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Research Article

Binding Studies of Lamotrigine with Sera of Different Animal Species

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Abstract

Purpose: The serum protein-binding characteristic of lamotrigine with sera from various species (horse, rat, rabbit and goat) was studied at different dielectric constants and temperatures to obtain an insight into the mechanism of interaction, evaluate the effect of dielectric constant on binding affinity, and to determine the effect of species variation on drug plasma-protein interaction.

Method: Binding data were generated by evaluating equilibrium dialysis of lamotrigine in methanol-water solvent (1:1 and 1:3) with horse serum at 20, 28 and 37 ^0C The equilibrium dialysis evaluation of lamotrigine with sera of other animal species - rabbit, goat and rat - was also performed at 37 ^0C.

Results: A Rosenthal plot obtained with the binding data at different temperatures showed that binding constant decreased with increasing temperature and that binding of lamotrigine to horse serum appeared to be saturation binding. The results also indicated that the binding process was characterized by negative ΔH^0 value and a small positive ΔS^0 values. The binding constant decreased as dielectric constant rose.

Conclusions: The results obtained suggest that hydrophobic interaction may have occurred and that van der Waals' forces were responsible for binding in the hydrophobic region. Data obtained for lamotrigine binding with horse, goat, rabbit and rat sera show that there are no significant changes in plasma protein binding in the above-mentioned species.

Keywords: Lamotrigine; Serum protein binding; Dielectric constant; Binding constant; Animal sera

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INTRODUCTION

Protein binding has long been considered one of the most important physicochemical characteristics of drugs, playing a potential role in pharmacokinetic (including hepatic metabolism rate, renal excretion, biomembrane permeation rate and steady state distribution volume) [1-3] and pharmacodynamic properties of drugs [4]. Plasma protein binding of drug molecules has implication in drug safety [5] and several adverse effects such as low clearance, low brain penetration, drug–drug interaction, etc [6,7]. It has also been pointed out that not only binding equilibrium but also offset rate may influence the efficacy/distribution of the compound [8]. In our previous works, studies were carried out to evaluate the effect of concentration, temperature, pH and ionic strength on highly albumin-bound drugs and bovine serum albumin (BSA) interaction to explore protein-binding characteristics of some drugs [9,10]. The influence of species on plasma protein binding of various drugs has also been reported [11-13].

The present study was designed to evaluate the effect of drug concentration, temperature and dielectric constant on horse serum binding of a moderately plasma protein-bound drug and also to determine the influence of the binding of the drug on the sera of different animal species. Lamotrigine, a phenyltriazine derivative, is as an anti-seizure drug with a degree of plasma protein binding of 56 % [14]. Some thermodynamic parameters [15,16] of lamotrigine and horse serum were also derived. The data obtained could help in gaining some insight into possible drug/protein interaction.

EXPERIMENTAL

Lamotrigine was obtained from Cadila, Ahmedabad, India as a gift. Dialysis tubing was purchased from Sigma Chemical Company, St. Louis, USA. All other chemicals and reagents used were of analytical grade.

The animal experiments were approved by the Animal Ethics Committee of Gupta College of Technological Sciences, Asansol, India (ref no. GCTS/AEC/2007/03) and carried out as per the requirements of the committee. The animals were housed in separate rooms at controlled temperature and relative humidity (25 ^{0}C and 45 %RH) and 12 h light/dark cycle for 3 days prior to blood collection. The rabbits (3) and rats (12) were fed normally with green leafy vegetables, milk and soaked Bengal gram (for the rabbits) and Nutrilab Rodent$^®$ (Vetcare Div., Tetragon Cheme Pvt Ltd, Bangalore, India). They also freely received water. Blood was withdrawn with heparinised needles from the marginal ear vein of the rabbits and the tail vein of the rats. The blood was pooled in each case and approximately 10ml was centrifuged at 3000×g to obtain serum which was stored at -20 ^{0}C till further use. Goat blood was collected from local slaughter house at Asansol while horse serum was kindly provided free of charge by Bengal Chemical and Pharmaceuticals, Kolkata, India and used as such

