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Dilutions of a Homeopathic Potency with Water do not Alter Number of Binding Sites During their Binding Interaction with a Protein

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Abstract Ultra high dilutions (UHD) of drugs, kept in 90% ethanol, are arbitrarily diluted with water and then administered orally on patients. It has been observed that dilutions do not interfere with the uniqueness of the remedy. UHDs initiate their action on proteins. The purpose is to see whether 4 different dilutions of *Sulphur* 200 cH and its medium aqueous ethanol show any change with respect to their binding reaction on a protein. *Sulphur* 200 cH was prepared in the laboratory from its 30th potency by the standard method and finally preserved in 90% ethanol. The same 90% ethanol was used as a control. Ethanol in the form of potencies, is used as a homeopathic drug. *Sulphur* 200cH and ethanol, both in 90% EtOH, were diluted with DD water 1 : 1000, 1 : 1200, 1 : 1400 and 1 : 1600. Using an isothermal titration calorimetry (ITC) instrument each dilution was tested at 25^{o} C on bovine serum albumin (BSA) to observe its interaction on the binding sites of the protein. *Sulphur* and ethanol dilutions act on single and 4 binding sites of BSA, respectively. Both the test and control ligands differ from each other with respect to thermodynamic properties. While ethanol dilutions showed exothermic reactions, *Sulphur* ones did both exo and endothermic reactions. All 4 dilutions of *Sulphur* 200cH and ethanol act on binding sites of BSA. They differ from each other with respect to binding properties. Dilutions did not alter number of binding sites.

Keywords Homeopathic potency, Dilutions, Binding interaction, BSA, ITC.

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Introduction

In homeopathy medicines are often used in ultra high dilutions (UHD) beyond Avogadro number. These medicines in UHD are in 90% ethanol. Patients are advised to further dilute the medicine with water before taking it and these dilutions are arbitrary. In an earlier experiment we observed that a UHD,

called a potency, retains its uniqueness with respect to free water molecules and hydrogen bond strength of OH groups even when diluted with water (Konar et al. 2018a). We further observed that homeopathic potencies initiate their action on binding sites of a protein (Konar et al. 2018c, Sarkar et al. 2017, 2018). The objective of the present study is to see whether 4 different dilutions of a homeopathic potency *Sulphur* 200 cH and its diluent medium aqueous ethanol could act on binding sites of a circulating protein bovine serum albumin (BSA). It is worth mentioning here that a potency in 90% EtOH, when diluted with water 1 : 1000, loses its alcohol effect but retains its specific biological effect (Sukul et al. 2012).

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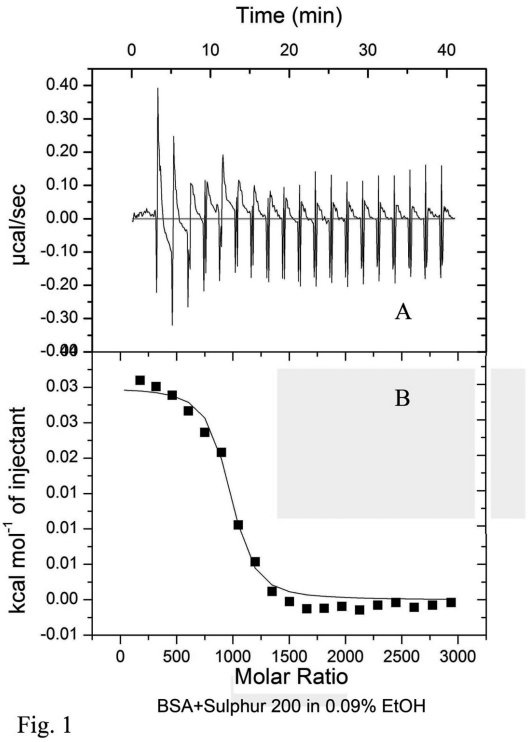


Fig. 1

Fig. 1. A : Raw data showing endothermic heat change (µcal/sec) in the form of peaks due to injection (2 µl) of ligand (*Sulphur* 200 cH in 0.09% EtOH) into 300 µl of 7 µM BSA in water. B; Non linear regression showing heat released per mole of ligand in relation to molar ratio ligand/BSA. ITC parameters in Table 1.

