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BINDING OF PIPRINHYDRINATE TO PLASMA PROTEINS AND THEIR SUBSTITUTES

M.S. Mohamed, F.S. Gazy, S.S. Abu-Zaid, B.W. Müller[1] and M.M. Abd ElRahman

Department of Pharmaceutics, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt
[1] Department of Pharmaceutics, Institute of Pharmacy, Kiel, Germany

هذه الدراسة استخدمت طريقة الديلزة المتوازية لدراسة ارتباط ببرينهيدرينات مع زلال بلازما البقر باستعمال محلول الفوسفات المنظم بعد ضبط الضغط الاسموزى مع الدم باستعمال كلوريد الصوديوم ، وقد أظهرت النتائج أن ببرينهيدرينات يتحد مع زلال بلازما البقر وأن معامل الربط الأولى K_1 والثانوى K_2 يساوى ٤,٦×١٠ ^{٤} م$^{-١}$ ، ٠,٧×١٠ ^{٤} م$^{-١}$ وأن عدد مواقع الربط n_1 ، n_2 تساوى ٠,٦٨ ، ٠,٦٩ على التوالى وقد دل الرسم البيانى للنتائج على وجود أكثر من موقع ربط على جزئى الزلال للعقار وإنه عند ثبات تركيز الزلال فإن النسبة المئوية للعقار المتحد مع الزلال تتناسب تناسبا عكسيا مع تركيز العقار المستخدم.

وقد أظهرت النتائج أن معامل الربط بين العقار وزلال بلازما البقر لا يتأثر بزيادة تركيز الزلال أو بزيادة الأس الايدروجينى ويتغير بتغير المحلول المنظم مثل محلول الفوسفات أو هيدروكلوريد التريس أو الخلات. وأوضحت النتائج أن أيون الكلوريد قلل قيمة معامل الربط بين العقار والزلال وهذا يمكن أن يعزى الى تأثير أيون الكلوريد.

وقد أوضحت التجارب أن العقار لا يرتبط مع الدكستران ذو وزن جزيئى مختلف وهو ٤٠٠٠٠ ، ٢٦٦٠٠٠ ، ٥٠٠٠٠٠ ، وكذلك الليفوزان والهيتاستارش فى وجود محلول الفوسفات المنظم.

The binding of piprinhydrinate to bovine serum albumin (BSA) was studied in isotonic Sorensen's phosphate buffer pH 7.4 at 25±1°C, using equilibrium dialysis technique. Piprinhydrinate was bound to BSA and the plot was curved indicating the presence of more than one binding site on the albumin molecule. At fixed protein concentration the percentage of bound drug to bovine serum albumin was inversely proportional to the concentration of the drug. But the binding parameters (binding constants as well as the number of binding sites) were found to be unaffected as the albumin concentration increased. However, binding parameters showed no significant difference due to pH change. Phosphate buffer, Gomori's tris-HCl buffer and Walpole's acetate buffer have different effect on the binding of piprinhydrinate to BSA; the values of the binding parameters can be arranged as follows: Sorensen's phosphate buffer > Gomori's tris-HCl > Walpole's acetate buffer. Also, it was found that chloride ions decrease the amount of the drug bound to BSA. The interaction of the drug with dextrans (40000, 266000 and 500000) in isotonic Sorensen's buffer pH 7.4 was investigated. No interaction was observed between the drug and the tested dextrans under the condition of the experiment. Also laevosan and hetastarch showed no significant interaction.

INTRODUCTION

Protein binding is an important factor in the distribution, metabolism, elimination and activity of many administered medicaments; only the free fraction of drug exerts pharmacological activity. The binding affinity may be important in the clinical setting[1-3].

Drug binding takes place in the plasma. It involves the combination of a drug with a plasma protein, usually albumin, to enhance its solubility and to facilitate its transport through the circulation. Protein binding becomes clinically important when involves a high

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proportion i.e. 90% of drug in the blood[4-6].

Since binding process is competitive in nature, any factor which modifies the nature of the binding system will modify the extent of binding of the drugs. Consequently, pH range, change in buffer ions composition, temperature change or presence of a second drug may have a significant effect on drug protein interaction[7-8].

Plasma protein binding, principally to albumin, may have a profound effect on overall drug activity. The following effects have become evident from protein binding investigations: (a) only free drug (unbound) is available for activity or tissue distribution; (b) tightly bound drugs tend to be distributed in a smaller body space or volume; (c) marked decrease in protein binding occur in uremia, hypoalbuminemia and hepatic failure; and (d) the delayed elimination of highly bound drugs is a result of glomerular filtration and hepatic uptake being directly proportional to free drug in the serum[9].

This study deals with the binding of piprinhydrinate, an antihistaminic drug, to bovine serum albumin, dextran, laevosan and to hydroxyethyl starch (hetastarch), as plasma substitutes. The factors that can affect the binding process were investigated. Comparison of equilibrium dialysis and ultrafiltration techniques was also studied.

