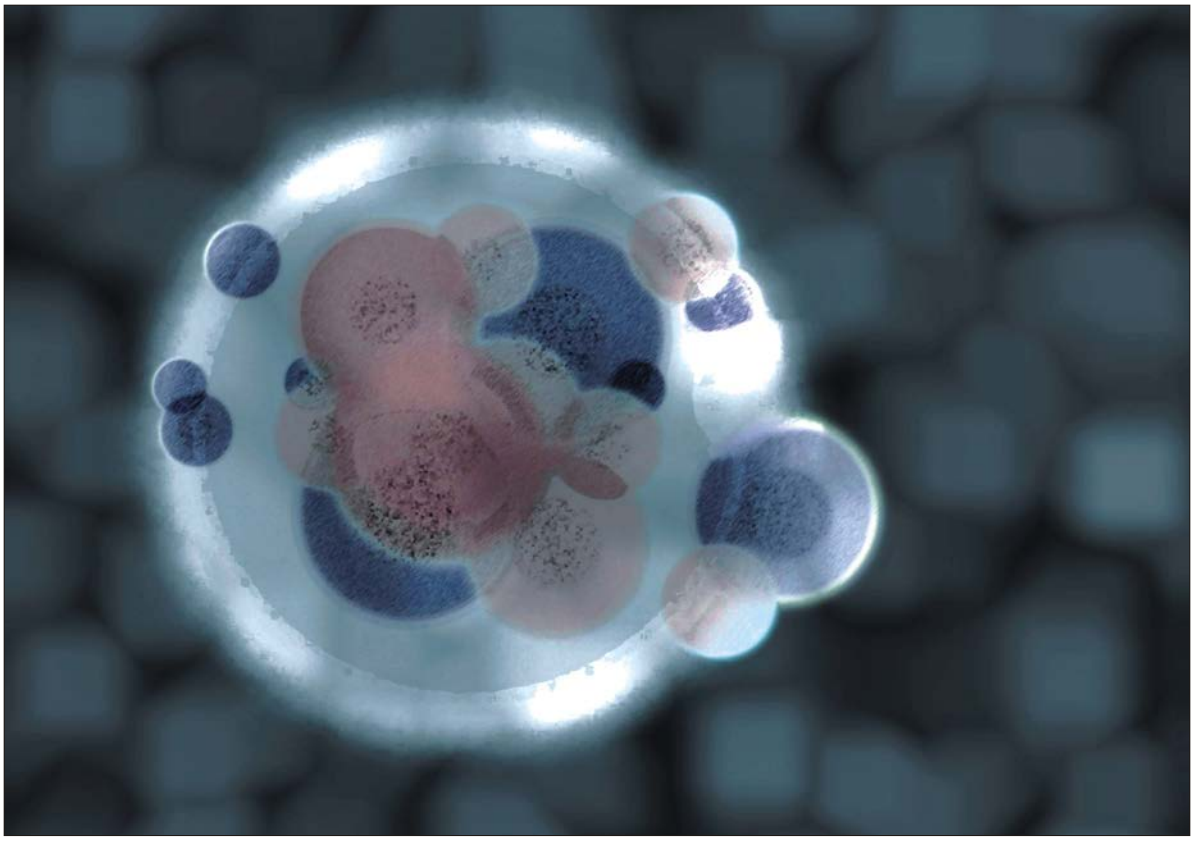


Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 6 (1): 167-172, 2006
ISSN 1727-3048

Interaction of Some Isoxazolyl Penicillins with Human Serum Albumin

Neelam Seedher and Pooja Agarwal
Department of Chemistry, Panjab University, Chandigarh-160014, India

