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The use of hollow fibre supported liquid membranes and Van't Hoff plots in the study of diphenylhydramine and chlorpheniramine drugs binding to human α_1-acid glycoprotein

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ABSTRACT

The study of the drug-protein binding using hollow fibre supported liquid membrane (HFSLM) and Van't Hoff plots is reported. Binding of diphenhydramine hydrochloride (DPH) and (±) – chlorpheniramine maleate salt (CPA) to human α_1-glycoprotein (AGP) was investigated at optimal conditions of the HFSLM. The binding was found to be effective within the first 400 minutes for DPH and about 2000 minutes for CPA after incubation at the concentration ranges studied. The effect of pH in the binding of the drugs to AGP was studied and showed that the optimal pH was 8 and 9 for DPH and CPA respectively. The kinetic patterns of the interactions at various temperatures as well as at physiological conditions of temperature and pH were carried out and by using Van't Hoffs' plot it was found that the association constant for DPH was 0.96 x 10^3 mol L^{-1} and that of CPA was 1.02 x 10^3 mol L^{-1}.

KEYWORDS

Hollowfibre;
Drug-protein binding;
vant Hoff plots.

INTRODUCTION

The study of the extent of interaction between drugs' active ingredients and plasma proteins is crucial as it reveals the effect of the interaction on the physiological, pharmacokinetic and the pharmacodynamic properties of a drug[1]. When the drug is ingested a fraction of the active ingredient will be bound to plasma protein displaying stereo selective properties due to the chiral stereochemistry of plasma proteins[2], while the remaining fraction will be free (unbound). It is this free fraction which has got the possibility to penetrate the cell membrane or walls of blood vessels to exert specific physiological conditions to the victim depending on the chemistry of the drug and the bound tissue[3].

The major drug binding proteins in plasma are human serum albumin (HSA), α_1-acid glycoprotein (AGP) and lipoproteins. HSA and AGP have structurally selective binding sites for drugs, in the same way that the active sites of enzymes are structurally selective for substrates[4]. The AGP also known as orosomucoid (ORM) is one of the plasma globulin glycoprotein found in human plasma at concentrations ranging between 0.6-1.2 mg/mL which in comparison to other plasma proteins makes 1-3 % plasma protein[5]. Since this protein is present in the body plasma and the fact that it has the

ability to bind organic molecules including those used in drug formulations it is important to investigate its interaction with these drug molecules. AGP interacts mainly with basic drugs with one binding site selective for basic drugs and like all glycoproteins it contains chains of glycans covalently linked to the main polypeptide side-chains as well as the carbohydrate molecule that is glycosilated to the protein.[6]

The drug-protein binding process may be represented by the following reaction equations:

$$\text{free drug} + \text{free protein} \leftrightarrow \text{drug} - \text{protein comple} \quad (1)$$

The free concentration of the drug and the unbound proteins are normally expressed as the sum of the fraction of the total drug and the total protein respectively.

$$\text{[Drug protein,DP]} + \text{[Free concentration of the drug,D}_f\text{]} = \text{[TotalDrug,D}_t\text{]} \quad (2)$$

The extent to which a drug is bound in plasma or blood is usually expressed as the fraction unbound (fu).

$$\text{Fraction unbound } (f_u) = \frac{\text{[Unbound drug]}}{\text{[Total drug, D}_t\text{]}} \quad (3)$$

The tighter the binding, the lower is the fraction unbound. There is a difference between the fraction of the drug that is unbound and unbound concentration of the drug. The fraction unbound of a drug is determined by a number of factors such as the affinity of the drug for the protein, the concentration of the binding protein and the concentration of drug relative to that of the binding protein. The free drug concentration is controlled by the free drug clearance which is independent of the plasma binding. Generally only the free (unbound) drug is metabolized and can access to the receptor and the free drug concentration is controlled by the free drug clearance which is independent of the plasma binding and it is expressed in % or by f_u (free fraction).

