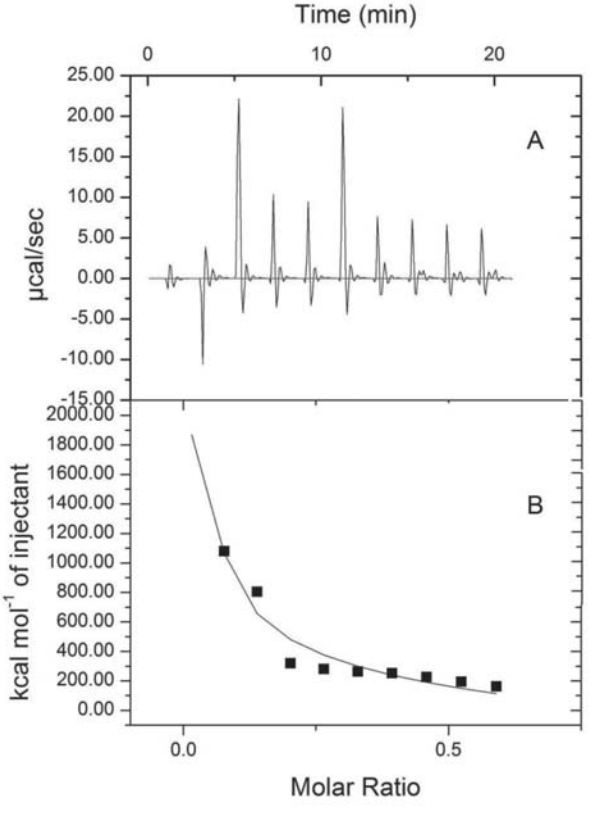

Fig 6x: HSA+ *Calcarea fluor* 6cH, 2 µl/injection

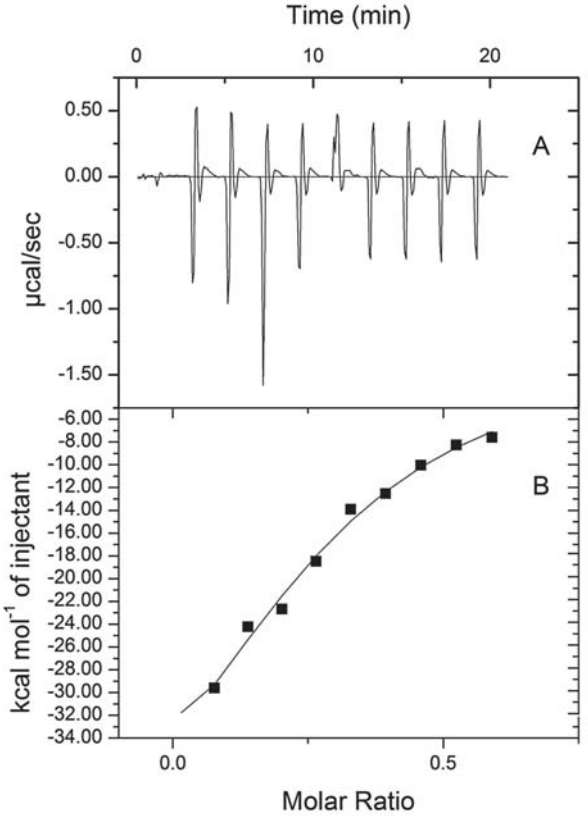

Fig 7x: HSA+ *Calcarea fluor* 30cH, 2 µl/injection

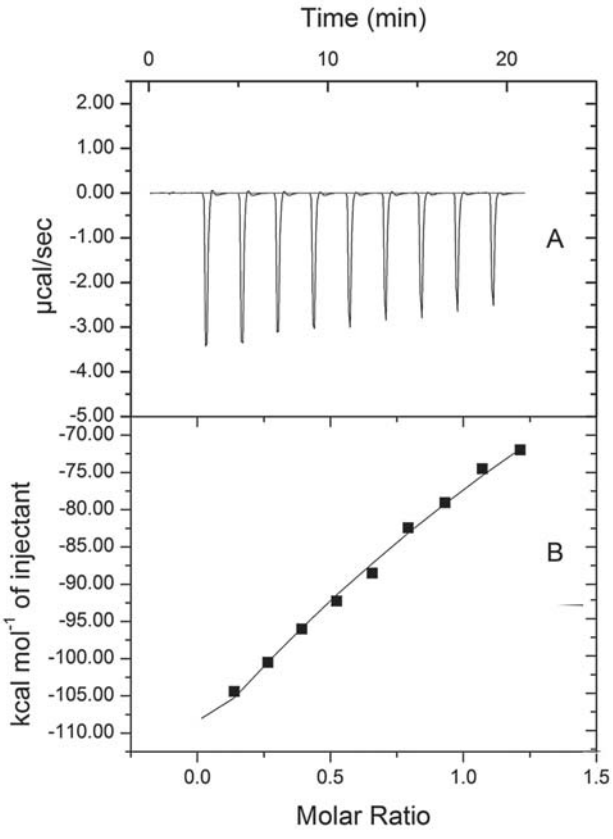

Fig 7y: HSA+ *Calcarea fluor* 30cH, 4 µl/injection

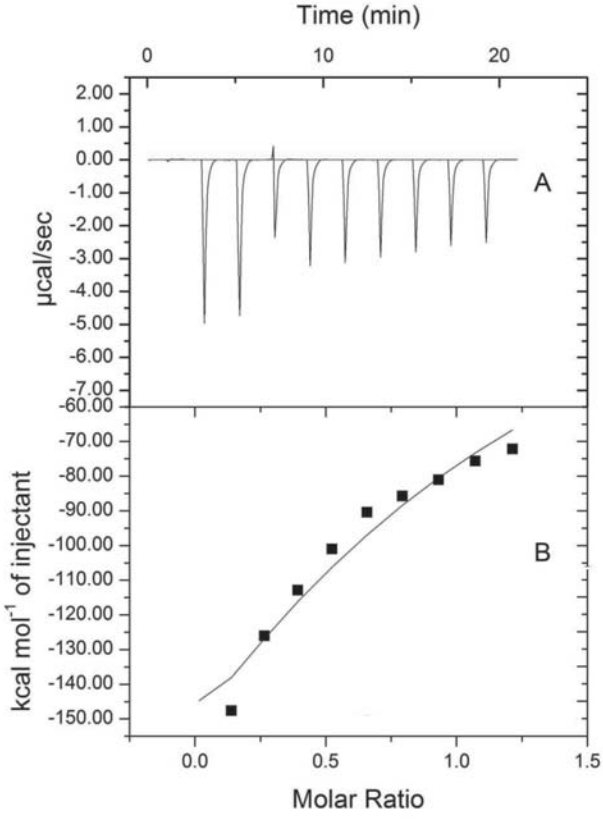

Fig 8y: HSA+ *Calcarea fluor* 200cH, 4 µl/injection

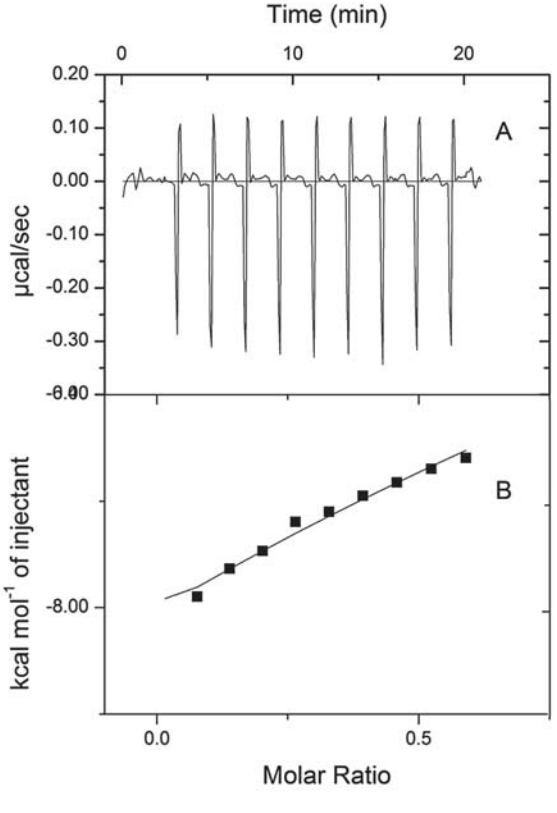

Fig 8x: HSA+ *Calcarea fluor* 200cH, 2 µl/injection

Discussion

Human body is under stress during any disease and growing tumour or cancer. The stressed cells or tissue take up albumin as a source of amino acids and energy 12. This may be the way by which potency-bound albumin molecules carry drug and deliver them at the site of infection, tumour etc. As the diseased cells take up albumin they may be acted upon by the potency.