EXPERIMENTAL

Materials

Piprinhydrinate: (Promanta-Germany), bovine serum albumin, purified and lyophilized, fraction V, molecular weight 67000 (Fluka-Switzerland). Dextran, molecular weight 40000, 266000 and 500000 (Sigma-U.S.A.). Laevosan, molecular weight 180.2 (Haes-Germany). Hetastarch (2-hydroxyethylstarch) molecular weight 450000 (Fresenius AG-Germany). Potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, hydrochloric acid, Tris-hydroxyaminomethane, acetic acid, sodium acetate (Merck-Germany). Sorensen's phosphate buffer pH 5.6, 6.2, 6.6, 7.0 and 7.4 were prepared as described in Docoumenta Geigy[10]. Walpole's acetate buffer[10]. Gomori's tris-HCl buffer 7.4[10]. Cellulose dialyzer membrane (Nadir dialyses schlauch, diamer 38 mm, pore size 25-80 Ao- Hochest-Germany). An amicon diaflo ultrafiltration membranes 10 Y M_{10} 25 mm Lot Ad 05525 A (Amicon corporation, Danvers, Ma)

Apparatus

Shaker with teflon dialysis cells (Christian - Albrechts University, Instit. of Pharmacy - Kiel - Germany). pH meter (Microprocessor pH/ion meter PM x 2000, Germany). Magnetic stirrer (Janke and Kunkel Ika-Werk Staufen-Germany). An Amicon Diaflo ultrafiltration apparatus (Model 3 Micro-volume stirred ultrafiltration cell [No. 5106] - England). UV spectro-photometer (Tegimenta AG., Uvikon 810, 243616, Switzerland.).

Methods

Determination of the equilibrium time

The dialysis cells containing the specified solutions were shaked for several hours in order to determine the time which is sufficient to attain the equilibrium: A 2.5 ml of isotonic Sorensen's phosphate buffer pH 7.4 containing $4x10^{-3}$ M (initial concentration) was injected in the upper compartments of the cell, 7.5 ml of bovine serum albumin solution $2.99x10^{-4}$ M was injected in the middle compartment of the cell and 2.5 ml of isotonic Sorensen's phosphate buffer pH 7.4 was injected in the lower compartment of the cell. The filling of each compartment of the cell was done through two stoppered side holes. The cells were rocked for specified periods at $25\pm1°C$ in order to determine the most appropriate period for carrying the experiment. Samples were withdrawn at specified periods for analysis. The absorbance of the free drug was measured spectrophotometrically at 248 nm. against the same buffer and plotted against time.

Binding studies of piprinhydrinate to bovine serum albumin

The binding of piprinhydrinate to bovine serum albumin was studied as a function of the concentration of the drug, pH buffer system and chloride ion.

The experiments were carried out using the equilibrium dialysis technique[11].

Binding of piprinhydrinate with different plasma substitutes

The procedure for binding studies of piprinhydrinate to bovine serum albumin was adopted here using dextrans (molecular weight 40000, 266000 and 500000) in a concentration 2%, 4% and 6%. Also, the binding studies of piprinhydrinate to laevosan (M.wt 180.2) and hetastarch in a concentration 2%, 4% and 6% were carried out.

Moreover, in order to verify the results obtained by the equilibrium dialysis technique, the ultrafiltration method was also used[10]. No significant differences between the results could be demonstrated. Accordingly the equilibrium dialysis technique was used.

RESULTS AND DISCUSSION

The experiment for equilibrium time was conducted at 25°C in Sorensen's phosphate buffer pH 7.4. The equilibrium time was determined for one concentration of piprinhydrinate $4.0x10^{-3}$ M (initial concentration). Figure 1 shows that the binding equilibrium was attained after 8 hours. Table 1 and Fig. 2 show the equilibrium dialysis data of interaction of piprinhydrinate with bovine serum albumin in Sorensen's phosphate buffer pH 7.4 at temperature 25 ± 1°C. It is clear that piprinhydrinate significantly binds to bovine serum albumin and that the degree of binding depends on drug concentration. Figure 3 shows that at a fixed protein concentration, the percentage of piprinhydrinate bound to bovine serum albumin decreases as the concentration of the drug increases. To determine the binding parameter K (the association constant) and n (the number of binding sites available on bovine serum albumin) the equilibrium dialysis data were plotted according to Scatchard[12] (Fig. 3). The plot is curved, therefore, the data were analyzed in terms of two classes of binding sites using the linear regression. The intercepts on the abscissa represents n_1 (number of primary binding sites) and n_2 respectively from which n_2 (number of secondary binding sites can be calculated). The slope of the first line represents K_1 (the primary association constant) and the slope of the second line represents K_2 (the secondary association constant). The primary association constant K_1 is found to be $4.60x10^{-4}$ M^{-1} and the number of primary binding sites is 0.68, the necessary association constant K_2 is $0.70x10^{-4}$ M^{-1} and n_2 is 0.69 (Table 1). These results indicate that piprinhydrinate is strongly bound to bovine serum albumin and are an important factor in predicting drug kinetics in the body. Drugs with a binding constant higher than 4 M^{-1} have a pharmacokinetic behavior that is dependent on the binding phenomena especially when their volume of distribution is small[6,7].

Figure 4 shows the effect of bovine serum albumin concentration on the binding of piprinhydrinate. The calculated K_1, n_1 and K_2, n_2 at different bovine serum albumin concentration are shown in table 2. It is clear that the binding of piprinhydrinate to bovine serum albumin is not affected by bovine serum albumin concentration. Similar results were obtained by Montero *et al.*[13] who studied the binding of HSA. On the other hand, Diem and Lentzer[14] reported that, significant changes in binding of drug to albumin or blood pH or local change in organ pH. In this work the binding of piprinhydrinate to bovine serum albumin at different pH values from 5.6 to 7.4 were carried out. As shown in Figs. 5, 6, 7 and 8 no significant effect of the pH changes could be demonstrated. This finding is in agreement with that of Bennet and Kirby[15] in their work on penicillin. On the other hand this result is in controversy to the finding of Abd Elbary *et al.*[16] in their work on phenylbutazone and oxyphenbutazone where the binding decreased with increasing pH. Newbould and Kilpatrick[17] also reported that the binding of sulfonamides to HSA is pH dependent.

Then the effect of buffer components on the binding process was investigated by replacing Sorensen's phosphate buffer by Gomori's tris-HCl buffer pH 7.4 and Walpole's acetate buffer pH 5.6[10]. All buffer solutions were made isotonic with sodium chloride. It was reported that different buffer systems vary in their interference with the binding of benzylpenicillin

Table 1: Equilibrium dialysis data of the binding of piprinhydrinate in isotonic Sorensen's phosphate buffer pH 7.4 at 25±1 °C.