Abstract: The nature and magnitude of the interaction of penicillins with serum albumin has important pharmacokinetic and pharmacodynamic implications. Mechanism of interaction of three isoxazolyl penicillins, cloxacillin sodium, dicloxacillin sodium and flucloxacillin sodium with Human Serum Albumin (HSA) has been studied using fluorescence spectroscopic technique. Quenching of tryptophan fluorescence of HSA under different conditions and fluorescent probe displacement studies were carried out. The stoichiometry of the interaction was found to be 1:1 in each case. The association constants were of the order of 10^4 in the case of all the drug samples. The order of association constants was dicloxacillin sodium > flucloxacillin sodium>cloxacillin sodium. The nature of drug-protein interaction could be predicted from the thermodynamic parameters for the binding. The binding was predominantly through hydrophobic interactions in the case of cloxacillin. In the case of dicloxacillin and flucloxacillin, hydrogen bonding as well as hydrophobic interactions contributed to the interaction. Binding studies carried out in the presence of hydrophobic probe, ANS showed that the drugs and ANS do not share common site on the albumin molecule. Displacement of DSS from its binding site on albumin showed that site II is involved in binding. Stern-Volmer analysis of fluorescence data revealed that the tryptophan residues of serum albumin are not fully accessible to drugs, drug binding site is in close proximity to the tryptophan residues and predominantly static quenching mechanism is operative.

Key words: Binding parameters, fluorescence, quenching mechanism, thermodynamics

INTRODUCTION

Isoxazolyl penicillins are β-lactamase-resistant semi-synthetic penicillins, indicated for staphylococcal infections. These are bactericidal agents with large acylamino side chains, which sterically hinder access to the β-lactam bond[1]. Isoxazolyl penicillins are acid-resistant and can be administered orally. Absorption of isoxazolyl penicillins after oral administration is rapid but incomplete; peak plasma concentrations are achieved in 1-1.5 h. Once absorbed, the penicillinase-resistant penicillins are highly bound to serum proteins, mainly albumin[2]. Serum albumin, the most abundant protein in plasma, serves as a storehouse for the drugs and it is the unbound moiety that is pharmacologically active. The nature and magnitude of the interaction of penicillins with serum albumin has important pharmacokinetic and pharmacodynamic implications and is a determinant factor in their therapeutic properties[3]. Serum protein binding becomes an important consideration when it exceeds 70-80%. This is because small changes in binding have a large effect on the proportion of free, pharmacologically active drug in plasma. High serum protein binding has several other major effects, including restricted tissue distribution, reduced penetration into interstitial spaces and inflammatory fluids, delayed elimination and interference with biological activity[4].

Isoxazolyl penicillins used in the present work are more than 95% bound to plasma proteins. Although these drugs are being frequently prescribed, no detailed study on the mechanism of interaction of isoxazolyl penicillins with serum albumin using spectroscopic methods is available. In the few reported studies, using other less specific physico-chemical techniques such as conductivity, electrokinetic behaviour, microcalorimetry and ultrafiltration[5-8] the views expressed by different authors regarding the nature of interaction are rather conflicting. In the present research, detailed studies on the mechanism of interaction of three isoxazolyl penicillins with serum albumin, including binding parameters, thermodynamics of the binding process, fluorescence quenching mechanism, the nature of forces involved in the interaction and the effect of pH and presence of salt on the concentration of biologically active free drug have been reported, using fluorescence spectroscopic technique.

Corresponding Author: Dr. Neelam Seedher, Professor, Department of Chemistry, Panjab University, Chandigarh-160014, India
Tel: 91-0172-2534431 Fax: 91-0172-2545074

MATERIALS AND METHODS

Pure drug samples (cloxacillin sodium, dicloxacillin sodium and flucloxacillin sodium) were obtained as gift from various manufacturers in Sept. 2004. Human Serum Albumin (HSA) and fluorescent probes; 1-amilinonaphthalene-8-sulfonate (ANS) and Dansyl Sarcosine piperidinium salt (DSS) were purchased from Sigma Chemical Co., U.S.A. All other reagents were of analytical grade. Water used was double distilled in all glass apparatus. HSA solutions were prepared based on molecular weight of 66,500. All experiments were carried out in 0.1 M phosphate buffer using fluorescence spectroscopic technique. Perkin Elmer fluorescence spectrophotometer (MPF 44B) equipped with a 150 W xenon lamp source was used.

