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RESEARCH PAPER

Using Difference Spectrophotometry to Study the Influence of Different Ions and Buffer Systems on Drug Protein Binding

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ABSTRACT

Difference spectrophotometry was used to investigate the effect of different ions and buffer systems on the binding of the anti-inflammatory drug tenoxicam to human serum albumin (HSA). Chloride anions, as well as sodium cations, were found to decrease the binding affinity. The effect of chloride ions was greater on the primary binding constant K_1*, while sodium ions had a greater effect on the secondary binding constant* K_2*. The number of binding sites* n_1 *and* n_2 *were not affected except at 0.12% HSA, for which the presence of sodium ions halved* n_2*. Potassium ions significantly increased* K_1*. The presence of potassium instead of sodium ions increased binding affinity at lower HSA concentrations. The number of binding sites* n_1 *and* n_2 *were fewer in presence of potassium than in the presence of sodium ions except at 0.12% HSA. The divalent calcium and magnesium cations increased the binding affinity of HSA to tenoxicam, with a greater effect on* K_1*. The effect of magnesium ions on* K_1 *occurred when the* $MgCl_2$ *concentration was increased to 3 and 9 mM, with the former seeming to be a critical concentration. The number of primary binding sites* n_1 *was not affected by calcium ions, but was halved by 1 mM* $MgCl_2$*. Both calcium and magnesium cations decreased* n_2*, which was halved when the concentration of either cation was increased to 9 mM. The effect of buffer systems on tenoxicam binding to HSA was dependent on HSA concentration. The value of* K_1 *was higher in Sorensen's phosphate buffer than in Tris [tris(hydroxymethyl) aminomethane HCl] buffer when the HSA concentration was 0.04% and 0.16%, while the reverse was observed at 0.08% and 0.12% HSA. The other binding parameters (*K_2*,* n_1*, and* n_2*) were higher in Sorensen's phosphate than in Tris buffer. However; at certain HSA concentrations, the values of such parameters were comparable in both buffer systems.*

INTRODUCTION

After reaching the circulation, many drugs are bound to different blood constituents, including cells and proteins. Within the plasma proteins, serum albumin is undoubtedly the most important carrier for drugs and other small molecules. Reversible binding of drugs has been realized for many years to have a profound influence on the pharmacodynamics and toxicity of drugs, as well as on their disposition. For example, most nonsteroidal antiinflammatory drugs (NSAIDs) exhibit a high degree of binding to albumin, and their binding characteristics are primary determinants of their pharmacokinetic properties (1).

Tenoxicam, a potent oxicam NSAID, is highly protein bound (2). It is reported to be more than 99% bound to human serum albumin (HSA) (3). In this study, the influence of different ions, as well as buffer systems, on the binding of tenoxicam to HSA was investigated. Metal ions are bound to serum albumin, which acts as a circulating reservoir that accepts or yields such ions on demand (4). There is evidence that metal ions protect against inflammation and are beneficial in the treatment of rheumatoid arthritis (5). Binding of antirheumatics to HSA was found to be affected by some metal ions. Ibuprofen (6) and benoxaprofen (7) binding was found to be reduced in the presence of calcium. The presence of calcium and magnesium at physiological pH decreases diclofenac binding (8). On the other hand, magnesium was shown to increase the binding of phenylbutazone and oxyphenbutazone to HSA, while chloride ion decreased it (9).

The N-B transition of HSA has been reported to affect its capacity to bind drugs (10). This transition is dependent on pH, but it may be affected to some extent by ionic strength of the buffer and buffer ion composition. Ionic strength was reported to have no effect on tenoxicam binding (3). Divalent ions such as calcium and magnesium, as well as chloride ions, affect the N-B transition and the binding of drugs to protein (10–14). However, buffer systems may affect drug protein interaction without affecting the N-B conformational change. This was noticed in the case of diclofenac sodium. Its binding to HSA, although not affected by the N-B conformational change, was higher in phosphate than in Tris [tris(hydroxymethyl) aminomethane HCl] buffer irrespective of HSA concentration (8).

Ultraviolet (UV) difference spectrophotometry (8,9, 15–19) was applied in this work as it provides valuable information on the drug protein interaction by allowing the determination of very low concentrations of free drug. Accordingly, a fair number of data can be obtained at low binding ratios ($r < 1$). This is very important if drug binding parameters with clinical significance are to be obtained. In many binding studies, low binding ratios (i.e., small r values) are seldom used, causing hazardous extrapolation to the various axes and negating the possibility of obtaining clinically significant primary binding constants (8,15,17). Besides, UV difference spectrophotometry is accurate, reproducible, and suitable for drugs of limited solubility.