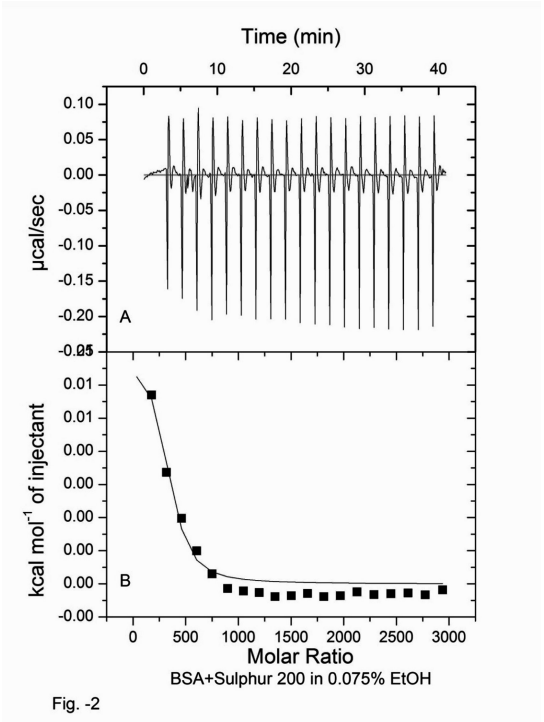


Fig. -2

Fig. 2. A : Raw data showing endothermic heat change (µcal/sec) due to injection of ligand (*Sulphur* 200 cH in 0.075% EtOH) into 300 µl of 7 µM BSA in water. B : Non linear regression showing heat released per mole of ligand in relation to molar ratio ligand / BSA. ITC parameters in Table 1.

Materials and Methods

The 30th potency of *Sulphur* was purchased from a local market at Kolkata. It was a product of Dr Reckeweg, Germany. This potency was diluted 1 : 100 with deionized and distilled (DD) water and succussed in the laboratory to prepare 200th potency following the standard method of preparation of homeopathic potencies (Konar et al. 2018b). The 200th potency was preserved in 90% ethanol. *Sulphur* 200cH was diluted with deionized and distilled (DD) water 1 : 1000 just before ITC experiment in order to reduce ethanol content to a negligible amount of 0.09%. At this dilution (1 : 1000) the ethanol effect would be negligible but the drug effected remains intact (Sukul et al. 2012, Sukul and Sukul 2004). Three more dilutions of *Sulphur* 200cH like 1 : 1200, 1 : 1400 and 1 : 1600 were prepared. Optical density of *Sulphur* 200

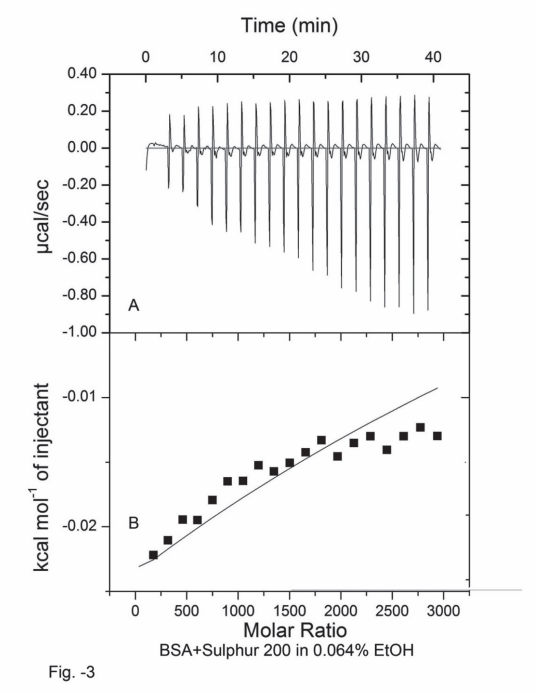


Fig. 3. A : Raw data showing exothermic heat change (µcal/sec) due to injection of ligand (*Sulphur* 200 cH in 0.064% EtOH) into 300 µl of 7 µM BSA in water. B : Non linear regression showing heat released per mole of ligand in relation to molar ratio ligand/ BSA. ITC parameters in Table 1.

cH after dilution with DD water 1 : 1000 was 0.03 at 220.5 nm as shown by UV-VIS spectrophotometer (Shimadzu, UV 2401-PC). All the four dilutions of *Sulphur* 200cH were tested on BSA for binding reaction using an isothermal titration calorimetry (ITC) instrument, ITC 200 GE Health care bioscience Ltd, Sweden. Four similar dilutions of the medium, 90% ethanol, were also prepared. The medium dilutions served as a control, although ethanol itself is used as a homeopathic drug (Farrington 1928).