The free fraction: f_u; may be mathematically presented by the following equations;

$$\text{Free fraction } (f_u) = \frac{\text{Free fraction concentration } (C_{free})}{\text{Total concentration } (C_{tot})} \quad (4)$$

$$\text{Total concentration, } C_{Tot} = \frac{C_{free}}{f_u} \quad (5)$$

A number of analytical methods have been devised and used for the measurements of drug-protein binding. Among these methods are dialysis which was used in the studies of drug-cyclodextrin stability constants[7,8], ultrafiltration which was employed in the studies of serum-protein binding of oxycodone and morphine as well as in the determination of human plasma binding of baicalin[9,10], spectrophotometric, which was used in the in vitro binding studies of fusidic acid[11], sulfoureas and phenothiazines to bovine serum albumin[12] as well as in the binding studies of amitriptyline and imipramine to bovine serum albumin[13]. However these methods are time consuming and the protocols demands many steps and labour intensive and they are also known to lack reproducibility[14]. The main problem with fluorescent is the lack of fluorescence change upon binding and/or a change of fluorescence due to a mechanism other than binding (e.g. photoxidation). The main limitation with spectrophotometric probes is that the methods are indirect and they can only be applied to drugs that can replace the probe drug. HPLC is another method that has been used successfully however; special columns need to be used[14].

In this particular study the drug-protein binding between AGP and two drugs (DPH and CPA), using hollow fibre supported liquid membrane extraction with high performance liquid chromatography is reported.

The use of HFSLM in the study of drug-protein binding is attractive in that it has high selectivity and specificity which makes the method more reliable[15,16]. The technique, involves the establishment of equilibrium between the aqueous plasma sample containing the drug in which the fibre is immersed, the organic phase impregnated in the hollow-fiber membrane pores and an aqueous receiving buffer filled in the hollow fibre lumen. With this system it is possible to measure the free fraction of the drug (unbound) present in the receiving aqueous buffer using suitable analytical instruments such as chromatographic systems and will thus give the measure of the drug-protein binding[17,18]. In the Determination of free fraction by HFSLM, the mass transfer is driven by the concentration difference of the uncharged species:

$$\Delta C = \alpha_D \times C_D - \alpha_A \times C_A \quad (6a)$$

or:

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$$\Delta C = C_F - \alpha_A \times C_A \quad (6b)$$

Where, C_F = free fraction of the analyte in sample, C_A = total (measured) conc. in acceptor, α_A = uncharged fraction of total conc. in acceptor, and α_A is not very near zero

At equilibrium, $\Delta C = 0$, but becomes near zero when the pH of acceptor phase ix very low. However, the free fraction is given by the following mathematical equation:

$$C_F = C_A \times \alpha_A \quad (7)$$

Since α_A is a known parameter it is then possible to control such that, the free fraction can be calculated as follows:

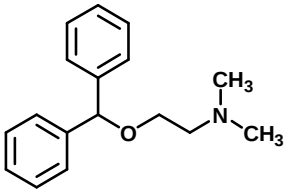
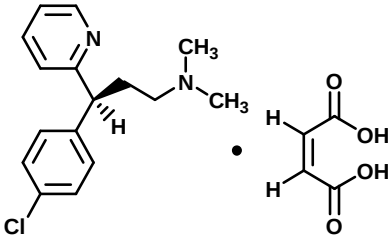
$$\alpha_A = \frac{1}{1 + 10^{(pKa - pH)}} \quad (8)$$

EXPERIMENTAL

Reagents and chemicals

Standards of DPH, and CPA were purchased from Sigma-Aldrich (St. Louis, MO, USA) (TABLE 1). A 100 uL Hamilton syringe was purchased from Perkin Elmer instruments. Analytical grade NaOH and HCl (32%) were purchased from Merck (Darmstadt, Germany) for adjusting the pH of the sample solutions. HPLC grade methanol was obtained from Merck (Darmstadt, Germany). Mobile phases were filtered through 0.45µm Millipore filter papers. Hollow fibre was Q3/2 Accurel polypropylene hollow fiber membrane (200 µm wall thickness, 600 µm inner diameter, 0.2 µm pore size) purchased from Membrane GmbH (Wuppertal, Germany).