EXPERIMENTAL

Materials and Equipment

Human serum albumin fraction V (Sigma Chemical Company, St. Louis, MO), tenoxicam (Epico Pharmaceutical Company, Cairo, Egypt), tris(hydroxymethyl) aminomethane (BDH Chemicals Ltd., Poole, England), potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, potassium chloride, calcium chloride, and magnesium chloride (Prolabo, Adwic, Egypt) were used for the study. The spectrophotometer was a Shimadzu PR 240 (Kyoto, Japan).

Methodology

Difference spectrophotometry employing the tandem cell technique in the split beam mode was used (18). The protein solution was placed in one cell, and buffer alone was placed in the other cell of the reference beam. The sample compartments contained exactly the same solutions. The baseline was obtained before any additions. Microliter aliquots of tenoxicam were added to the buffer cell in the reference beam, as well as to the protein cell in the sample beam (at equal concentrations). To maintain a constant protein concentration throughout the titration, each addition of drug to the protein solution in the sample cuvette was accompanied by a concomitant addition of an equal volume of albumin solution at a concentration twice that in the cuvette. Similarly, drug concentration was maintained equally in both beams during titration by the addition of an equal volume of buffer to the cuvette containing drug and buffer in the reference compartment. All experiments were done at 25°C ± 1°C (8,19).

Isotonic Sorensen's phosphate (20) or Tris (21) buffer of pH 7.4 was used to prepare both drug and protein solutions. HSA solutions were used at the concentrations of 0.58×10^{-5} M (0.04%), 1.16×10^{-5} M (0.08%), 1.73×10^{-5} M (12%), and 2.32×10^{-5} M (0.16%). Tenoxicam solution (10×10^{-4} M) was used for the difference spectrophotometric titration processes, resulting in a final concentration range of 0.098×10^{-4} M to 0.614×10^{-4} M. The concentrations of drug and protein used in

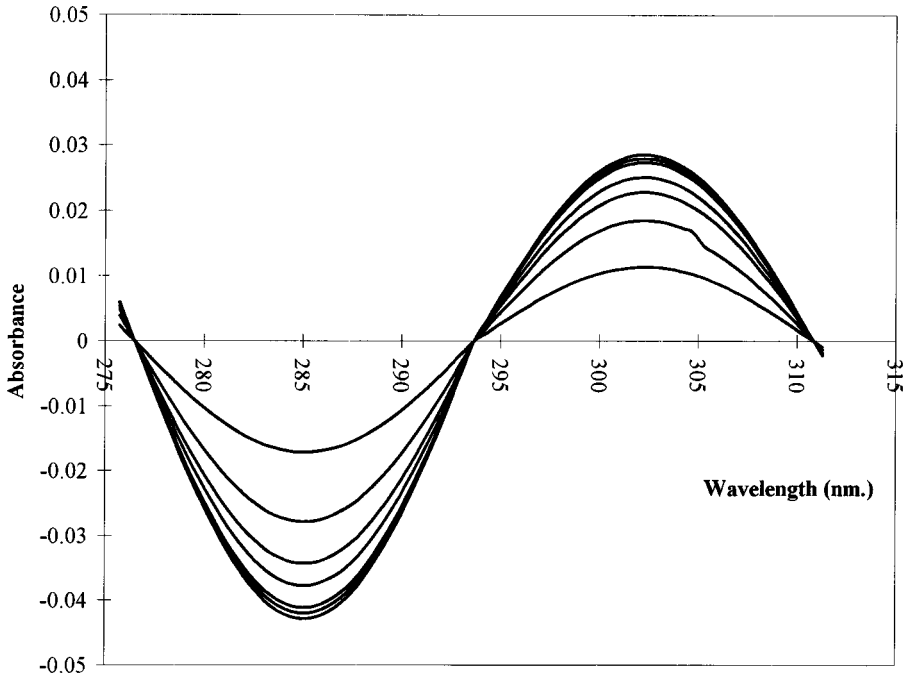

Figure 1. Difference spectrophotometric titration of 1.15×10^{-5} M (0.08%) HSA with tenoxicam in pH 7.4 isotonic Sorensen's phosphate buffer. Each curve represents the addition of a 10 μl increment of 10×10^{-4} M tenoxicam.

difference spectrophotometry should be low to permit light scattering and fluorescence to be ignored (17).