Determination of binding parameters: Binding parameters were determined using Ward method[9,10]. Two milliliters of 10 μM Human Serum Albumin (HSA) solution was taken in a quartz cell and increasing amounts of drug stock solution (1 mM) was added. HSA concentration was kept fixed at 10 μM by adding the same volume of 20 μM HSA solution to the cell. Fluorescence spectra were recorded in the range 300-400 nm after excitation at 295 nm in each case. Intrinsic fluorescence of protein was measured at 334 nm; drugs used did not have any fluorescence at the emission wavelength of protein. No correction for inner filter effect was applied since the drugs had very low absorbance (less than 0.05) at the excitation and emission wavelengths.

Stoichiometry of the drug-protein interaction was determined by the method of continuous variations[11], keeping drug plus protein concentration constant at 10 μM.

Data analysis: Data was analysed as follows using Ward method[9]. The fractional occupancy of the total protein binding sites by drug was obtained from the ratio, $\theta = \Delta F/\Delta F_{max}$[10], where, $\Delta F = F_0 - F$; F_0 and F are the fluorescence intensities of human serum albumin in the absence and presence of drug, respectively. ΔF_{max} values were obtained from the double reciprocal plots ($1/\Delta F$ versus $1/D_t$).

If P_t is the total protein concentration and n is the number of binding sites, the total number of sites on protein is given by nP_t and the concentration of bound sites on protein is given by $n\theta P_t$[9], which is also equal to the concentration of the bound drug (D_b). D_f, the number of moles of free drug, was obtained from the difference, $D_t - D_b$, where D_t is the total drug added. The amount bound was expressed as moles of drug bound per mole protein, r (= D_b/P_t). The binding parameters were computed directly by fitting the experimental data (r and D_f values) to the following general equation (Scatchard equation) using an iterative non-linear least squares regression program, developed for this purpose.

$$r = \sum_{i=1}^{i=m} n_i K_i D_f / 1 + K_i D_f \quad (1)$$

The association constant (K) and the number of binding sites (n) were determined at three different temperatures (17, 27 and 37°C) and the thermodynamic parameters for binding were calculated using equations:

$$\Delta G^0 = -RT \ln K \quad (2)$$

$$\ln K = -\Delta H^\circ/RT + \Delta S^\circ/R \quad (3)$$

Drug-albumin interaction in the presence of fluorescent probes: Experiments were also carried out in the presence of hydrophobic probe, ANS[12,13] and site II-specific probe, dansylsarcosine, DSS[14,15].

RESULTS AND DISCUSSION

Drug-serum albumin interaction: HSA is a single-chain protein containing 585 amino acids and has an isoelectric point of 4.7. The protein has three homologous domains (I-III) and each of these is comprised of two subdomains (A and B). It contains a single tryptophan residue at position 214 in subdomain IIA[16]. Intrinsic fluorescence of HSA is usually measured by selectively exciting the tryptophan residues at 295 nm. Cloxacillin, dicloxacillin and flucloxacillin are structurally similar. Dicloxacillin differs from cloxacillin by the presence of an additional chlorine atom and flucloxacillin by the presence of a fluorine atom, on the aromatic ring in the acylamino side chain. The structures of drugs used are shown in Fig. 1. They are anionic molecules with pK_a values in the range 2.7-2.8 and will be fully ionised in aqueous solution at pH 7.4[7].

All the three drugs were found to quench the intrinsic fluorescence of serum albumin. However, there was no observable shift in the wavelength for maximum emission. The stoichiometry of the interaction was determined by the method of continuous variations, described in the experimental section. The maximum in the fluorescence change in Job's plot occurred at 0.5 mole fraction of drug in each case, corresponding to 1:1 stoichiometry. One representative plot for flucloxacillin sodium is shown in Fig. 2.