TABLE 1 : Structures of the compounds studied, their pKa values and CAS numbers

Compound	Structure	pKa	LogP	Solubility at 25 °C	CAS #	Ref
Diphenylhydramine hydrochloride		9.12 (base)	3.75	0.363 mg/mL	147-24-0	24-26
S-(+)-Chlorpheniramine maleate salt		3.86(pyridine ring) 9.18 (base)	3.74	160.0 mg/mL	2438-32-6	24-27
α-1-glycoprotein (AGP)	Mwt 42 000 Conc g/L → 0.4-1.0 mM → 9-23					

Preparation of standards

Stock solutions of 1000 mg/L for both DPH and CPA were prepared by dissolving appropriate amounts in double distilled water in volumetric flasks. From these stock solutions standards of lower concentrations were made by diluting aliquot amounts from the stocks. AGP was dissolved in Sörensen's phosphate buffer according to Urien (1995)[19]

Hollow fibre supported liquid membrane extraction of the drugs and drug-protein binding studies

The study of DHP/AGP and CPA/AGP binding using HFSLM proceeded in three stages. In the first set of experiment, the drug standards were used to establish the best set of HFSLM optimal conditions for extraction. Therefore optimization of parameters such as best organic solvent, sample pH, extraction time, stirring speed and acceptor buffer were studied. The second part involved the study the binding pattern of DPH and CPA to the protein (AGP), whereby the drugs (DPH as well as CPA) were each mixed with AGP at a 1:1 ratio and then adjusted to basic conditions (pH 12 for DPH and pH 13 for CPA) before being incubated at a selected temperature prior to extraction using

HFSLM. The incubation time was varied such that sampling was being done for the analysis of the unbound faction at specified time intervals. The unbound fraction of the drugs crossed the organic membrane comprising of 5% tri-n-octylphosphine oxide (TOPO) in isooctane and were extracted inside the lumen of the hollow fibre containing acceptor phase which was adjusted to acidic conditions using acetate buffer (pH ~ 3.00 for DPH and pH ~ 0.5 for CPA) at stirring speed of 310 rpm. Each extraction proceeded for 45 minutes. Then the lumen contents of the HFSLM was flushed out using a syringe into a 100 µL sample insert which was fitted into HPLC sample vials. Volumes of about 7µL were obtained and 5 µL of this extract was injected into the HPLC for analysis. The third set of experiment involved extraction of DPH and CPA from cough syrups, CS1, containing DPH and CS2 containing CPA) and use the extracts to study the binding behaviour with AGP. The experimental set up used in this work is similar to the one reported earlier[20].

Chromatographic conditions

Shimadzu LC-20AT prominence HPLC with DGU-20A5 prominence degasser, SIL-20A prominence autosampler and SPD-M20A prominence DAD detector was used for all separation and detection of extracted compounds. The column used was an XTerra MS C_{18} 3.5µm x 3.0mm x 150mm column from Waters (Ireland). The mobile phase was 75% methanol and 25% water. The isocratic mode of elution was used throughout at a flow rate of 0.3 ml/min.

RESULTS AND DISCUSSION

Effect of pH on drug-protein binding

To study the effect of pH on the drug protein binding, the drugs (2 µg/L of each of DPH and CPA) were mixed with AGP (1:1, v/v) and these solutions were adjusted to a range of pH from pH 6 to pH 11 and incubated at room temperature. DPH was incubated for ~ 240 minutes while CPA was incubated for ~ 2000 minutes. The free as well as the bound fraction were then determined. The results for this experiment are shown in Figure 1 whereby the pH seems to control the binding process. The fraction bound was increasing with pH from pH 6 to 8 for DPH and to 9 for CPA.

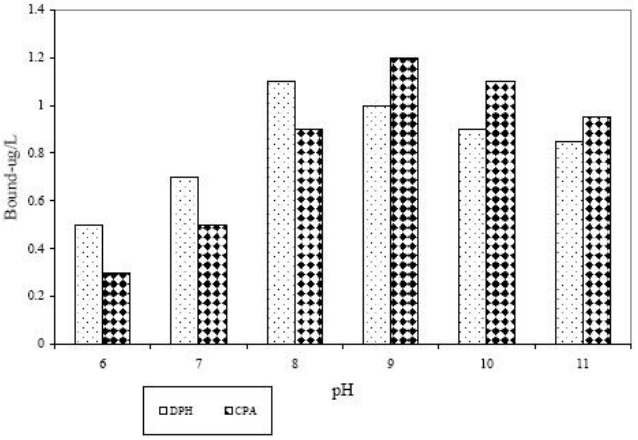


Figure 1 : Effect of pH on drug-protein binding.