The effect of chloride ions was explored using pH 7.4 Sorensen's phosphate buffer containing no sodium chloride (9). The effect of sodium ions was studied in pH 7.4 Tris buffer containing no sodium chloride. The effect of potassium ions was investigated in pH 7.4 Tris buffer containing 0.1 M potassium chloride instead of sodium chloride. To study the effect of calcium and magnesium ions, pH 7.4 Tris buffer containing calcium chloride or magnesium chloride at concentrations of 1 mM, 3 mM, and 9 mM (8) was used.

Experiments were repeated until difference absorption spectra were obtained in the required pattern, as demonstrated in Fig. 1.

RESULTS AND DISCUSSION

Difference absorption spectra were obtained for the association of tenoxicam with HSA. Figure 1 shows that the spectra were characterized by an isosbestic point at 294 nm, a positive peak at 302 nm, and a negative trough at 285 nm. Such characteristics did not change throughout this work. Each curve represents the addition of 10 μl aliquot of 10×10^{-4} M tenoxicam. The absorption titration curve, represented by Fig. 2, was generated by plotting the change in absorbance ΔA (measured as the difference in intensity between each peak and the corresponding trough) versus the corresponding concentration of tenoxicam resulting after each drug aliquot addition during the difference spectrophotometric titration. Calculation of the binding parameters was done by adopting a technique that depends on calculating the amounts of free and bound drug from the titration curve after drawing a tangent at the origin (8,16,17). Plotting the resulting information according to Scatchard (22) resulted in curved plots that showed the presence of more than one binding site. Figure 3 is an example of the nonlinear Scatchard plots obtained.

The effects of some ions, as well as buffer systems, on the binding of tenoxicam to HSA are reflected by the primary binding parameters K_1 and n_1, as well as the secondary parameters K_2 and n_2, obtained from the corresponding Scatchard plots and tabulated. Such effects are discussed below.

Effect of Chloride Ions

Table 1 shows that chloride ions decreased both K_1 and K_2, with a much more prominent decrease in K_1. The

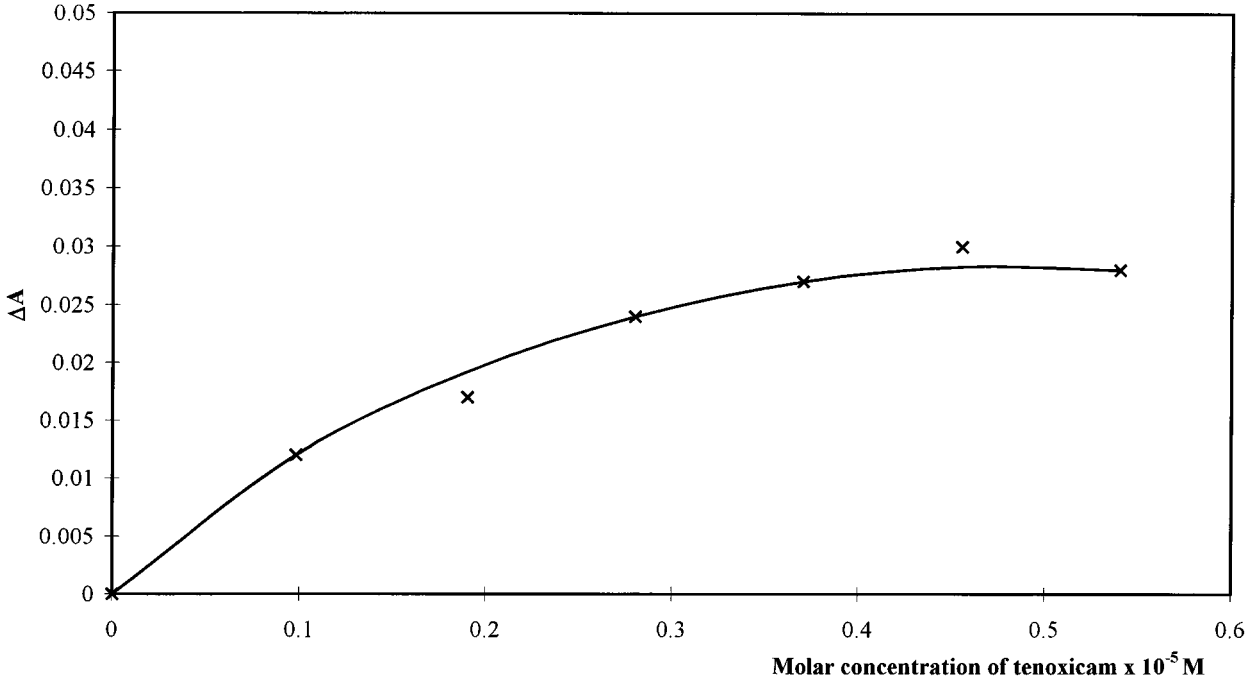

Figure 2. Absorption titration curve of tenoxicam-HSA interaction in pH 7.4 isotonic Tris buffer using 1.16×10^{-5} M HSA.