The experimental data could be fitted into an equation for only one class of binding sites (m = 1). Association constants were of the order of 10^4 in each

Table 1: Binding parameters for the interaction of isoxazolyl penicillins with human serum albumin at pH 7.4 and different temperatures

Temperatur	Cloxacillin		Dicloxacillin		Flucloxacillin	
(K)	K x 10^{-4*}	n*	K x 10^{-4*}	n*	K x 10^{-4*}	n*
290.15	1.525	0.99	5.560	0.97	4.122	0.94
300.15	1.978	0.98	4.006	0.97	3.370	0.95
310.15	2.347	1.00	3.249	1.11	2.718	0.98

*K and n are the association constant and the number of binding sites

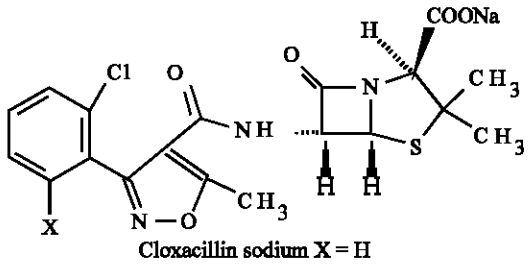
Table 2: Thermodynamic parameters for the interaction of isoxazolyl penicillins with human serum albumin

Drug sample	Free energy change (ΔG°)* kJ mol^{-1}	Enthalpy change (ΔH°) kJ mol^{-1}	Entropy change(ΔS°) J mol^{-1}
Cloxacillin	−25.941	+16.179	+135.980
Dicloxacillin	−26.779	−20.160	+21.180
Flucloxacillin	−26.320	−5.590	+34.650

*ΔG° values have been calculated at 37°C

case. The order of association constants was found to be dicloxacillin sodium>flucloxacillin sodium> cloxacillin sodium. The relatively higher association constants of dicloxacillin and flucloxacillin as compared to cloxacillin show that the binding affinity increases as the size and hydrophobicity of the acylamino side chain in drug molecule increases (Table 1). Thus acylamino side chain is involved in binding. Ruso *et al.*[7], using dynamic light scattering experiments, have shown that the hydrodynamic radius of HSA-dicloxacillin complex is larger than that of the HSA-cloxacillin complex. Barbosa *et al.*[8] using microcalorimetric technique have also shown significant differences in the physico-chemical characteristics of HSA-cloxacillin and HSA-dicloxacillin complexes.

It is seen from Table 2 that the standard free energy change (ΔG°) is lower for cloxacillin as compared to dicloxacillin and flucloxacillin. In the case of dicloxacillin and flucloxacillin, the standard enthalpy change, ΔH° was negative and standard entropy change, ΔS° was positive. In the case of cloxacillin, however, both ΔH° and ΔS° were found to be positive with high ΔS° value. The nature of drug-protein interaction could be predicted from the thermodynamic parameters for the binding. For electrostatic interactions, ΔH^0 has a very small negative value or is nearly zero[9,17]. High magnitude of ΔH° in the case of all the drugs suggested that electrostatic interactions are not present. This can also be inferred from the fact that at physiological pH, drugs as well as HSA are in predominantly anionic form. Positively charged side chain residues of protein do not appear to be involved in binding. In the case of cloxacillin sodium, ΔS° had a large positive value and ΔH° was also positive. Thus hydrophobic interactions are predominantly involved in the binding of cloxacillin to HSA[18]. In the case of



Cloxacillin sodium X = H

Dicloxacillin sodium X = Cl

Flucloxacilli sodium X =F

Fig. 1: Structures of isoxazolyl penicillins

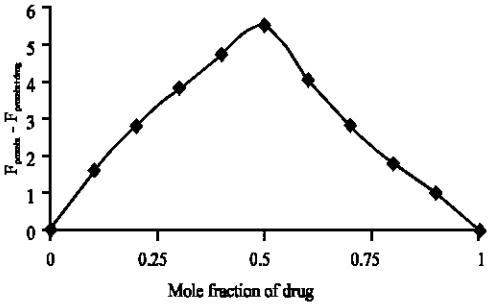


Fig. 2: Job's plot for the binding of flucloxacillin sodium to human serum albumin

dicloxacillin and flucloxacillin, ΔS° is again positive indicating the participation of hydrophobic interactions in the binding. However, negative ΔH^0 values in the case of these drugs indicate that hydrogen bonding interactions also contribute to the binding[19]. Substituents Cl and F on the phenyl group of the acylamino side chain in dicloxacillin and flucloxacillin, probably increase the ionic character of the C-Cl and C-F bonds, thereby facilitating the formation of hydrogen bonds.