The pH was also found to have control on the association constant, as it increased with pH to optimal levels of 8 and 9 for DPH and CPA respectively, when the temperature was kept constant at 37 ºC. At these pH values nearly half of the drug molecules are neutral since their pKa values are within the pH range. Below and beyond this pH range more than half molecules are ionized and this causes the low values of the association constants. Generally, the association constants of the neutral forms of ligands are higher than those of the ionized forms of ligands[21]. This may be due to the type of interaction that exist between these drugs and the protein, which could be hydrophobic and/or van der Waals[21] Another plausible explanation could the fact that, the complexation of neutral ligands with AGP may be favoured because neutral molecules are less hydrated than ionized ones[22] and this leaves more sites available for drug binding.

Effect of concentration on drug protein binding

The effect of concentration on the drug-protein binding was studied by gradually increasing the concentration of the drugs, at levels, 2, 4, 6, 8 and 10µg/L. The results are shown in Figures 2a and b. At increased drug concentration, the bound fraction diminishes presumably due to the depletion of the binding sites.

Drug-protein interaction

The process of binding of drug to plasma (and tissue) proteins is very important as it governs the process of drug disposition and distribution in the body system. Binding of drugs to proteins has also a very important effect on drug dynamics as only the free (unbound) drug interacts with specific proteins.

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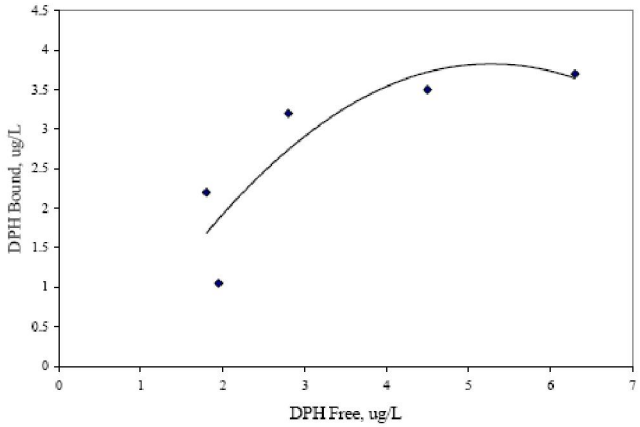

Figure 2a : Effect of concentration on the DPH-AGP binding.

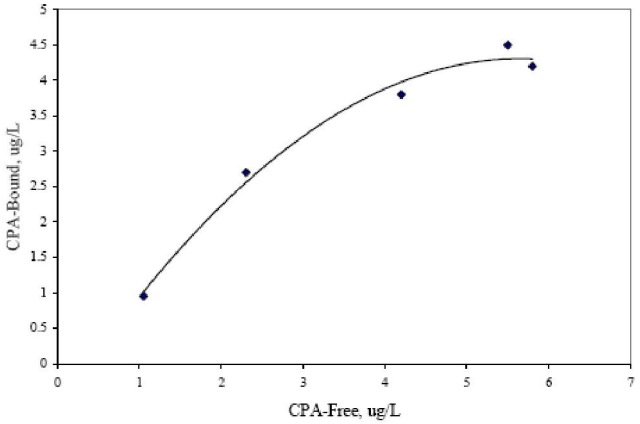

Figure 2b : Effect of concentration on the CPA-AGP binding.

From the results of drug-protein binding for DPH/AGP and CPA/AGP (Figures 3a and 3b), it shows that interaction actually does exists.

Figures 3a and 3b show that after about 200 minutes of incubation at a total concentration is 2 mg/L and the unbound concentration of DHP was reduced to half while the same proportion was achieved after ~ 1300 minutes in the case of CPA.