Determination of extent of lamotrigine - horse serum protein binding

The extent of protein binding of lamotrigine with horse serum binding was determined by equilibrium dialysis method [17-18]. Equilibrium dialysis was chosen for this study because of its ability to reasonably approximate physiological conditions. The drug bound was determined indirectly by measuring the unbound drug fraction spectrophotometrically at 308 nm (Thermo Spectronic UV1, UK.). Dialysis bags were prepared from dialysis tube as described in literature (after treating according to the manufacturer's instruction) and then soaked for 24 h in phosphate buffer solvent (pH 7.4) prior to use [19]. The equilibrium dialysis study of concentrated solutions of lamotrigine (7.24×10^{-5} and 32.57×10^{-5} M/L) using 61.5 µM/L horse serum was performed using 1:3 methanol-water system. Incubation was for 1, 4, 8, 20, 24 and 28 h, respectively, at 20 ^{0}C

with continuous stirring at very slow speed. A control, containing drug in dialysing medium, was run simultaneously to determine loss due to membrane binding of drugs in each case. Another control without drug was also run to determine probable albumin transfer across the membrane, by measuring the UV absorbance of the solvent at 280 nm to check for albumin leakage. In all determinations, the horse serum albumin (HSA) concentration used was 0.4% w/v, i.e., 61.5 µM/L. The unbound drug fraction was measured spectrophotometrically at 308 nm.

Effect of drug concentration on *in vitro* horse serum-drug binding

A dialysis bag containing 5 ml of horse serum and 2 ml of lamotrigine solution (of various concentrations) in 1:3 methanol-water system was lowered into 20 ml of 1:3 methanol-water dialyzing solution. The dialysis procedure was performed for 20 h with continuous stirring at very slow speed at 20, 28 and 37 ^{0}C. In all determinations, HSA concentration used was 0.4 %w/v, i.e., 61.5 µM/L. All control studies were also performed as described above. The bound lamotrigine was detected indirectly by measuring the unbound fraction of lamotrigine at 308 nm.

### Effect of dielectric constant on lamotrigine - HSA binding

Equilibrium dialysis of different concentrations of lamotrigine with has at different dielectric contants was also evaluated using 1:3 methanol-water and 1:1 methanol-water solvent systems at 20 ^{0}C. Other conditions were constant. All control studies were similarly performed as described above. The bound lamotrigine was detected indirectly by measuring its unbound fraction at 308 nm. Protein binding (%) was calculated from Eq. 1.

$$Bound\ drug\ (\%) = 100 \times (1 - \frac{concentration\ of\ free\ drug\ in\ test\ sample}{concentration\ of\ free\ drug\ in\ control\ sample}).....(1)$$

RESULTS

Binding equilibrium

The results of the experiments to determine the amount of lamotrigine bound to horse serum protein at various times indicate that the minimum binding time requirement to reach equilibrium was 20 h (Fig 1). Based on these results, all equilibrium dialysis studies were performed for 20 h. From the results of the control studies, it was observed that not more than 1.6 % drug was bound with the membrane and the limit of detection of albumin leakage by this method was 0.03 % albumin. Both of these correction factors were taken into account in calculating drug protein binding.
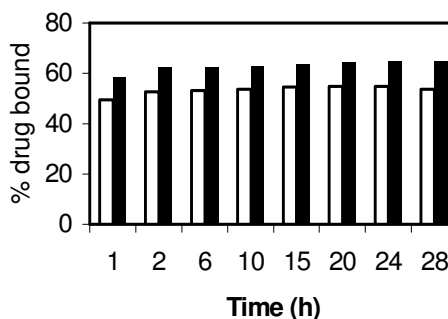
Fig 1: Binding of lamotrigine to horse serum at different time intervals at 20 ^{0}C. (Key: , drug conc. = 0.17 µmol/L; ■, drug conc. 5.25 µmol/L)

Binding characteristics of lamotrigine with horse serum protein

The equilibrium dialysis study data for the binding of lamotrigine to horse serum at three different temperatures were analyzed using Graph Pad Prism 3-version software to perform a regression for Rosenthal plot [20] which is expressed in Eqs. 2 - 4:

(Bound x K_d) + (Bound x Free) = (Free x B_{max}).......(2)

$$\frac{Bound \times K_d}{Free} + Bound = B_{max} \ldots\ldots\ldots\ldots(3)$$

or,

$$\frac{Bound}{Free} = \frac{B_{\max}}{K_d} - \frac{Bound}{K_d} \dots\dots\dots\dots\dots\dots\dots(4)$$

where 'Bound' and 'Free' means bound and free drug concentrations, respectively, after equilibration with protein; K_d is the dissociation constant; and B_{max} is saturation binding.

