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***In-vitro* interaction of verapamil hydrochloride with magnesium sulphate (anhydrous) and its influence on protein binding of verapamil hydrochloride**

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ABSTRACT

The interaction between Verapamil Hydrochloride and Magnesium Sulphate (anhydrous) has been studied in an aqueous system at pH 7.4 and 2.4. From spectrophotometric study, it has been found that Verapamil Hydrochloride form 1:1 complex with Magnesium Sulphate (anhydrous). Spectral studies helps to detect the initial complexation between drug and metal. Job's plot at 7.4 and 2.4 provides same type of information. The Ardon's spectrophotometric method confirmed the 1:1 complexation and the value of stability constants was calculated using Ardon's plot. An *in vitro* study of protein binding of Verapamil Hydrochloride and their 1:1 mixture with Magnesium Sulphate (anhydrous) has been conducted by equilibrium dialysis method at $(37 \pm 0.5)^{\circ}\text{C}$ and at pH 7.4. The Scatchard plots were prepared to reveal the number of binding sites and the affinity for protein binding. It has been found that interaction of the drug with Magnesium Sulphate (anhydrous) results into increasing the affinity and increasing the protein binding of Verapamil Hydrochloride.

Key words: Verapamil Hydrochloride, Protein binding, Equilibrium dialysis, Scatchard plot, Magnesium Sulphate (anhydrous), Ardon's method.

INTRODUCTION

Verapamil Hydrochloride is an oral and intravenous calcium-channel blocking (CCB) agent. It is useful for the treatment of angina, hypertension, supraventricular tachyarrhythmias (Bertram G. Katzung, 1997). It inhibits the influx of extra cellular calcium across the myocardial and vascular smooth muscle cell membranes. Magnesium sulfate is commonly used as a saline laxative. It can also be used to treat hypomagnesemia, in cardiac arrest, asthma, eclampsia. Plasma protein binding is one of the important and useful pharmacokinetic parameters of a drug. There are multiple binding sites on a protein molecule. The pharmacokinetic and pharmacodynamic behavior of a drug is governed by the strength of a complex formed with the protein. Drugs generally form reversible complexes with the plasma proteins that act as a reservoir, releasing the free drug to the circulation. Free form of the drug shows pharmacologic response is metabolized and excreted. Bound drug is gradually released to maintain the equilibrium and thus the pharmacologic response is maintained. Protein binding of a drug is a limiting factor for drug effect. Simultaneous administration of two or more drugs into the systemic circulation can modify the affinity of the drug to bind with plasma protein and thus percentage of protein binding. Due to this modification, the combined therapy can change the volume of distribution, renal and hepatic clearance, and hence drug effect (Donald E. Cadwallader, 1983). This study was aimed to evaluate the influences

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of interaction of Verapamil hydrochloride with Magnesium Sulphate on protein binding of drug in physiological pH and temperature.

MATERIALS AND METHOD

Materials

Verapamil Hydrochloride and Magnesium Sulphate (anhydrous) were kind gifts from department of pharmacy, USTC. Bovine serum albumin (Fatty acid free, fraction V, 96-98%, Sigma) and semipermeable membrane (Medicell, England) and all other reagents were purchased from BDH (England).

Spectral studies

Initial detection of complexation of Verapamil hydrochloride and Magnesium sulphate (anhydrous) has done from the nature of spectra of pure compounds as well as their 1:1 mixtures in chloride buffer 2.4 and phosphate buffer solution of 7.4 at a fixed concentration (0.1×10^{-5}) M.

Job's spectrophotometric method of continuous variation (Vogel, 1978)

In this method, series of absorbance of Verapamil Hydrochloride and Magnesium Sulphate (anhydrous) mixtures with different molar ratios at pH 2.4 and pH 7.4 were measured by keeping the total moles constant. The absorbance of Verapamil Hydrochloride and Magnesium Sulphate (anhydrous) solutions was measured at 278 nm. The observed absorbance of the mixtures at various mole fractions were subtracted from the sum of the values for free Verapamil Hydrochloride and free Magnesium Sulphate (anhydrous). The absorbance difference (D) was then plotted against the mole fractions of drugs in the mixtures. A curve, thus, obtained showed a maximum at a point, which indicated the molar ratios of Verapamil Hydrochloride and Magnesium Sulphate (anhydrous) in the complex.