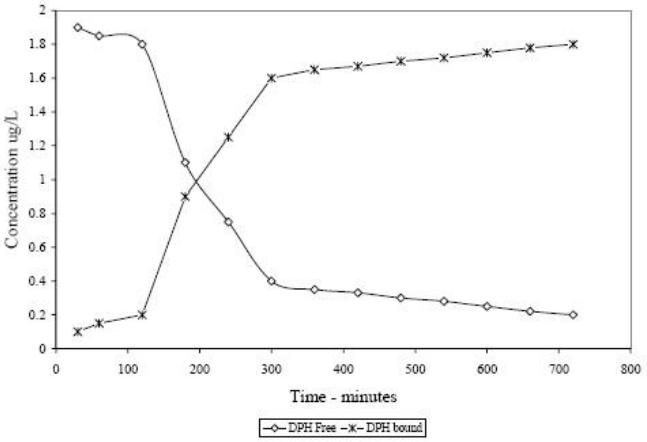

Figure 3a : Free and bound fractions in DPH-AGP binding and the kinetics studies at physiologic conditions (pH 7.4 and 37 ºC).

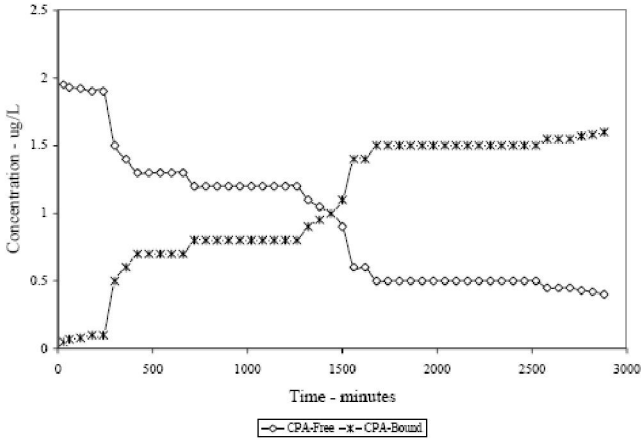

Figure 3b : Free and bound fractions in CPA-AGP binding and the kinetics studies at physiologic conditions (pH 7.4 and 37 ºC).

Measurements of association constant between the drugs and AGP at physiological conditions

The measurements of association constants and other thermodynamic parameters were conducted at physiological pH of 7.4 and temperatures of 37 °C. It is known that the protein bound fraction and the free fractions can be related by the law of mass action which states that when two species (X and Y) reacts at a particular temperature the affinity that may exist between them, is proportional to the active masses, that is the concentration of X as well as that of Y, raised to a particular power. A mathematical model for this relationship may be given as follows:

$$\text{Affinity} = k[X]^x[Y]^y \quad (9)$$

Where, k is the association constant

Therefore at equilibrium this reaction may be represented mathematically by the following equation:

$$k[X_1]_{eq}\,[Y_1]_{eq} \rightleftharpoons k[X_2]_{eq}\,[Y_2]_{eq} \quad (10)$$

But, at equilibrium, the forward reaction = backward reaction, therefore,

Forward reaction $\rightleftharpoons$ backward reaction, that is,

$$k_+[X_f][Y_f] \rightleftharpoons k_-[X_b][Y_b] \quad (11)$$

Therefore, the equilibrium constant

$$(K) = k_+/k- \rightleftharpoons [X_b][Y_b]/[X_f][Y_f] \quad (12)$$

However, the association constant is related to free

Gibbs energy (ΔG°) by the following equation

$$k = e^{-\Delta G°/RT} \quad (13a)$$

or;

$$\Delta G = -RT\ln k;\ \Delta G/RT = -\ln k \quad (13b)$$

Where, R = Universal gas constant (1.987 cal/mol -K) and T = temperature in deg K.

Effect of temperature on binding behavior of the DPH and CPA to AGP

The binding tendency of DPH and CPA to AGP was investigated at various temperatures (10, 25, 37 and 42) ºC at physiological pH of 7.4. The results showed that, the association constant was decreasing with increase in temperature (Figures 4a and 4b). When ln k was plotted against inverse temperature (1/T) (see Figure 4c), a linear relationship was obtained. This implies that there is a higher affinity of drugs to protein at lower temperatures rather than at higher temperatures. Generally the affinity as observed from the ln k values (TABLE 2) are very low which indicates that the association was very weak and as explained previously short lived. This may not be termed as binding per se but rather association between drugs and the protein. The values for k obtained in this study are 0.96 x 10^3 mol L^{-1} and 1.02 x 10^3 mol L^{-1} for DPH and CPA respectively. NB: The values for ΔGº used for the calculations of ln k were obtained from Zeiss and Bauer-Brand, (2006)[23].