In a binding interaction negative value of ΔG (Gibbs free energy) indicates an equilibrium state at constant temperature and pressure of a protein-ligand complex. Decrease in total Gibbs free energy is the global driving force for binding reaction [7]. Binding becomes tighter when ΔG is increasingly negative (TA instrument application note). Our results show higher negative value of ΔG with the 4 µl dose of a drug than with the 2 µl dose (Fig 1-8, Table 1) except with *Calcarea carb* 6. In any ITC experiment heat change due to an injection of a ligand should be lower in magnitude than that of the previous injection 13. In this respect the 4 µl dose satisfies the condition in a better way than the 2 µl dose. Moreover, 4 µl dose always shows single binding site (Fig 1-8, Table 1).

ΔH shows negative value in all the test samples except water and *Calcarea carb* 200. This negative value of ΔH is much higher with 4 µl dose than with 2 µl dose (Table 1). The 4 µl dose shows exothermic reaction in all the cases. In this reaction atoms interact in a favourable non-covalent way during protein ligand binding. In endothermic reaction energetically favourable non covalent interactions are disrupted. The change in enthalpy reflects a global energy change involving many individual interactions like van der waals contacts, increase or decrease of hydrogen bonds, ion pairs, other polar and apolar interactions. Besides protein and

ligand, solvent also plays an important role in these interactions 7. So we can say that 4 µl dose mostly promotes all these individual interaction. Addition of a homeopathic potency vis-à-vis specific water structure would reorganize the solvent of HSA in such a way that the entire solvent now assumes a modified water structure. The recognized solvent would interact with HSA. Since a homeopathic potency does not contain any original drug molecules, the HSA-ligand binding in this case involves mainly the interactions between the reorganized solvent and the protein. In our earlier experiments we demonstrated that the effect of a homeopathic potency is transferrable from one plant to another[14-15], one animal to another[16-17], and also from one test tube to another through capillary water[18]. All these transfer experiments suggest that water structures in different interconnected bodies are amalgamated into one unified form, and the guiding force here is the water structure in the homeopathic potency. The reorganization involves hydrogen bond strength, number of hydrogen bonds, free and bound water molecules [19].

Entropy (S) measures the randomness of different molecules in a system. While the negative value of ΔS indicates lesser randomness, the positive one does greater randomness of the molecules. The change in entropy includes three components like change in solvent entropy, change in conformational entropy of protein/ligand, and change in rotational and translational freedom of protein and ligand molecules during binding and complex formation 7. In our study 4µl dose shows higher negative value of all the test samples except *Calcarea carb* 200. This means the 4 µl dose provides lesser degree of freedom of protein and ligands. The 2 µl dose shows both positive and negative value of ΔS indicating higher degree of randomness (Table 1). Conformational changes involve folding and unfolding of HSA. Using fluorescence spectroscopy we observed that different homeopathic potencies bring about different degrees of quenching which suggest different levels of unfolding of a protein[19]. So we can assume that the 4µl dose produced lesser unfolding of HSA as compared to 2µl dose.

Different potencies of drugs show different degrees of unfolding as revealed by their ΔS values (Table 1). This may be related to the variation of the biological effects of the potencies. It is reported that the activation energies of the enzyme α-amylase show marked differences corresponding to the thermal unfolding rates of the protein[20].

A homeopathic potency has two major components, namely (i) free water molecules and (ii) hydrogen bond strength of OH groups[21-23]. Let us see how these two factors influence ΔG, ΔH and ΔS. The more the ΔG is negative, the stronger is the binding between HSA and the potency. Here binding occurs between structured water in a potency and amino acid residues at their binding site of HSA[24]. A binding site has amino acid residues where both hydrophobic and hydrophilic components exist[25]. Free water molecules tend to bind to OH groups of amino acids of HSA. The potency-induced unfolding of HSA would expose more binding sites of the protein. A change in entropy reflects a change in unfolding, and consequently the release of bound water molecules into the bulk solvent. This occurs when the hydrophobic groups in the binding sites of the protein and those in the ligand interact[26].

Conclusion

1. Human serum albumin (HSA) shows binding interaction with ligands like high dilutions (HD) of ethanol, *Calcarea carbonica* (carbonate of lime) and *Calcarea fluorica* (fluoride of lime)
2. Of the two doses used 4 µl dose shows stronger binding interaction than 2 µl dose in terms of heat change, binding affinity, stoichiometry, gradual saturation, change in enthalpy, entropy and Gibbs free energy
3. In case of high dilutions of ligand the binding interactions involve mainly the solvent (water), and HD induced conformational change of HSA. Free water molecules are thought to interact with the OH group of amino acids in the hydrophobic binding pockets of HSA.

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