Each diluted ligand, *Sulphur* 200 cH or ethanol, was injected at 2 µl every 2 min into a measurement cell containing 300µl of 7 µM BSA. Both the sample and reference cell containing water only were maintained at a constant temperature of 25°C. When thermal equilibrium was established injections of ligand was started. After each experiment with a ligand the instrument was washed thoroughly with DD water. Binding reaction between the ligand and BSA was recorded in terms of absorption of heat (endothermic) or release of heat (exothermic). The raw data in the form of peaks were analyzed by a software origin 7 and best fit parameters like binding constant (K), change in enthalpy (ΔH) and entropy (ΔS) were obtained. Change in Gibb's free energy was calculated from the equation $\Delta G = \Delta H - T\Delta S$, where T represents absolute temperature in Kelvin.

Results

Results are represented in Figs. 1—8. Each figure has two panels A and B. A shows raw data in terms of heat rate (µcal/sec) versus time in min. B displays heat change per mole of ligand during interaction of BSA in relation to molar ratio, ligand/protein, in the form of non-linear regression. ITC parameters like K, ΔH, ΔS, ΔG, heat rate, number of binding sites

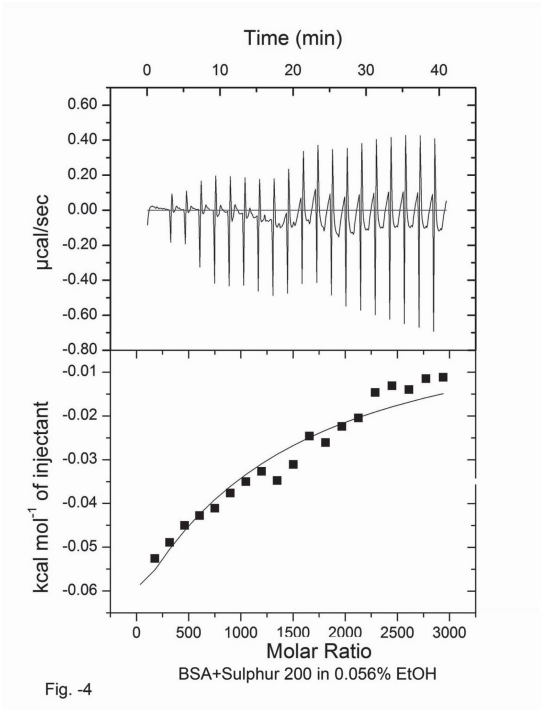
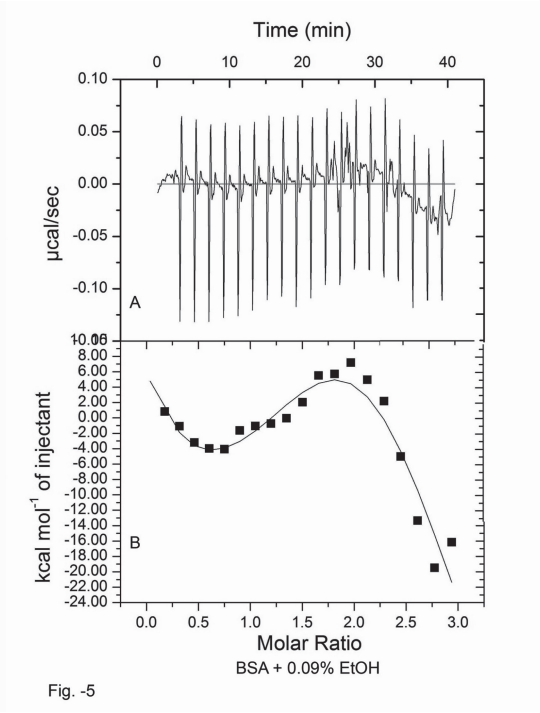


Fig. 4. A : Raw data showing exothermic heat change (µcal/sec) due to injection of ligand (*Sulphur* 200 cH in 0.056% EtoH) into 300 µl of 7 µM BSA in wate. B : Non linear regression showing heat released per mole of ligand in relation to molar ratio ligand/ BSA. ITC parameters in Table 1.