This is an equation for a straight line (Y = c - mX) which was first derived by Rosenthal, where Y = Bound/Free and X = Bound. The Rosenthal plot obtained using the equilibrium dialysis study data for the binding of lamotrigine to horse serum protein (HSA) at three different temperatures is shown in Fig 2. The data suggest that the binding of lamo-
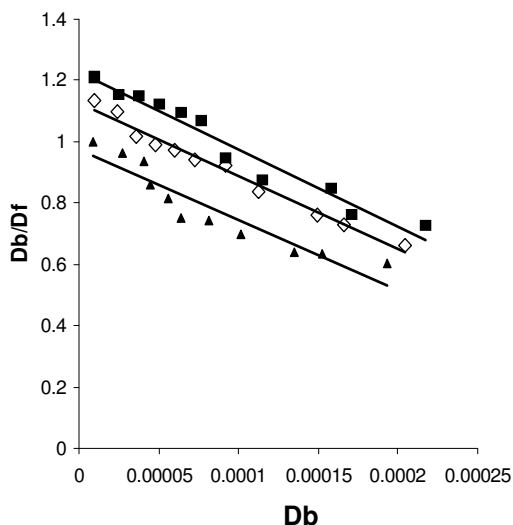
Fig 2: Lamotrigine-horse serum protein interaction at different temperatures in methanol: water (1:3) solvent system. (Key: **Db** = bound drug; **Df** = free drug; ■ = 20 ^{0}C; ◇ = 28 ^{0}C and ▲ = 28 ^{0}C

trigine to HSA is a saturation-binding phenomenon. The bound fraction decreased with increasing drug concentration, and binding occurred more with the unionised drug. The dialysis medium used was 1:3 methanol-water system. The binding constant decreased with increasing temperature. To determine the effect of dielectric constant of the dialysis medium on the binding affinity,

the binding studies were performed in two different solvent systems, viz, 1:3 methanol-water and 1:1 methanol-water. The results are shown in Fig 3. Decreasing dielectric constant resulted in a fall in polarity and increased the affinity of the drug to the solvent. The best fit linear regression equations are as shown in Eqs 5 - 8.
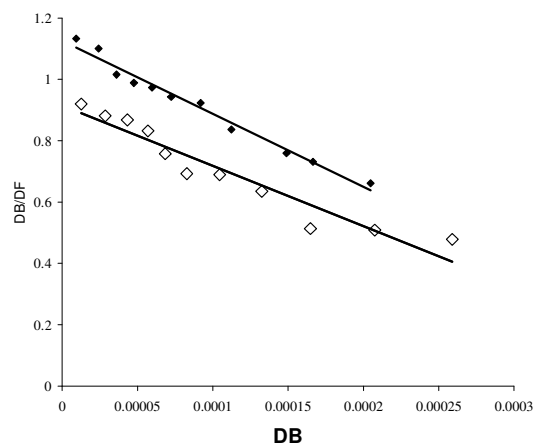
Fig 3: Lamotrigine-horse serum protein interaction at 20 ^{0}C in two solvent systems (**Key:** DB = bound drug; DF = free drug; ♦ = 1:3 methanol-water; and ◇ = 1:1 methanol-water)

At 20 ^{0}C (1:3 methanol-water system),

$$\frac{D_b}{D_f} = -2520.1 D_b + 1.2274 \dots\dots\dots\dots\dots(5)$$

$$(r^2 = 0.9548, \quad P < 0.0001)$$

At 28 ^{0}C (1:3 methanol: water system),

$$\frac{D_b}{D_f} = -2382.5 D_b + 1.1259 \dots\dots\dots\dots\dots(6)$$

$$(r^2 = 0.9798, \quad P < 0.0001)$$

At 37 ^{0}C (1:3 methanol: water system),

$$\frac{D_b}{D_f} = -2277.1 D_b + 0.9728 \dots\dots\dots\dots\dots(7)$$

$$(r^2 = 0.8759, \quad P < 0.0001)$$

At 20 ^{0}C (1:1 methanol:water system),

$$\frac{D_b}{D_f} = -1963 D_b + 0.9142 \dots\dots\dots\dots\dots(8)$$

$$(r^2 = 0.9223, \quad P < 0.0001)$$

where D_b and D_f are bound and free drugs, respectively. The calculated values of B_{max}, K_{ass} (association constant = $1/K_d$) and standard free energy change are reported in Table 1.

Table 1: Binding of lamotrigine with horse serum protein under various conditions

Equilibrium conditions	K_{ass} lt/mol	B_{max} (mol) x 10^{-5}	ΔG^0 Kcal/mol
20 ^{0}C, 1:3 methanol:water	2520	48.7	-4.62
28 ^{0}C, 1:3 methanol:water	2383	47.26	-4.63
37 ^{0}C, 1:3 methanol:water	2277	42.7	-4.70
20 ^{0}C, 1:1 methanol:water	1963	46.57	-4.43

Binding of lamotrigine with various animal sera

Table 2 shows the results of the interaction of lamotrigine with the sera of horse, goat, rabbit and rat. We observed that the extent of serum protein binding of lamotrigine with different species is within 47 – 51 % at low concentration of lamotrigine but it is within 39-41% at higher concentration.