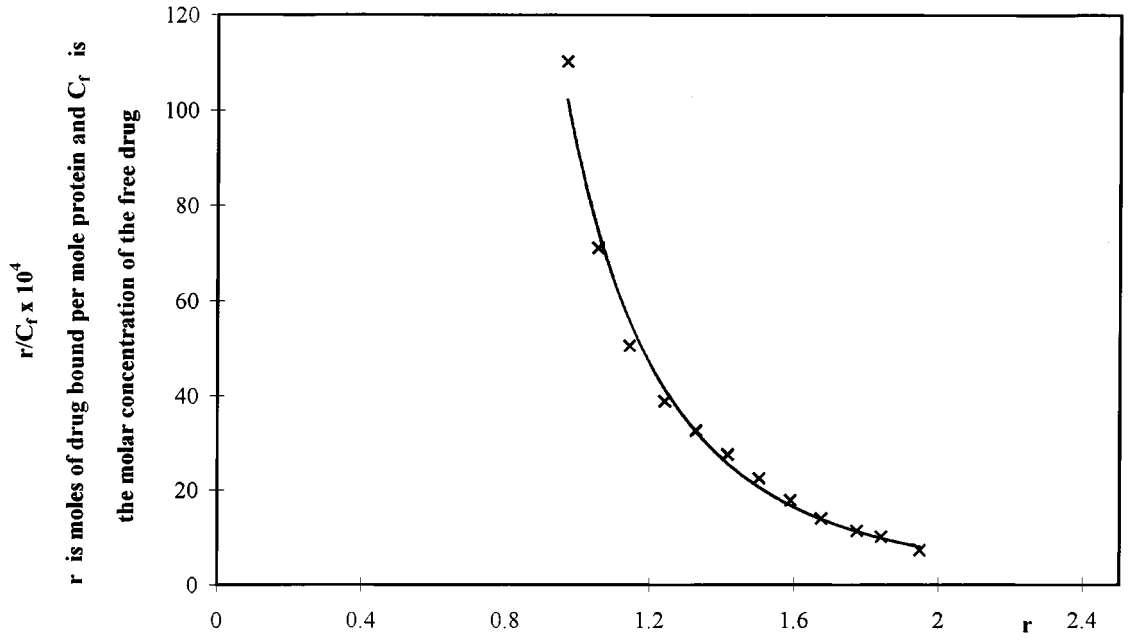

Figure 3. Scatchard plot of UV difference spectrophotometric data of tenoxicam-HSA interaction in pH 7.4 isotonic Tris buffer using 1.16×10^{-5} M (0.08%) HSA.

Table 1

Effect of Chloride Ion on the Binding Parameters of Tenoxicam to 1.16 × 10^{-5} M (0.08%) HSA at pH 7.4 in Sorensen's Phosphate Buffer

Binding Parameters	In Presence of 0.055 M NaCl	In Absence of NaCl
K_1	13.857 × 10^5	470.000 × 10^5
n_1	1.130	0.940
K_2	2.453 × 10^5	7.089 × 10^5
n_2	1.920	1.580

values of n_1 and n_2 did not show a significant change. It was reported that chloride ions reduced the binding of phenylbutazone and oxyphenbutazone to HSA, and that this effect was greater on the high-affinity sites (9). The suggested mechanism is the displacement of drug from albumin (10–12). It was also reported that chloride ions affect the N-B transition (10–13).

Effect of Calcium Ions

Table 2 shows that the presence of calcium ions increased both K_1 and K_2, with the increase in K_1 more prominent. Increasing the Ca^{++} concentration increased its effect. The value of n_1 was not significantly affected, but n_2 decreased. The value of n_2 was halved when $CaCl_2$ was increased to 9 mM.

The observed effect of Ca^{++} may be due to the effect of divalent cations (e.g., Ca^{++} and Mg^{++}) on the N-B transition (10–14). It was reported that this transition occurs over a narrower pH range in the presence of calcium ions (11,14,23). This is in accordance with a previous finding that the B form of HSA favors tenoxicam binding (24).