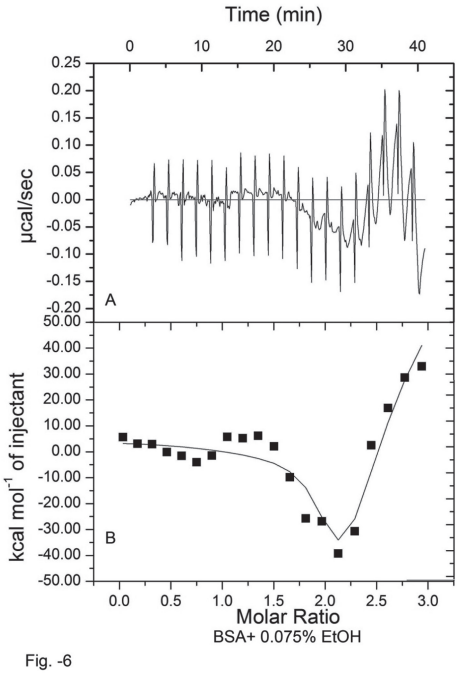


Fig. 5. A : Raw data showing exothermic heat change (µcal/sec) due to injection of ligand (0.09% EtOH) into 300 µl of 7 µM BSA in water. B : Non linear regression showing heat released per mole of ligand in relation to molar ratio ligand/ BSA. ITC parameters in Table 1.

Fig. 6. A : Raw data showing exothermic heat change (µcal/sec) due to injection of ligand (0.075%EtOH) into 300 µl of 7 µM BSA in water. B : Non linear regression showing heat released per mole of ligand in relation to molar ratio ligand/BSA. ITCparameters in Table 1.

are presented in Table 1.

Fig. 1 (A, B) shows interaction between *Sulphur* 200 cH (ligand) in 0.09% EtOH and BSA. Details of ligand protein interaction are given in Table 1. Fig. 2 (A, B) shows interaction between *Sulphur* 200cH (ligand) in 0.075% EtOH and BSA. Details of interaction are given in Table 1. Fig. 3 (A, B) displays binding interaction between *Sulphur* 200cH (ligand) in 0.064% EtOH and BSA. Details of reaction are

Table 1. Thermodynamic parameters of binding interactions between 7 µM bovine serum albumin (BSA) in water and 4 different dilutions of the same ligand like *Sulphur* 20cH in 0.09, 0.075, 0.064 and 0.056% ethanol. Each dilution of ligand was injected 20 times at 2 µl every 2 min into 7 µM BSA in water at 25°C in an isothermal titration calorimetry (ITC) instrument. Data analyzed by Origin 7.

Ligands injected, 2µl every 2 min	Binding constant $KM^{-1}\times10^4$	Change in enthalpy ΔH Cal/mol×10^3	Change in entropy ΔS Cal/mol/deg×10	Change in free energy ΔG Cal/mol × 10^2	No. of binding sites, maximum heat change, reaction
Sulphur 200 in 0.09% EtOH	1.20	0.02997	1.88	-51.0544	1, 0.4 µcal/sec, Exothermic saturation after 8 injections
EtOH 0.09%	K_1 : 8.41 ×10 K_2 : 4.93 ×10 K_3 : 4.74 K_4 : 6.23	ΔH_1 : 6.570 ΔH_2 : 5.25 × 10 ΔH_3 : 5.65 × 10^2 ΔH_4 : 1.67 × 10^3	ΔS_1 : 4.91 ΔS_2 : 1.50 ×10^2 ΔS_3 : 1.92 × 10^2 ΔS_4 : 5.6 × 10^2	ΔG_1 : 1.34 ×10 ΔG_2 : 4.09 × 10^2 ΔG_3 : 5.18 × 10^2 ΔG_4 : 1.51 × 10^3	4, 0.1 µcal/sec, Exothermic, no.saturation
Sulphur 200 in 0.075% EtOH	0.391	0.00713	1.65	-45.0001	1,0.1 µcal/sec, Endothermic, saturation after 5 injection

Table 1. Continued.