Table 2: Binding of lamotrigine with serum proteins of various animal species at 37 ^{0}C, pH 7.4 and μ = 0.1

Animal species	Drug concentration used mol/L (x 10^5)	Absorbance*		Bound drug (%)
		Control sample test sample	Test sample	
Horse	6.2	0.051	0.102	50.0
	24.2	0.265	0.45	41.1
Rat	6.2	0.052	0.102	49.02
	24.2	0.270	0.45	40.0
Rabbit	6.2	0.523	0.102	48.72
	24.2	0.2693	0.45	40.2
Goat	6.2	0.054	0.102	47.1
	24.2	0.274	0.45	39.1

* Mean of three readings.

DISCUSSION

Binding equilibrium

The principle of protein binding is that when a drug and protein are initially mixed together, the amount of drug-protein complex formed will exceed the dissociation of the drug-protein complex [21]. Subsequently, the system reaches equilibrium, following which maximum drug protein interaction will occur. Therefore, the time taken to reach equilibrium is an important factor in equilibrium dialysis. In this work, the equilibrium time was found to be 20 h and hence this was the time used in all the evaluations.

Thermodynamic analysis and mechanism of interaction

Assuming that there was no significant temperature dependence of enthalpy change within the temperature range in which the interaction was carried out, the standard free energy change (ΔG^0), the standard enthalpy change (ΔH^0) and the standard entropy change (ΔS^0) for drug-protein binding were obtained from van't Hoff plot (not shown) based on Eqs 9 and 10.

$$\Delta G^0 = -2.303\ RT \log K \quad \text{...................} \quad (9)$$
$$\text{Log } K = -\Delta H^0/2.303RT + \Delta S^0/2.303R \text{... (10)}$$

The standard enthalpy change, ΔH^0, and the standard entropy change, ΔS^0, can also be obtained from the above relationship. A best-fit linear plot of log K vs. 1/T (van't Hoff plot) for primary binding site was made and the regression equation obtained is shown in Eq 11.

$$\text{Log } K = 303.13/T + 2.3715 \text{ .... (11)}$$
$$(R^2 = 0.997)$$

The values of ΔH^0 and ΔS^0 for primary binding site were obtained from the slope and intercept of the above equation. The values of ΔG^0, ΔH^0 and ΔS^0 were – 4.65 Kcal/mol (mean calculated from Table 1), –1.38 Kcal/mol, and 10.81 cal/mol deg,

respectively. The decrease in the association constant of lamotrigine - horse serum interaction process with increasing temperature shows that the binding was an exothermic reaction and the negative sign for ΔG^0 means that the binding process was spontaneous. A high negative ΔH^0 value indicates the absence of electrostatic interaction between drug and protein molecules [16, 22]. The positive value of ΔS^0 associated with many reactions involving proteins is usually attributed to disorientation and unfolding of the protein molecule. The moderately high negative value of enthalpy change observed in this work shows that this is not a satisfactory explanation. Rather a small positive ΔS^0 suggests that hydrophobic interaction [23] may be responsible and that van der Waals forces [24] are involved in binding in the hydrophobic region [25-26].

Effect of dielectric constant on lamotrigine - horse serum protein binding

Decrease in dielectric constant of the binding medium lowered the intrinsic binding affinity between lamotrigine and horse serum. The effect of solvent on drug-protein binding has been estimated by various researchers [27-29]. Decrease in binding constant with decrease in dielectric constant (ε = 55.6 for 1:1 methanol-water system compared to ε = 67 for 1:3 methanol-water system) may be attributed to the fact that the drug is less polar than water. Thus, their affinity to the medium would increase as dielectric constant decreases. This observation indicates that hydrophobic interaction was the major force in the binding of lamotrigine with horse serum protein [30].

Binding of lamotrigine with other animal sera

The interaction of lamotrigine with the proteins of horse, goat, rabbit and rat sera did not vary significantly, thus indicating that protein binding with the drug in the sera of the animal species was similar.

CONCLUSION

Protein binding of the moderately serum protein-bound drug, lamotrigine, was dependent on temperature and the dielectric constant of the solvent medium. The mechanism of binding was mainly due to hydrophobic interaction arising from van der Waals forces as evident in the fact that binding constant fell with decrease in dielectric constant.

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