Starting Conc. $x10^{-5}$ M	$C_f x10^{-5}$ (M)	$C_b x10^{-5}$ (M)	r	r/C_f (M^{-1})
1.869	0.175	1.694	0.057	32571
3.878	0.441	3.4347	0.115	26077
5.698	0.735	4.963	0.166	22585
7.756	1.092	6.664	0.223	20072
9.611	1.491	8.120	0.272	17741
11.445	2.051	9.394	0.314	15310
13.104	2.604	10.563	0.353	13556
14.917	3.157	11.760	0.393	12449
16.877	3.927	12.950	0.433	11026
19.152	0.075	14.077	0.471	9281
28.721	9.618	19.103	0.639	6096
38.171	16.366	21.805	0.729	4274
47.894	23.226	24.668	0.825	3482
57.554	31.101	26.453	0.885	2846
67.032	37.597	29.435	0.984	2617
74.529	43.008	31.521	1.054	2451
83.818	51.555	32.263	1.079	2222

Bovine serum albumin concentration $2.99x10^{-4}$ M.
C_f= Free drug concentration.
C_b= Bound drug concentration.
r= Moles of drug bound per mole of albumin.

Table 2: Binding parameters of piprinhydrinate to bovine serum albumin in isotonic Sorensen's phosphate buffer pH 7.4 at 25±1 °C.

Albumin conc. (M)	K_1x10^4 (M^{-1})	n_1	K_2x10^4 (M^{-1})	n_2
$2.99x10^{-4}$	4.60	0.68	0.70	0.69
$1.49x10^{-4}$	4.14	0.72	0.67	0.71
$7.46x10^{-5}$	4.16	0.79	0.69	0.68

and phenoxymethyl penicillin to bovine serum albumin[18]. From the data in Figures 9 and 10, it is clear that the tested buffer systems have different effects on the binding of piprinhydrinate to bovine serum albumin. The primary association constant K_1, decreased from $4.60x10^4$ M^{-1} to $3.51x10$ M^{-1} when Sorensen's phosphate buffer was replaced with Gomori's tris-HCl buffer. The primary association constant K_1, decreased from $4.0x10^4$ M^{-1} to $0.62x10^4$ M when Sorensen's phosphate buffer pH 5.6 was replaced with Walpole's acetate buffer pH 5.6. This finding is in agreement with Naoki Nambu and Tsuneji[19] they reported that the effect of ion species on the binding of 13 kinds of phenothiazines to bovine serum albumin

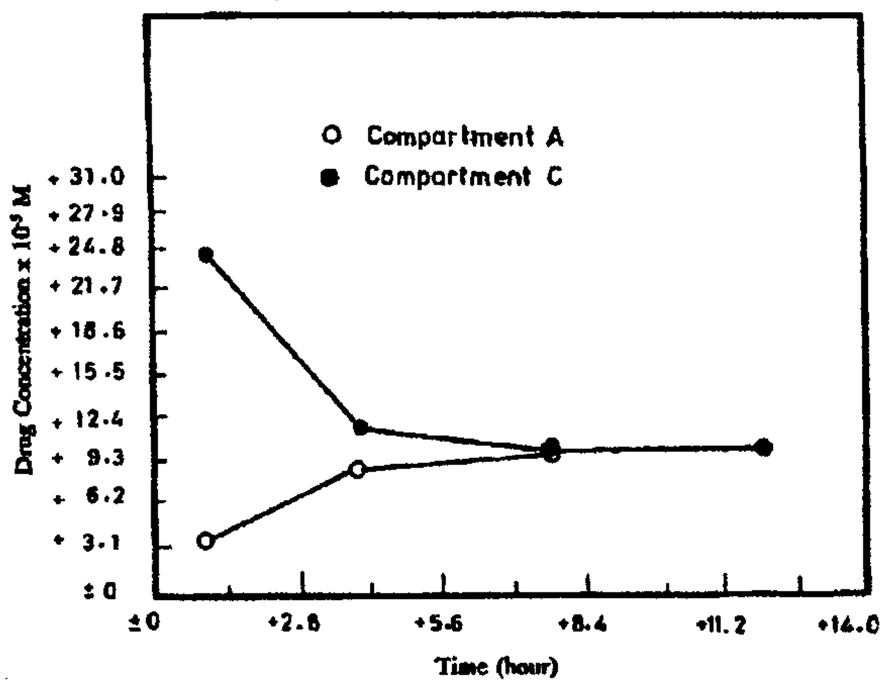

Fig. 1: The equilibrium time for the binding of piprinhydrinate to BSA.
○ Compartment A ● Compartment C.