Effect of Magnesium Ions

It is observed from Table 2 that the increase in K_1 occurred only when $MgCl_2$ was present at a concentration of 3 mM and 9 mM, being more prominent at 3 mM, which seems to be a critical concentration. The value of n_1 was nearly halved in the presence of 1 mM $MgCl_2$. When the concentration of $MgCl_2$ was increased, n_1 showed no further considerable decrease. The effect of Mg^{++} on K_2 and n_2 did not show a significant difference from that of Ca^{++}.

It was reported that magnesium ions also increased the binding of phenylbutazone and oxyphenbutazone (9).

Effect of Sodium Ions

Table 3 shows that Na^+ decreased both K_1 and K_2, with a greater effect on the latter. The decrease was more prominent at 0.08% HSA than at 0.12%. The number of binding sites n_1 and n_2 was not significantly affected except at 0.12% HSA, at which n_1 was nearly halved.

Effect of Potassium Ions

Table 4 shows that potassium ions significantly increased the primary binding constant K_1. This effect increased by increasing HSA concentration from 0.08% to 0.12%. At 0.08% HSA, K_2 slightly increased, but at 0.12% HSA, it decreased. The number of binding sites n_1 and n_2 decreased at 0.08% HSA, but increased at 0.12% HSA.

Comparing the effect of K^+ to that of Na^+, Table 5 shows that at 0.16% HSA, there is no significant difference when potassium ions were present instead of sodium ions. At lower HSA concentrations, K_1 increased significantly, and it was more than 6 times higher when the HSA concentration was 0.04%. At 0.08% and 0.12%, K_1

Table 2

Binding Parameters of Tenoxicam to 1.16 × 10^{-5} M (0.08%) HSA in Presence of Calcium Chloride and Magnesium Chloride at pH 7.4 in Tris Buffer

Binding Parameter	Buffer Containing 0.1 M NaCl	Buffer Containing Different Concentrations of $CaCl_2$			Buffer Containing Different Concentrations of Mg/Cl_2		
		1 mM	3 mM	9 mM	1 mM	3 mM	9 mM
K_1	50 × 10^5	81.667 × 10^5	120 × 10^5	137.778 × 10^5	26.667 × 10^5	266.667 × 10^5	99.535 × 10^5
n_1	1.190	1.060	1.095	0.945	0.590	0.690	0.608
K_2	1.415 × 10^5	2.957 × 10^5	4.500 × 10^5	6.157 × 10^5	2.305 × 10^5	4.237 × 10^5	5.534 × 10^5
n_2	2.460	1.725	1.800	1.210	1.920	1.770	1.265

Table 3

Effect of Sodium Ion on the Binding of Tenoxicam to HSA at pH 7.4 in Tris Buffer

	Buffer Containing 0.1 M NaCl		Buffer Without NaCl	
Binding Parameter	1.16×10^{-5} M (0.08%) HSA	1.73×10^{-5} M (0.12%) HSA	1.16×10^{-5} M (0.08%) HSA	1.73×10^{-5} M (0.12%) HSA
K_1	50×10^5	294.118×10^5	85.909×10^5	311.111×10^5
n_1	1.190	0.318	1.170	0.722
K_2	1.415×10^5	1.900×10^5	4.856×10^5	4.207×10^5
n_2	2.460	1.763	2.080	1.450

was nearly doubled. The increase in K_2 is slight, except at 0.08% HSA, at which it was 4 times higher. The number of binding sites n_1 and n_2 decreased, except at 0.12% HSA, at which n_1 increased 2.5 times when potassium ions were present instead of sodium.

Effect of Buffer System

Table 6 shows that, in Tris buffer, K_1 for the binding of 10×10^{-4} M tenoxicam to 0.04%, 0.08%, and 0.16% HSA did not change significantly. An exceptionally prominent increase in K_1 is observed at 0.12% HSA. This also occurred in Sorensen's phosphate buffer, but it was less, while a decrease is observed at 0.08%. Such variation is due to the effect of HSA concentration (discussed in Ref. 24). Accordingly, K_1 was higher in Sorensen's phosphate buffer at 0.04% and 0.16% HSA and was lower at 0.08% and 0.12% in comparison to Tris buffer.

At 0.04% HSA, K_2 was comparable in both Tris and Sorensen's phosphate buffers. At higher HSA concentrations, K_2 did not change significantly in the case of Sorensen's phosphate buffer, but decreased in the case of Tris buffer, except at 0.12% HSA. Accordingly, K_2 was less in Tris than in Sorensen's phosphate buffer.