The Ardon's spectrophotometric methods (Ardon, 1957)

In this method, concentrations of Verapamil Hydrochloride varied while keeping the concentrations of Magnesium Sulphate (anhydrous) fixed at 2×10^{-3} M. The whole experiment was performed in the buffer at pH 2.4 and pH 7.4. The absorbances were measured at 278 nm by using UV-VISIBLE spectrophotometer. For calculation, the Ardon's equation was used. This equation is given below:

$$\frac{D}{(D - \epsilon_A C)} = \frac{1}{KC(\epsilon_{com} - \epsilon_A)[B]} + \frac{1}{C(\epsilon_{com} - \epsilon_A)}$$

Where,

D = Absorbance of the mixture.

B = Molar concentration of the Verapamil Hydrochloride.

C = Molar concentration of the Magnesium Sulphate (anhydrous).

C_{com} = Molar extinction co-efficient of the complex.

C_a = Molar extinction co-efficient of the Verapamil Hydrochloride.

The value of C_a was chosen as 1, which is an essential condition for validation of the method. The value 1 or 1/(D - C_aC) were plotted versus 1/[C] to get the straight lines. The concentration of Magnesium Sulphate was kept constant at 2×10^{-3} M (denoted by C in the equation) & the concentration of interacting species Verapamil Hydrochloride was varied (denoted by B in the equation). The 1:1 complex gave a straight line in the plots with an intercept and a slope. The stability constant of the complex was given by the relation:

$$K = \text{intercept} / \text{slope}$$

Equilibrium dialysis (Singlass, 1987)

Equilibrium Dialysis is one of the methods used for the determination of the protein binding of any compound. This method was developed by E.Singlass. Membrane was cut into small pieces usually 4 cm in length and taken in a 500 ml beaker containing de-ionized water. The pieces of membrane were dipped into de-ionized water and heated for more than 8 hours in order to remove sulfur as sulfur may interfere in the overall binding process. The temperature was maintained between 65-70°C, and hot water was replaced by fresh de-ionized water. The activated membrane pieces were filled with BSA solution with different concentrations of drug and their (1:1) drug-metal mixture, keeping the total volume 4ml. The membrane bags were immersed in conical flasks containing 60 ml of phosphate buffer solution having pH 7.4. Conical flasks were shaken gently at (37±0.5) °C for about 6 hours in metabolic shaking incubator. After proper shaking, the absorbance of buffer (outside the membrane bags) was measured at 278 nm using the UV-VIS spectrophotometer and the concentrations of the bound and unbound drugs were found using a standard curve.

Calculation of the percentage of protein binding:

The percentage of protein binding (F) is given by:

$$F = B / (B - A) \times 100$$

Where,

A = Molar concentration of free drug in buffer compartment

B = Molar concentration of total drug in protein compartment

Calculation of number of protein binding sites and the affinity constants

The Scatchard method (Goldstein, A et al., 1974; Scatchard, G. 1949) was used for this purpose and a curve was thus produced by plotting 'r/[A]' versus 'r' using the equation

$$r = \frac{[B] - [A]}{[\text{Protein}]}$$

Where, r = the ratio between the molar concentration of the bound drug and the molar concentration of protein. The curve so obtained when extrapolated gave an intercept on the Y axis

representing nK_a , the intersection on the X-axis representing n and the slope of the line being K_a , Where, n = the number of binding sites on the protein available to bind drug molecule or its complex, K_a = affinity constant (or, binding force) for the binding of drug or its complex.

RESULTS

Spectral study

In spectral studies, it was seen that Verapamil Hydrochloride gives a sharp peak at 278 nm when magnesium Sulphate salt mixed with Verapamil Hydrochloride in 1:1 ratio. The intensity of the peak of Verapamil hydrochloride changes remarkably i.e. absorption characteristics are altered due to interaction but the position of the compound do not shift (Fig.1 & 2).