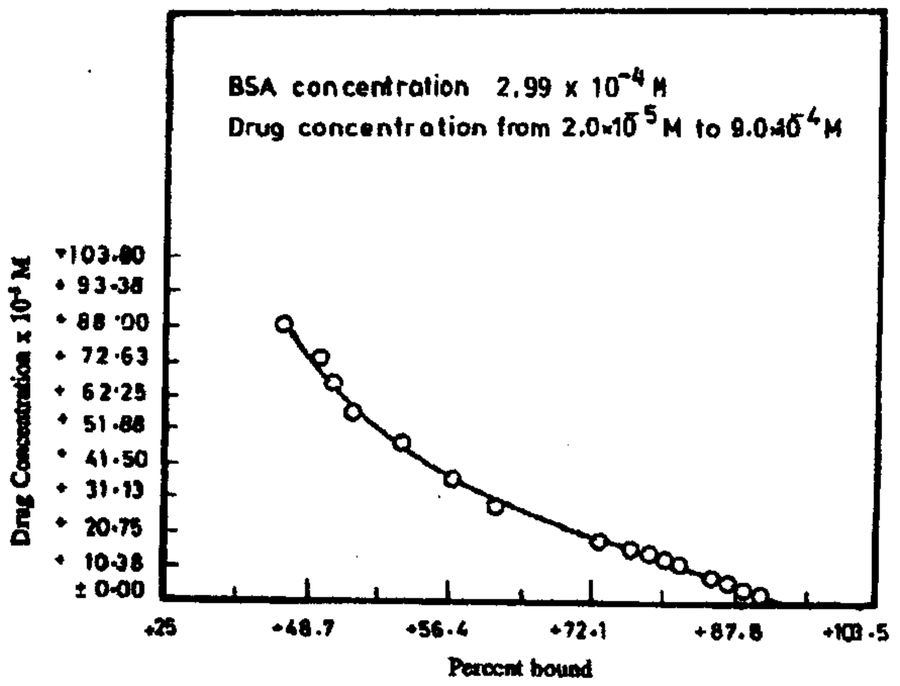

Fig. 3: Binding of piprinhydrinate to BSA in isotonic Sorensen's phosphate buffer pH 7.
BSA concentration 2.99×10^{-4} M
Drug concentration from 2.0×10^{-5} to 9.0×10^{-4} M.

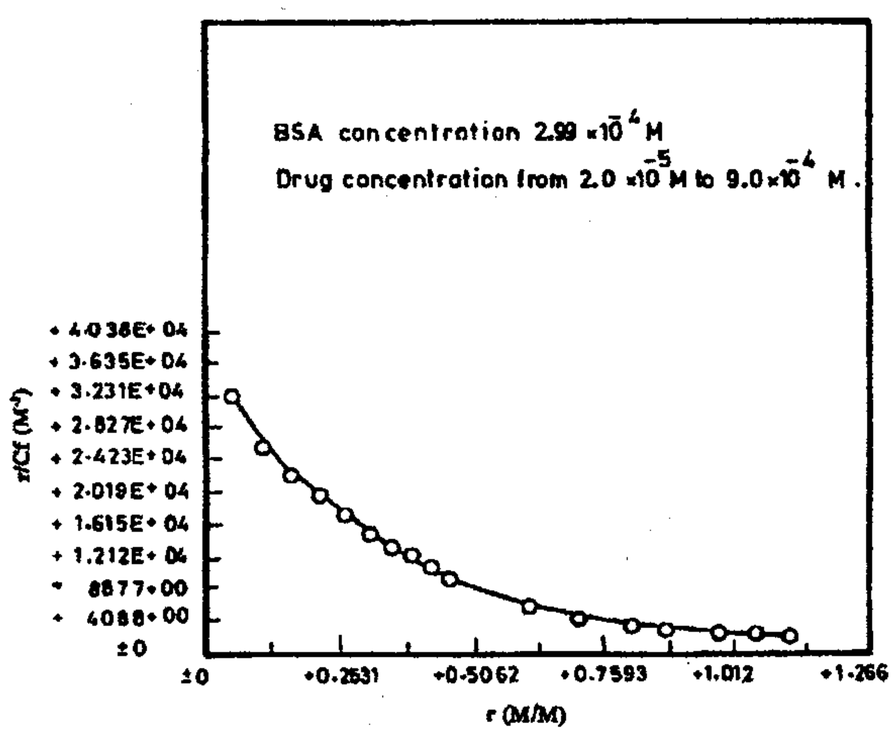

Fig. 2: Scatchard plot of dialysis data for the binding of piprinhydrinate to BSA in isotonic Sorensen's phosphate buffer of pH 7.4.
BSA concentration 2.99×10^{-4} M
Drug concentration from 2.0×10^{-5} to 9.0×10^{-4} M.

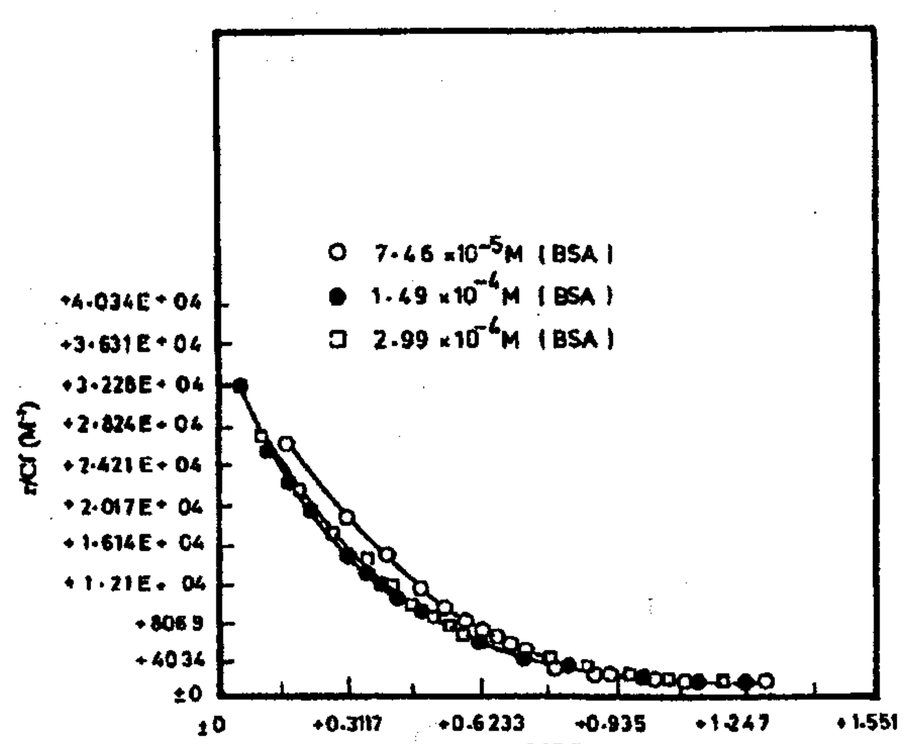

Fig. 4: Scatchard plot of dialysis data for the binding of piprinhydrinate to BSA in isotonic Sorensen's phosphate buffer pH 7.4.
○ 7.46×10^{-5} M (BSA) ● 1.49×10^{-4} M (BSA)
□ 2.99×10^{-4} M (BSA).