Ligands injected, 2 µl every 2 min	Binding constant $KM^{-1} \times 10^4$	Change in enthalpy ΔH Cal/mol × 10^3	Change in entropy ΔS Cal/mol/deg × 10	Change in free energy ΔG Cal/ mol × 10^2	No. of binding sites, maximum heat change, reaction
EtOH 0.075%	K_1 : 1.77 × 10^4 K_2 : 2.19 × 10^3 K_3 : 2.08 × 10 K_4 : 1.31 × 10	ΔH_1 : 0.00395 ΔH_2 : -2.815 ΔH_3 : -1.69 × 10^2 ΔH_4 : -6.29 × 10^2	ΔS_1 : 4.84 ΔS_2 : 2.42 ΔS_3 : -5.42 × 10 ΔS_4 : 0.213	ΔG_1 : 1.32 × 10 ΔG_2 : -6.63 ΔG_3 : -1.46 × 10^2 ΔG_4 : 5.70	4, 0.15 µcal/sec, Exothermic, no. saturation
Sulphur 200 in 0.064% EtOH	0.0000028	-1.11 × 10^5	-3.70 × 10^4	14669.1122	1,0.9 µcal/sec, Exothermic, increasing interaction
EtOH 0.064%	K_1 : 5.79 × 10^2 K_2 : 2.06 K_3 : 3.13 K_4 : 8.20	ΔH_1 : 2.090 ΔH_2 : 5.17 × 10 ΔH_3 : -2.95 × 10^3 ΔH_4 : -3.98 × 10^3	ΔS_1 : 3.8 ΔS_2 : 1.93 × 10 ΔS_3 : -9.9 × 10^2 ΔS_4 : 1.34 × 10^3	ΔG_1 : 1.03 ×10 ΔG_2 : -5.22 × 10 ΔG_3 : 2.67 × 10^3 ΔG_4 : -3.62 × 10^3	4, 0.4 µcal/sec, Exothermic, no. saturation
Sulphur 200 in 0.056% EtOH	0.00384	-2.20 × 10^2	-73.0	1961.4171	1, 0.8 µcal/sec, Exothermic, tending to gradual satura tion upto 12 injections then increasing
EtOH 0.056%	K_1 : 1.15 × 10^5 K_2 : 7.73 × 10^4 K_3 : 8.83 × 10^4 K_4 : 1.15 × 10^5	ΔH_1 : 3573 ΔH_2 : -9.94 × 10^4 ΔH_3 : -2.59 × 10^5 ΔH_4 : -4.66 × 10^5	ΔS_1 : 35.1 ΔS_2 : -311 ΔS_3 : 890 ΔS_4 : -1.54 × 10^5	ΔG_1 : 9.55 ΔG_2 : -8.5 × 10 ΔG_3 : 2.40 × 10^2 ΔG_4 : -4.16 × 10^2	4, Exothermic, 0.8 µcal/sec, heat increasing, no.saturation

given in Table 1. Fig. 4 (A, B) displays interaction between *Sulphur* 200cH (ligand) in 0.056% EtOH and BSA. Details of reaction are given in Table 1. Figs. 5-8 show interaction between BSA and 4 different concentration of the ligand, ethanol like 0.09% (Fig 5), 0.075% (Fig. 6), 0.064% (Fig. 7), 0.056% (Fig. 8). Details of ligand protein interaction are given in Table 1. While *Sulphur* 200 cH solutions have one binding sites, ethanol solutions have four binding sites. Both *Sulphur* and ethanol solutions show marked variation in heat change (µcal/sec) and other binding properties like K, ΔH, ΔS and ΔG values (Figs. 1—8, Table 1).