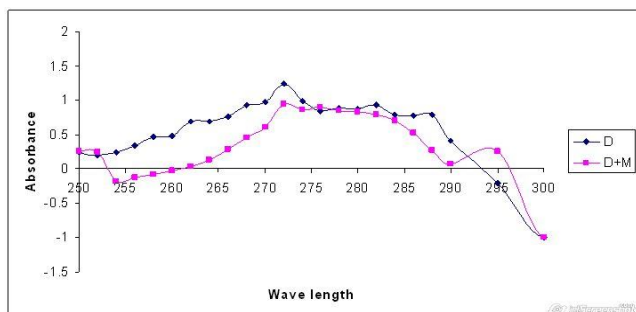


Fig. 1: Comparison study of Verapamil Hydrochloride alone and with $MgSO_4$ at pH 2.4.

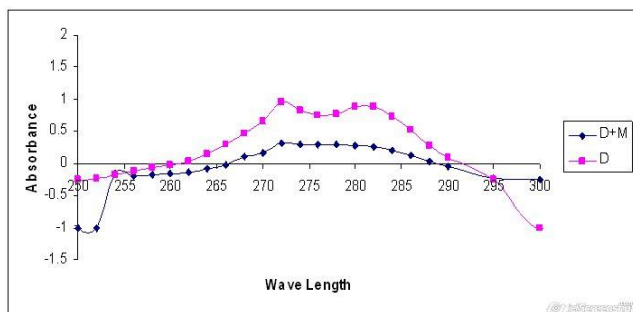


Fig. 2: Comparison study of Verapamil Hydrochloride alone and with $MgSO_4$ at pH 7.4.

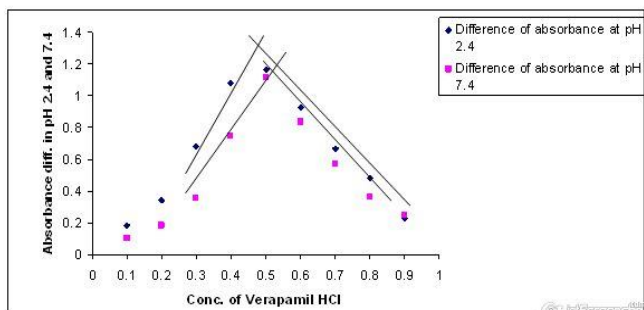


Fig. 3: Job's Plot for Complexation of Verapamil Hydrochloride with $MgSO_4$ at pH 2.4 & 7.4

Study of job's method

The molar ratios of the complexes of Verapamil Hydrochloride with Magnesium Sulphate (anhydrous) were

estimated by job's method of continuous variation. The observed absorbance values were measured in pH 2.4 and 7.4 at various concentrations (0.1×10^{-5} to 0.9×10^{-5} M) of Verapamil HCl and magnesium Sulphate at 278 nm. The Job's plots at pH 2.4 and 7.4 were obtained by plotting absorbance differences against the mole fraction of the drug (Verapamil Hydrochloride) which are presented in Fig 3.

Study of Ardon's method

Ardon's plot confirmed the formation of 1:1 complex of Verapamil hydrochloride and Magnesium Sulphate at pH 2.4 and 7.4, since the method is valid for only 1:1 complexes.

The Ardon's plots gave straight lines intercept which are presented in Fig.4 indicating the formation of 1:1 complexes at both pH.

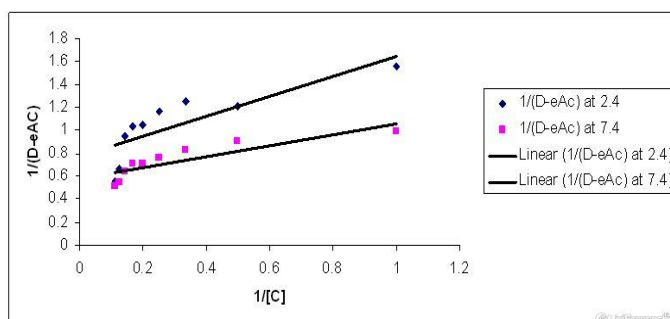


Fig. 4: Ardon's plot for Verapamil Hydrochloride with $MgSO_4$ at pH 7.4 and 2.4.