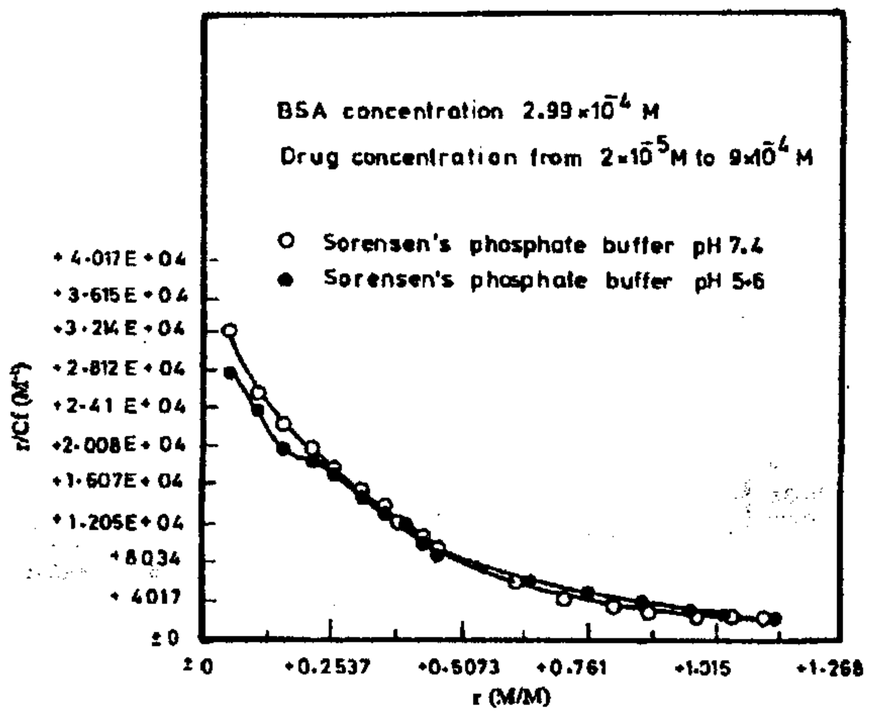

Fig. 5: Scatchard plot of dialysis data for the binding of piprinhydrinate to BSA in isotonic Sorensen's phosphate buffer pH 5.6.
BSA concentration 2.99×10^{-4} M
Drug concentration from 2.0×10^{-5} to 9.0×10^{-4} M.
○ Sorensen's phosphate buffer pH 7.4
● Sorensen's phosphate buffer pH 5.6.

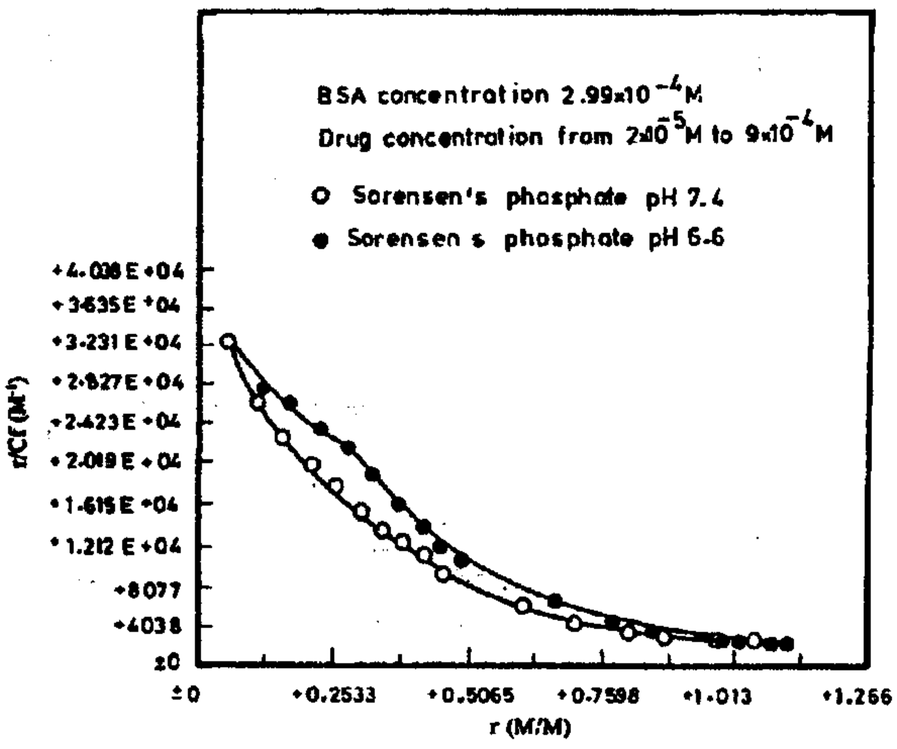

Fig. 7: Scatchard plot of dialysis data for the binding of piprinhydrinate to BSA in isotonic Sorensen's phosphate buffer pH 6.6.
BSA concentration 2.99×10^{-4} M
Drug concentration from 2.0×10^{-5} to 9.0×10^{-4} M.
○ Sorensen's phosphate pH 7.4
● Sorensen's phosphate pH 6.6.