Drug-albumin interaction in the presence of fluorescent probes: In order to further understand the nature of interaction involved, binding was also studied in the presence of hydrophobic probe, ANS and site II-specific probe, dansylsarcosine (DSS)[14,15]. Under identical conditions, at the highest quencher concentration used (12 μM), whereas ANS could quench about 55% of protein fluorescence, the drugs used could quench only 6-9% of the protein fluorescence. The percentage quenching of protein fluorescence by drugs was much less as compared to the quenching by ANS. It, thus, appears that the mode of interaction of drugs and ANS with HSA are different. ANS fluorescence was also measured in albumin-ANS mixture in the absence and presence of increasing amounts of drug. It was found that

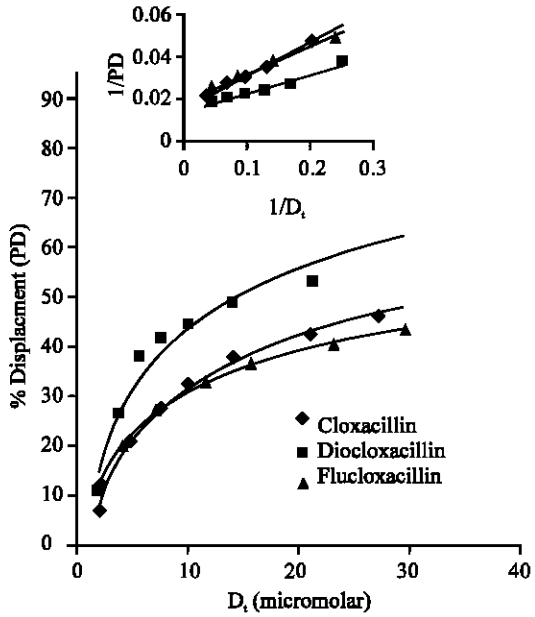


Fig. 3: Percentage displacement of DSS by penicillins

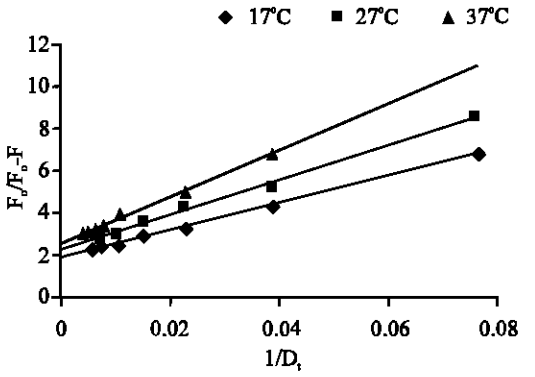


Fig. 4: Stern-Volmer plots for the binding of flucloxacillin sodium to HSA at different temperatures.

in each case, the presence of drug caused only a small decrease in the fluorescence of ANS. The effect was quantitatively studied by determining the percentage displacement (D) of probe, ANS. The results showed that up to a drug:protein ratio of 5:1, only 8-11% of drugs were displaced, which leads to the conclusion that the drugs and ANS do not share common site on albumin.