Estimation of Stability Constant

The value of stability constant for the complexation of Verapamil Hydrochloride with Magnesium Sulphate at pH 2.4 and 7.4 were obtained from the spectral data using Ardon's plot. The values for stability constant were calculated from the slopes and intercepts of the straight lines from these plots. It was seen from the Ardon's equation that the values of stability constant was given as (Intercept)/(slope). The value of intercept and slope were calculated by Least Squares Method using the following equation:

$$y = mx + C$$

The values of stability constants for the drug-metal system at pH 2.4 and 7.4 are given in the Table 1.

Table1: Values of stability constant.

System		Stability Constant, K
Verapamil Hydrochloride & Magnesium sulphate	pH 2.4	0.8947
	pH 7.4	1.2178

Study of protein binding

The *In Vitro* determination of percentage of protein binding of Verapamil Hydrochloride and their 1:1 mixture with Magnesium Sulphate (anhydrous) was done by equilibrium dialysis method at physiological temperature (37 ± 0.5)°C and at pH 7.4. The observed values for drug alone and with metal are given in Fig. 5.

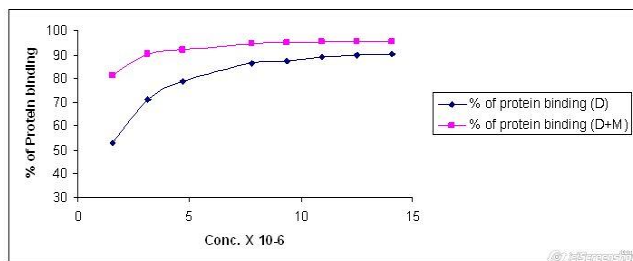


Fig. 5: % of protein binding of drug alone & in presence of metal (1:1 mixture) to BSA at pH 7.4.

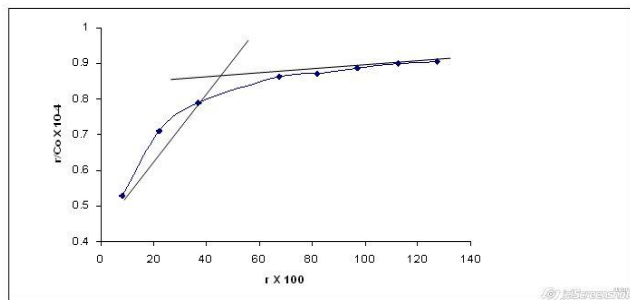


Fig. 6: Scatchard plot for protein binding of Verapamil Hydrochloride to BSA.

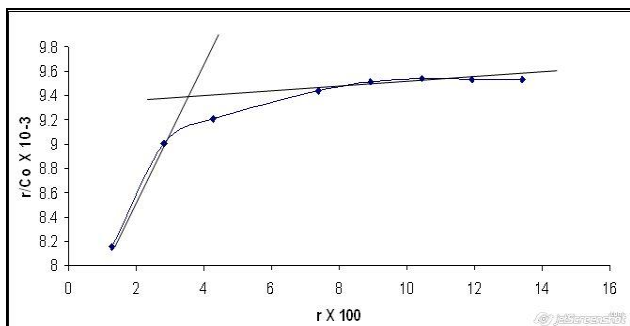


Fig. 7: Scatchard plot for protein binding of Verapamil Hydrochloride to BSA in presence of Magnesium Sulphate.