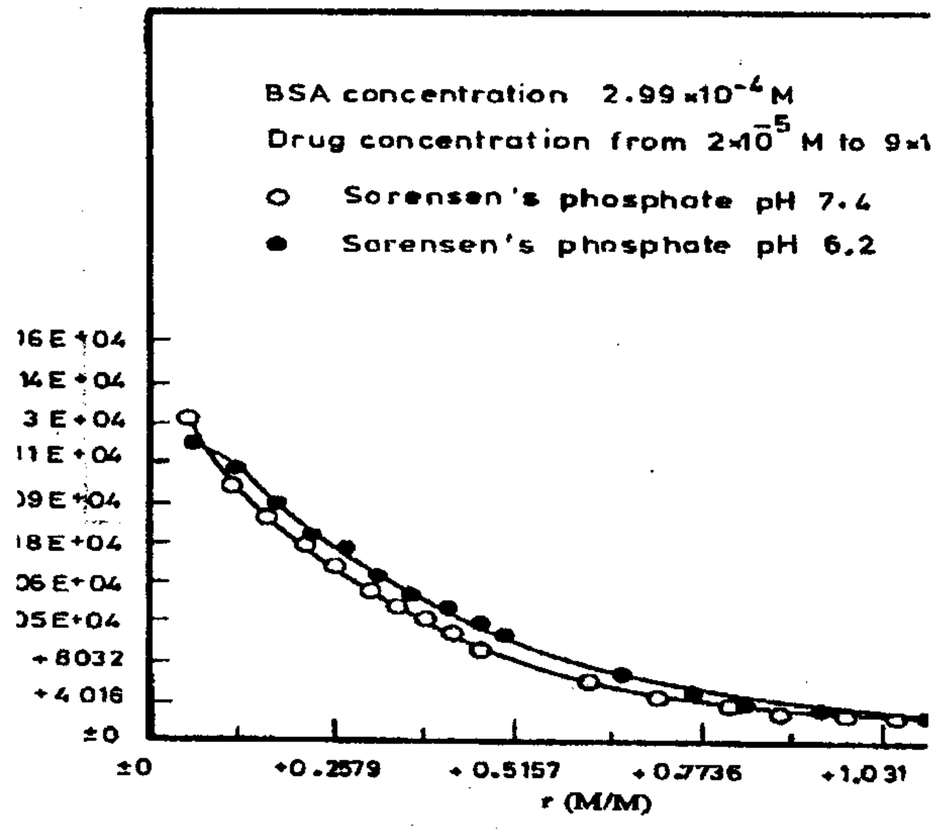

Fig. 6: Scatchard plot of dialysis data for the binding of piprinhydrinate to BSA in isotonic Sorensen's phosphate buffer pH 6.2.
BSA concentration 2.99×10^{-4} M
Drug concentration from 2.0×10^{-5} to 9.0×10^{-4} M.
○ Sorensen's phosphate pH 7.4
● Sorensen's phosphate pH 6.2.

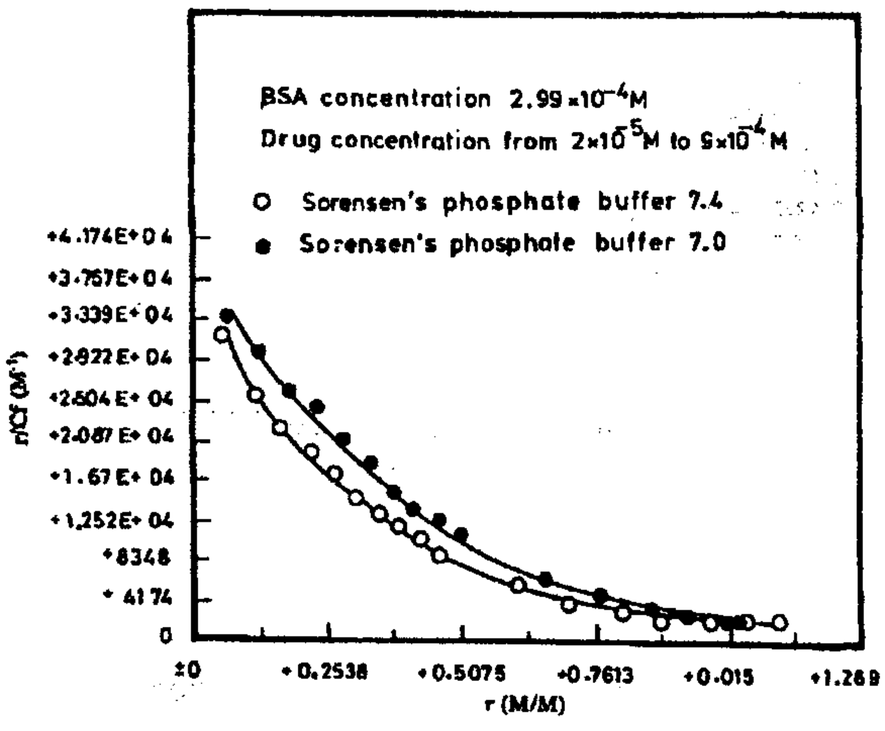

Fig. 8: Scatchard plot of dialysis data for the binding of piprinhydrinate to BSA in isotonic Sorensen's phosphate buffer pH 7.0
BSA concentration 2.99×10^{-4} M
Drug concentration from 2.0×10^{-5} to 9.0×10^{-4} M.
○ Sorensen's phosphate pH 7.4
● Sorensen's phosphate pH 7.0.