Site-specific probe, dansylsarcosine (DSS) shows greatly enhanced fluorescence as a result of interaction with proteins and other macromolecules[14,15]. The addition of increasing amount of drug (2-30 μM) to HSA-DSS mixture (1:1, 5 μM each) resulted in decrease of DSS fluorescence, indicating thereby that the drugs displace DSS from its binding site. Percentage displacement (PD) of probe, determined using equation, $(F_1-F_2)/F_1$, Where, F_1 and F_2 are the fluorescence intensities of the probe + HSA without and with drug, respectively has also been plotted against drug concentration in Fig. 3. The maximum percentage displacement, obtained from the double reciprocal plot (1/PD versus $1/D_t$, Inset Fig. 3), was found to be 72% in the case of dicloxacillin, 62% in the case of cloxacillin and 52% in the case of flucloxacillin. Since DSS is a site II-specific probe, displacement of DSS from its binding site on albumin showed that site II is involved in the binding of isoxazolyl penicillins to HSA. Crystallographic analyses have assigned site II in HSA to subdomain IIIA and among the individual amino acid residues in this subdomain, ^{410}Arg and ^{411}Tyr are usually assumed to be important[20,21].

Earlier studies regarding the nature of interaction of penicillins with serum albumin, are rather conflicting. Some researchers believe that only hydrophobic interaction contributes to the complex formation[6], other authors think that the electrostatic interactions between the carboxyl group of penicillin thiazolidine ring and positively charged groups on the protein surface occur together with hydrophobic interactions[3,22]. The presence of hydrogen bonds in the penicillin-albumin complex has also been reported[23]. However, present results clearly indicated that in the case of cloxacillin, the interactions are predominantly hydrophobic in nature. In the case of dicloxacillin and flucloxacillin, hydrogen bonding as well as hydrophobic interactions are involved in the binding.

Stern-Volmer Analysis: Stern-Volmer analysis of fluorescence data is useful in the estimation of the accessibility of tryptophan residues in proteins to the drug (quencher) molecules. Simple Stern-Volmer equation was not applicable. The plots showed a downward curvature, indicating the presence of buried tryptophan residues. Fluorescence quenching data was, therefore, analysed by the modified Stern-Volmer equation[24].

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a} + \frac{1}{[Q] f_a K_q} \quad (4)$$

where, $[Q] = D_t$ is the total drug concentration, F_0 and F are the steady state fluorescence intensities at 334 nm in the absence and presence of quencher (drug), respectively, K_q is the Stern-Volmer quenching constant and f_a is the fraction of fluorophore accessible to the quencher. From the linear $F_0/(F_0 - F)$ versus $1/D_t$ plots (Fig. 4), K_q and f_a values were calculated. K_q values were of the order of 10^4 in the case of all the drugs and f_a values were found to be close to 0.5 in most cases (Table 3). The variation of K_q and f_a values are due to the structural differences in the acylamino side chains in three

Table 3: Parameters f_a and K_q of the Stem-Volmer equation for the interaction of isoxazolyl penicillins with human serum albumin

	Cloxacillin		Dicloxacillin		Flucloxacillin	
Temperature (K)	$K_q \times 10^{-4}$	f_a	$K_q \times 10^{-4}$	f_a	$K_q \times 10^{-4}$	f_a
290.15	1.384	0.47	3.869	0.49	2.970	0.53
300.15	1.693	0.65	3.297	0.52	2.660	0.45
310.15	2.056	0.39	2.636	0.53	2.290	0.39

penicillins. It is known that the accessibility of the tryptophan residues of HSA varies with the nature and size of the molecules of interacting species[25]. The results show that the tryptophan residues of protein are not fully accessible to drugs. It appears that the tryptophan residue of HSA is not part of the drug binding site. However, drug binding site is in close proximity to the tryptophan residues, since nearly 50% of HSA fluorescence was quenched by the drugs. Kragh-Hansen[26] and Steinhardt *et al.*[27] have suggested that the albumin domains have hydrophobic interior and polar exterior and the environments of the tryptophan residues in albumin solutions are relatively polar. Isoxazolyl penicillins used in the present work appear to shield the tryptophan residues by binding at a site close to these residues. The observation that the quenching constants are close to the association constants for the interaction indicate that the quenching mechanism essentially involves static quenching[28].