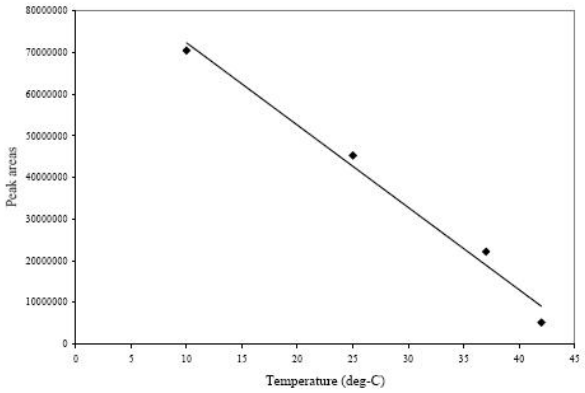

Figure 4a : Effect of temperature on the binding of CPA to AGP.

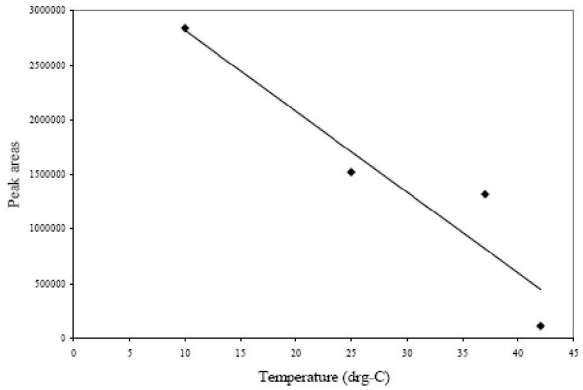

Figure 4b : Effect of temperature on the binding of DPH to AGP.

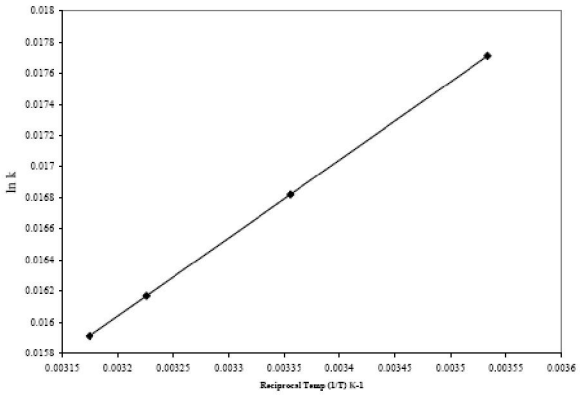

Figure 4c : Van't Hoff plot for the interaction of CPA with AGP at pH 7.4.

TABLE 2 : Van't Hoff parameters for CPA.

Temp (K)	1/T (K^{-1})	ΔG kJ mol^{-1} [Ref 23]	RT(cal/mol)	ΔG/RT = lnk
283	0.003533568	-9.96	562.321	0.017712303
298	0.003355704	-9.96	592.126	0.016820744
310	0.003225806	-9.96	615.970	0.016169618
315	0.003174603	-9.96	625.905	0.015912958

CONCLUSIONS

A combined HFSLM and HPLC-UV/DAD has shown success in the study of drug-protein binding. Important factors influencing drug-protein binding have been studied. It was observed that at high concentra-

tions the binding is affected due to saturation of the binding sites in the protein (AGP). pH also is important in influencing binding as well as the association constants. The two drugs studied DPH and CPA has different optimal binding optimal pH values. The association constants revealed that the binding is too weak to such an extent that it may not be called binding but merely an association and which cannot last long and hence a conclusion that these drugs may be distributed and eliminated faster than many others. This study has also shown that DPH is being distributed and eliminated faster than CPA.

To the best of my knowledge to binding studies involving CPA and DPH has so far been reported and therefore there are data for comparison.

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