Discussion

It is evident from the results that 4 different dilutions of *Sulphur* 200 cH show interactions on a single binding site of BSA, but the interactions vary with respect to rate of heat change, types of reaction (exo- or endothermic) and other binding properties like K, ΔH, ΔS and ΔG values. They also differ from each other with respect to saturation of the binding site. Two higher dilutions (3rd and 4th) show increasing interactions (Table 1). Dilutions of aquous ethanol show marked difference from those of *Sulphur* 200 cH with respect to all binding properties. None of them saturated binding sites within 20 injections. In fact all of them showed increasing interaction towards the end of interactions. But binding site was same (4) with all 4 dilutions (Table 1). These results indicate that dilutions of the test samples (*Sulphur* 200 cH and ethanol) did not interfere with the number of binding sites, but greatly modified binding interactions irrespective of the amount of water added. The results indicate that 1 : 1000 dilution of a potency or control is the most suitable one for ITC experiments. This is because with higher dilutions of *Sulphur* 200 cH like 1 : 1400 onwards there was a change in type of reaction from endo to exothermic one and increase in heat change instead of gradual decrease (Figs. 1-4, Table 1). For ethanol the heat change is good for the first 2 dilutions 1 : 1000 and 1 : 1200 (Figs. 5-8, Table 1).

Conclusion

All the 4 dilutions of *Sulphur* 200cH and ethanol act on binding sites of BSA. The dilutions differ from each other with respect to binding properties. Number of binding sites in BSA with *Sulphur* 200 cH or ethanol remains unchanged with different dilutions.

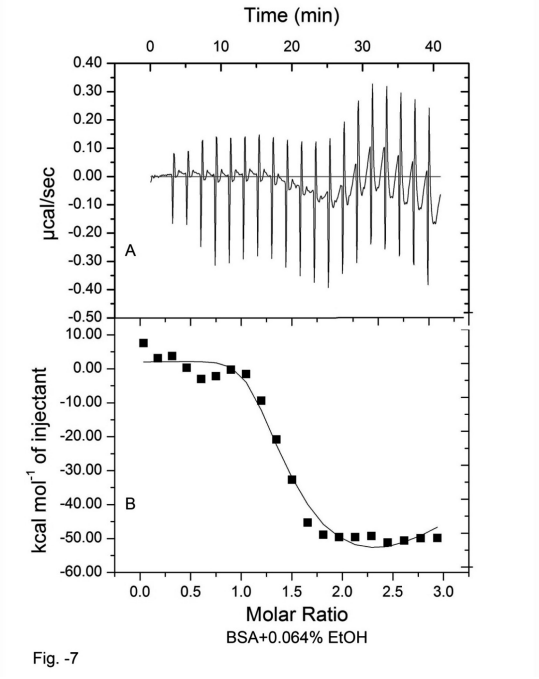


Fig. -7

Fig. 7. A : Raw data showing exothermic heat chage (µcal/sec) due to injection of ligand(0.064% EtOH) into 300 µl of 7 µM BSA in water. B : Non linear regression showing heat released per mole of ligand in relation to molar ratio ligand/BSA. ITC parameters in Table 1.

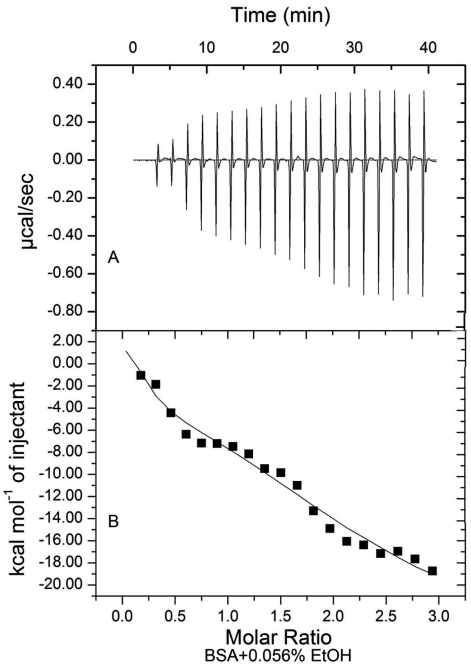


Fig. -8

Fig. 8. A : Raw data showing exothermic heat change (µcal/sec) due to injection of ligand (0.056% EtOH) into 300 µl of 7 µM BSA in water. B : Non linear regression showing heat released per mole of ligand in relation to molar ratio ligand/ BSA. ITC parameters in Table 1.

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