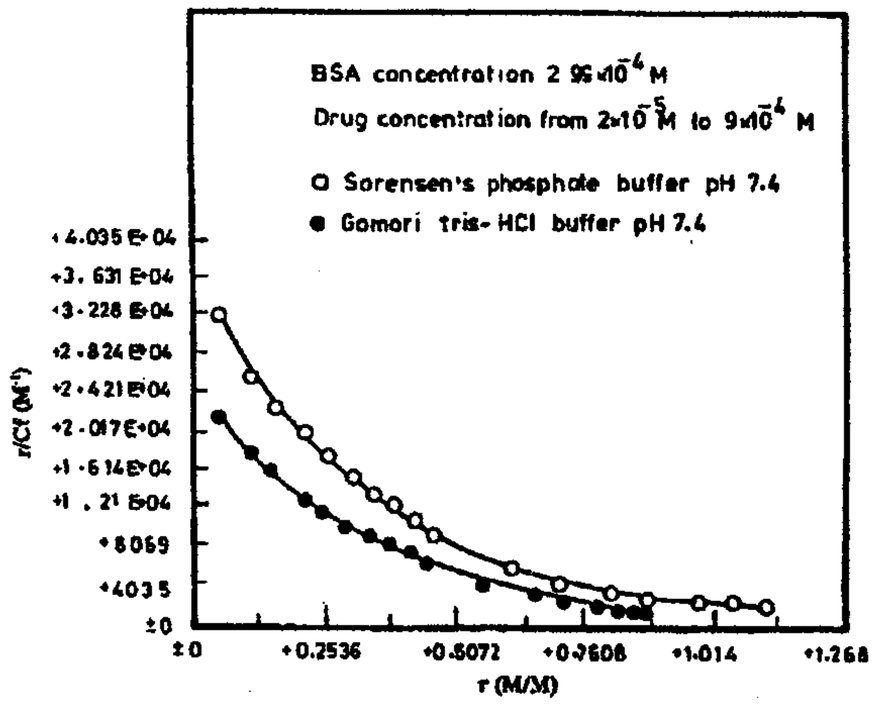

Fig. 9: Scatchard plot of dialysis data for the binding of piprinhydrinate to BSA in isotonic Gomori's tris-HCl buffer pH 7.4.
BSA concentration 2.99×10^{-4} M
Drug concentration from 2.0×10^{-5} to 9.0×10^{-4} M.
○ Sorensen's phosphate buffer pH 7.4
● Gomori tris-HCl buffer pH 7.4.

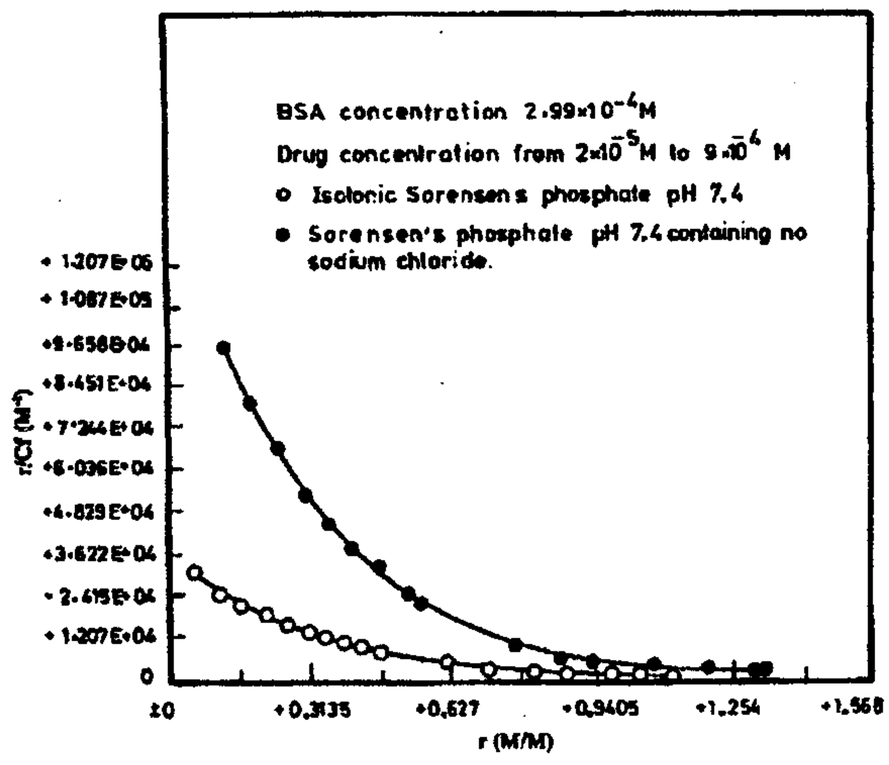

Fig. 11: Scatchard plot dialysis data for the binding of piprinhydrinate to BSA in Sorensen's phosphate buffer pH 7.4 containing no sodium chloride.
BSA concentration 2.99×10^{-4} M
Drug concentration from 2.0×10^{-5} to 9.0×10^{-4} M.
○ Isotonic Sorensen's phosphate pH 7.4
● Sorensen's phosphate pH 7.4 containing no sodium chloride.