Both n_1 and n_2 decreased on increasing the concentration of HSA whether Tris buffer or Sorensen's phosphate buffer was used. In the case of Sorensen's phosphate buffer, both n_1 and n_2 were halved when the concentration of HSA was doubled (24). In Tris buffer, when the HSA concentration was increased four times (viz. from 0.04% to 0.16%), n_2 was halved, while n_1 decreased to one-fifth its value. The value of n_1 was higher in Sorensen's phosphate buffer than in Tris buffer, except at 0.08% HSA, at which n_1 had comparable values. The values of n_2 were slightly higher in Sorensen's phosphate buffer at 0.04%, but slightly lower at 0.08%, 0.12%, and 0.16%. At 0.12% and at 0.16%, the difference is negligible. It can be observed that the effect of buffer systems on tenoxicam binding parameters is dependent on HSA concentration. The binding of diclofenac sodium was reported to be higher in phosphate than in Tris buffer, but this was irrespective of HSA concentration (8).

CONCLUSION

Changes in the proportions of chloride, sodium, potassium, calcium, and magnesium ions were found to affect the binding parameters of tenoxicam to HSA. Accord-

Table 4

Effect of Potassium Ion on the Binding of Tenoxicam to HSA at pH 7.4 in Tris Buffer

	Buffer Without KCl		Buffer Containing 0.1 M KCl	
Binding Parameter	1.16×10^{-5} M (0.08%) HSA	1.73×10^{-5} M (0.12%) HSA	1.16×10^{-5} M (0.08%) HSA	1.73×10^{-5} M (0.12%) HSA
K_1	85.909×10^5	311.111×10^5	128.571×10^5	636×10^5
n_1	1.170	0.722	0.810	0.825
K_2	4.856×10^5	4.207×10^5	5.890×10^5	1.917×10^5
n_2	2.080	1.450	1.630	2.400

Table 5

Binding Parameters of Tenoxicam to Different Concentrations of HSA at pH 7.4 in Tris Buffer Containing 0.1 M Potassium Chloride Compared to 0.1 M Sodium Chloride

	Molar Concentrations of HSA in Presence of 0.1 M Na/Cl				Molar Concentrations of HSA in Presence of 0.1 M K/Cl			
Binding Parameters	0.58×10^{-5} (0.04%)	1.16×10^{-5} (0.08%)	1.73×10^{-5} (0.12%)	2.32×10^{-5} (0.16%)	0.58×10^{-5} (0.04%)	1.16×10^{-5} (0.08%)	1.73×10^{-5} (0.12%)	2.32×10^{-5} (0.16%)
K_1	48.485×10^5	50×10^5	294.118×10^5	53.750×10^5	335×10^5	128.571×10^5	636×10^5	49.60×10^5
n_1	1.730	1.190	0.318	0.340	0.540	0.810	0.825	0.237
K_2	2.375×10^5	1.415×10^5	1.900×10^5	0.990×10^5	2.557×10^5	5.890×10^5	1.917×10^5	1.279×10^5
n_2	3.326	2.460	1.763	1.491	1.940	1.630	2.400	1.075

Table 6

Binding Parameters of Tenoxicam to HSA at pH 7.4 in Isotonic Sorensen's Phosphate Buffer Compared to Tris Buffer

	Molar Concentrations of HSA in Sorensen's Phosphate Buffer				Molar Concentrations of HSA in Tris Buffer			
Binding Parameters	0.58×10^{-5} (0.04%)	1.16×10^{-5} (0.08%)	1.73×10^{-5} (0.12%)	2.32×10^{-5} (0.16%)	0.58×10^{-5} (0.04%)	1.16×10^{-5} (0.08%)	1.73×10^{-5} (0.12%)	2.32×10^{-5} (0.16%)
K_1	70×10^5	13.857×10^5	178.524×10^5	80×10^5	48.485×10^5	50×10^5	294.118×10^5	53.750×10^5
n_1	2.400	1.130	0.745	0.590	1.730	1.190	0.318	0.340
K_2	2.500×10^5	2.453×10^5	2.333×10^5	2.583×10^5	2.375×10^5	1.415×10^5	1.900×10^5	0.990×10^5
n_2	3.950	1.920	1.660	1.310	3.326	2.460	1.763	1.491

ingly, it is recommended to adjust tenoxicam dose in cases of diseases that induce electrolytic imbalance.

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