DISCUSSION

In the present work, the interaction of an important anti-hypertensive drug, Verapamil Hydrochloride, with Magnesium Sulphate has been studied in the aqueous system at pH 2.4 and 7.4 by a variety of physical method like inspection of spectral behavior, Job's method of continuous variation and Ardon's straight line plots by spectrophotometry. The protein binding experiments of the free drugs as well as the combined systems were studied by equilibrium dialysis method. From spectral study, it has been seen that Verapamil Hydrochloride gives a sharp peak at 272 nm. When magnesium Sulphate salt mixed with Verapamil Hydrochloride at 1:1 ratio, the intensity of the peak of Verapamil hydrochloride changes remarkably (absorbance decreases) i.e. absorption characteristics are altered due to interaction but the position of the compound do not shift. Job's plot has given the molar ratio of complexes of Verapamil Hydrochloride and Magnesium Sulphate. At pH 2.4 and 7.4 Verapamil Hydrochloride forms strong 1:1 complexes with Magnesium sulphate indicated as 'Λ' shaped curves. These curves may indicate strong kinetics of complexation between Verapamil Hydrochloride & Magnesium

sulphate. The Ardon's spectrophotometric plots also confirm the phenomenon of 1:1 complexation which is indicated by straight lines. The stability constant of the complex has estimated from this straight line plots using Ardon's equation.

In protein binding studies it is found that at a low drug concentration the percentage of protein binding attains a steady state plateau condition (87%). This may be the saturated zone of protein binding of the drug. In our experiment, the highest percentage of binding of Verapamil Hydrochloride with BSA has found to be 90% and 52% at high and low concentration range respectively. In presence of $MgSO_4$, the percentage of protein binding of drug increased (52 to 81) % at lower concentration range and (90 to 95)% at higher concentration zone. In brief, $MgSO_4$ causes an increase in protein binding of Verapamil Hydrochloride leading to the formation of stable 1:1 Verapamil Hydrochloride-Magnesium Sulphate complex. This means that the increase in percentage of protein binding may be due to the capture of binding sites in the protein by Magnesium Sulphate or Verapamil Hydrochloride & Verapamil Hydrochloride-Magnesium Sulphate complex. Thus, possibility of adverse effect of Verapamil Hydrochloride may become prominent in presence of Mg or similar drugs in the body system. It can therefore be inferred that a careful consideration is needed during concurrent administration of Verapamil hydrochloride with magnesium sulfate.

The Scatchard plots (Fig. 6, 7) shows that there are at least two classes (Class I and Class II) of binding sites in BSA for Verapamil Hydrochloride and its (1:1) complex with Magnesium Sulphate. The lines represent the individual plots of the two classes of sites. The number of binding sites of Verapamil Hydrochloride alone in BSA is found to be 0.052 and 1.162 for class I and class II respectively. The affinity constants k_1 and k_2 associated with these respective classes of binding sites were 9 and 0.7 respectively.

The number of binding sites of Verapamil Hydrochloride — Magnesium Sulphate system in BSA is found to be 0.020 and 0.694 for class I and class II respectively. The affinity constants k_1 and k_2 associated with these respective classes of binding sites were 352.2 and 13.5 respectively.

From the data stated in the Table 2, in class I binding sites, it is obvious that a value of affinity constants for Verapamil Hydrochloride alone is much lower than its 1:1 complexes with Magnesium Sulphate i.e., the presence of Magnesium Sulphate with Verapamil Hydrochloride at physiological temperature and pH conditions, cause an increase in values of affinity constant. The values of affinity constants in class II binding sites revealed the same results. i.e., decrease in volume of distribution of Verapamil Hydrochloride (Hossain, et al., 1994).

Table2: Binding parameters of Verapamil Hydrochloride.

Systems	Class I		Class II			
	$n_1 \times 10^3$	$k_1 \times 10^{-3}$	$n_1 k_1$	$n_2 \times 10^3$	$k_2 \times 10^{-3}$	$n_2 k_2$
Verapamil HCl alone	0.052	9	0.4747	1.162	0.7	0.8137
Verapamil HCl – Magnesium Sulphate	0.0221	352.2	7.7488	0.694	13.5	9.3703

It was observed that the affinity for protein is higher for 1:1 complexes of Verapamil Hydrochloride with Magnesium Sulphate than that of Verapamil Hydrochloride alone. As a result, the intake of Verapamil Hydrochloride as Magnesium Sulphate complex or the concurrent therapy can decrease both hepatic and renal clearance of the drug as well as it can increase the half-life of the drug (Hansten, et al., 1989).

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