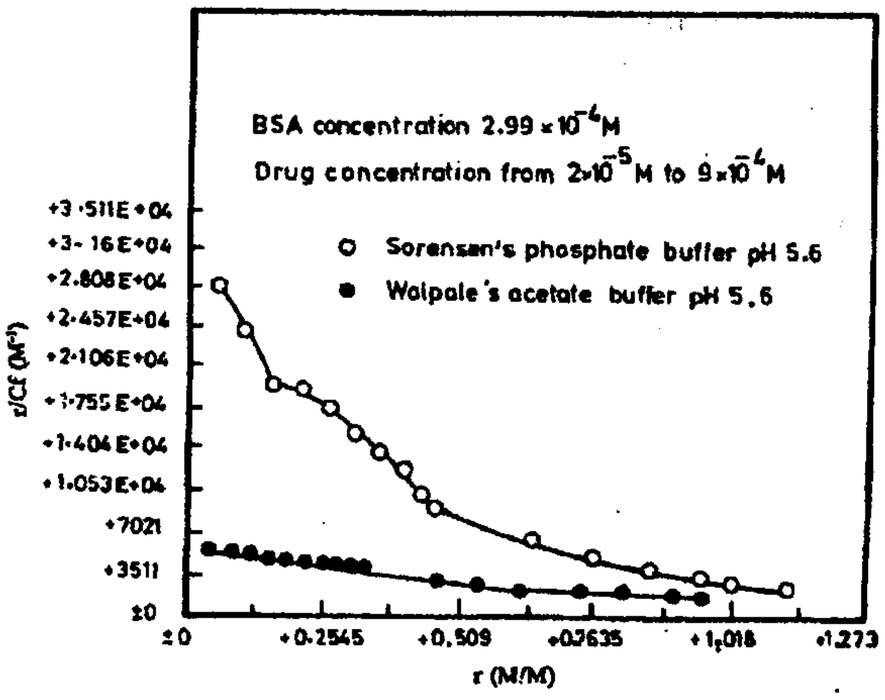

Fig. 10: Scatchard plot of dialysis data for the binding of piprinhydrinate to BSA in isotonic Walepole's acetate buffer pH 5.6.
BSA concentration 2.99×10^{-4} M
Drug concentration from 2.0×10^{-5} to 9.0×10^{-4} M.
○ Sorensen's phosphate buffer pH 5.6
● Walpale's acetate buffer pH 5.6.

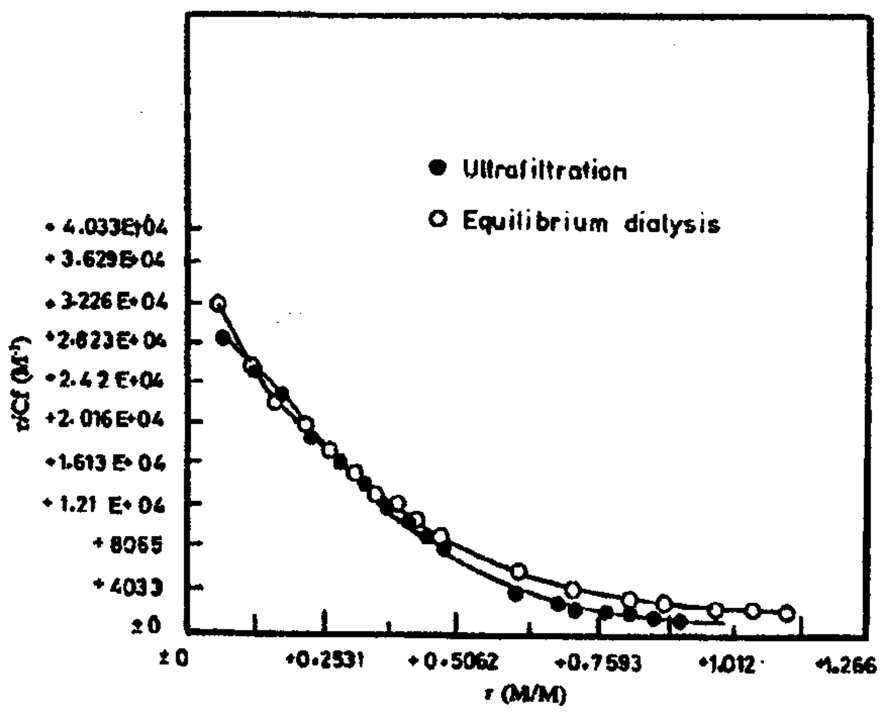

Fig. 12: Scatchard plot of dialysis data and ultrafiltration for the binding of piprinhydrinate to BSA in isotonic Sorensen's phosphate buffer pH 7.4.
● Ultrafiltration ○ Equilibrium dialysis.

was as follows: citrate > succinate > phosphate > acetate. The binding of phenoxymethylpenicillin and benzylpenicillin to bovine serum albumin has been studied by Keen[18] over the pH range 1.5-10.0 in a variety of buffers. There was a marked reduction in binding above pH 9.0.

Moreover, chloride ion has been reported as an endogenous ion affecting the extent of protein binding of many drugs[20]. In the present work, the effect of the chloride ion on the binding of piprinhydrinate to BSA was investigated. Figure 11 indicates a significant effect of the presence of chloride ions. It is clear that the presence of chloride ions decreased the amount of drug bound to BSA. The primary association constant K_1 was found to be higher in Sorensen's phosphate buffer than in isotonic Sorensen's phosphate buffer, where the latter was made isotonic with sodium chloride. This finding is in good agreement with that of Janssen and Nelen[21] who found that the binding of sulfaethidzole to both bovine and human serum albumin decreased by chloride ions. Brown and Crooks[22] reported similar finding when worked on the binding of tolbutamide to HSA in presence of sodium chloride, where the percentage of drug bound was decreased by chloride ions. Whereas, Brock[23], reported that the binding of digoxin to HSA was not affected by chloride ions.

Lastly the interaction of piprinhydrinate with certain polymers was carried out. The interaction of piprinhydrinate with dextran 40000, dextran 266000 and dextran 50000, was studied. Results revealed that no interaction of piprinhydrinate occurred with any of these macromolecular compounds at any of the tested concentrations. Brorchardt *et al.*[24] demonstrated the interaction of many drugs with dextran of different grades. The binding of drugs with dextrans was negligible and not of clinical relevance (<20%). Also, no interaction of piprinhydrinate in the presence of laevosan and hetastarch was noticed. Comparison of equilibrium dialysis and ultrafiltration techniques for measuring the free fraction of piprinhydrinate gave the same results as shown in Fig. 12.

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