For a bimolecular quenching process, $K_q = k_q \tau_0$ where, τ_0 is the lifetime in the absence of quencher and k_q is the rate constant for quenching. As τ_0 value for tryptophan fluorescence in proteins is known to be of the order of 10^{-9}s[29], the rate constant, k_q would be of the order of $10^{13}\ M^{-1}s^{-1}$. The upper limit of k_q expected for a diffusion-controlled bimolecular collisional quenching constant is $10^{10}\ M^{-1}s^{-1}$[30]. The high magnitude of k_q in the present study ($10^{13}\ M^{-1}s^{-}$)[1] also shows that the quenching is not initiated by dynamic collision, but originates from the formation of a complex. However, collisional quenching mechanism is also involved since the magnitude of quenching constants are smaller than the association constants for the interaction.

CONCLUSIONS

Isoxazolyl penicillins are bound to human serum albumin on a single site with moderate affinity. Acylamino side chain is involved in the binding. The binding is predominantly through hydrophobic interactions in the case of cloxacillin. In the case of dicloxacillin and flucloxacillin, hydrophobic interactions as well as hydrogen bonding interactions are involved. Studies carried out in the presence of hydrophobic probe showed that drugs and ANS do not share common site on albumin. Displacement of DSS from its binding site on albumin showed that site II is involved in the binding. Stern-Volmer analysis of the fluorescence data showed that the tryptophan residues of HSA is not fully accessible to the drugs, the drug binding site is in close proximity to the tryptophan residues and predominantly static quenching mechanism is involved.

ACKNOWLEDGMENTS

Pooja Agarwal is thankful to the Council of Scientific and Industrial Research (CSIR), New Delhi, India for financial assistance. The authors are also thankful to M/s. Surya Medicare Ltd., Derabassi, India for gift samples of the drugs.

REFERENCES

1. Lacey, R., 1982. Stability of the isoxazolyl penicillins to staphylococcal beta-lactamase. J. Antimicrob. Chemother., 9: 239-240.
2. Terasaki, T., H. Nouda and A. Tsuji, 1992. Relationship between lipophilicity and binding affinity with human serum albumin for peniciliin and cephem antibiotics. J. Pharmacobio-Dyn., 15: 99-106.
3. Keen, P.M., 1966. The binding of penicillins to bovine serum albumin. Biochem. Pharmacol., 15: 447-463.
4. Craig, W.A. and C.M. Kunin, 1976. Significance of serum protein and tissue binding of antimicrobial agents. Ann. Rev. Med., 27: 287-300.
5. Ullmann, U., 1977. The binding of isoxazolyl penicillins to human serum proteins. Arzneimittel. Forschung., 27: 2136-2138.
6. Markovich, M.N., L.G. Isakovich and V.F. Klinichev, 1986. Microcalorimetric study of the parameters of dicloxacillin binding with human serum albumin at different temperatures. Antibiot. Med. Biotekhnol., 31: 603-606.
7. Ruso, J.M., D. Attwood, M. Garcia, P. Taboada, L.M. Varela and V. Mosquera, 2001. A study of the interaction of the amphiphilic penicillins cloxacillin and Dicloxacillin with human serum albumin in aqueous solution. Langmuir, 17: 5189-5195.

8. Barbosa, S., P. Taboada, D. Attwood and V. Mosquera, 2003. Thermodynamic properties of the complex formed by interaction of two anionic amphiphilic penicillins with human serum albumin. Langmuir, 19: 10200-10204.
9. Ward, L.D., 1985. Measurement of ligand binding to proteins by fluorescence spectroscopy. Methods in Enzymol., 117: 400-414.
10. Maruyama, T., M. Otagiri and S.G. Schulman, 1990. Binding characteristics of coumarin anticoagulants to human-α_1-acid glycoprotein and human serum albumin. Intl. J. Pharm., 59: 137-143.
11. Rahman, M.H., T. Maruyama, T. Okada, K. Yamasaki and M. Otagiri, 1993. Study of interaction of carprofen and its enantiomers with human serum albumin-I. Mechanism of binding studied by dialysis and spectroscopic methods. Biochem. Pharmacol., 46: 1721-1731.
12. Daniel, E. and G. Weber, 1966. Cooperative effects in binding by bovine serum albumin. I. the binding of 1-anilino-8-naphthalenesulfonate. Fluorimetric titrations. Biochemistry, 5: 1893-1900.
13. Seedher, N. and S. Bhatia, 2005. Mechanism of interaction of non-steroidal anti-inflammatory drugs meloxicam and nimesulide with serum albumin. J. Pharm. Biomed. Anal., 39: 257-262.
14. Sudlow, G., D. Birkett and D. Wade, 1975. The characterization of two specific drug binding sites on human serum albumin. Mol. Pharmacol., 11: 824-832.
15. Sudlow, G., D. Birkett and D. Wade, 1976. Further characterization of specific drug binding sites on human serum albumin. Mol. Pharmacol., 12: 1052-1061.
16. Kragh-Hansen, U., V.T.G. Chuang and M. Otagiri, 2002. Practical aspects of the ligand-binding and enzymatic properties of human serum albumin Biol. Pharm. Bull., 25: 695-704.
17. Ross, P.D. and S. Subramanian, 1981. Thermodynamics of protein association reactions. Forces contributing to stability. Biochemistry, 20: 3096-3102.
18. Aki, H. and M. Yamamoto, 1989. Thermodynamics of the binding of phenothiazines to human plasma, human serum albumin and alpha-acid glycoprotein. A calorimetric study. J. Pharm. Pharmacol., 41: 674-679.
19. Miyoshi, T., K. Sukimoto and M. Otagiri, 1992. Investigation of the interaction mode of phenothiazines neuroleptics with α_1-acid glycoprotein. J. Pharm. Pharmacol., 44: 28-33.
20. He, X.M. and D.C. Carter, 1992. Atomic structure and chemistry of human serum albumin. Nature, 358: 209-215.
21. Sugio, S., A. Kashima, S. Mochizuki, M. Noda and K. Kobayashi, 1999. Crystal structure of human serum albumin at 2.5 A resolution. Protein Eng., 12: 439-446.
22. Philips, G.O., D.W. Power, C. Robinson and J.V. Davies, 1970. Ion binding of penicillins to protein. Biochim. Biophy. Acta., 215: 491-502.
23. Landau, M.A., M.N. Markovich and L.A. Piruzyan, 1977. Studies on the thermodynamics and nature of interaction between serum albumin and penicillins. Biochem. Biophys. Acta., 493 : 1-9.
24. Lehrer, S.S., 1971. Solute perturbation of protein fluorescence. The quenching of tryptophan fluorescence of model compounds and of lysozyme by iodide ion. Biochemistry, 10: 3254-326.
25. Weber, G. and L.B. Young, 1964. Fragmentation of bovine serum albumin by pepsin. I. The origin of the acid expansion of the albumin molecule. J. Biol. Chem., 239: 1415-1423.
26. Kragh-Hansen, U., 1981. Molecular aspects of ligand binding to serum albumin. Pharmacol. Revs., 33: 17-53.
27. Steinhardt, J., J.D. Leidy and J.P. Mooney, 1972. Effects of n-alkyl ligands on the difference spectra of bovine and human serum albumin. Biochemistry, 11: 1809-1817.
28. Gonzalez-Jimenez, J., G. Frutos and I. Cayre, 1992. Fluorescence quenching of human serum albumin by xanthines Biochem. Pharmacol., 44: 824-826.
29. Cui F.L., J. Fan and W. Li *et al.* 2004. Fluorescence Spectroscopic studies on 5-aminosalicylic acid and zinc 5-aminosalicylate interaction with human serum albumin. J. Pharm. Biomed. Anal., 34: 189-197.
30. Pang, Y.H., L.L. Yang, S.M. Shuang, C. Dong and M. Thompson, 2005. Interaction of human serum albumin with bendroflumethiazide studied by fluorescence spectroscopy. J. Photochem. Photobiol. Biol., 80: 139-144.