RESEARCH PAPER

Accepted 14 April 1998
Received 6 October 1997
Indian J. Pharm. Sci., 1998, 60(5) pp. 270-274

In vitro protein binding studies on aztreonam : Temperature effects and thermodynamics

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The effect of temperature on protein binding of aztreonam was investigated at pH 7.4 by equilibrium dialysis method using bovine serum albumin (BSA) with the temperatures ranging between 25° and 42°. Binding parameters like albumin-bound fraction (β), number of binding sites (n), association (binding) constant (K) and the thermodynamic parameters like standard free energy (ΔG°), enthalpy (ΔH°) and entropy (ΔS°) were computed on an in-house PC using PROBE software developed by the authors. The binding of aztreonam was found to decrease with an increase in temperature, the effect being more pronounced at temperatures above 37°. The subsequent factorial analysis on the computed β values, confirmed the effect of temperature on binding to be statistically significant. The magnitude and sign of the thermodynamic parameters indicated the interaction between aztreonam and BSA to be predominantly ionic in nature.

BINDING of drugs to plasma proteins have important pharmacokinetic and pharmacodynamic implications[1-2]. A variation in temperature is an important factor amongst a myriad of variants which generally affect the magnitude of the protein-bound faction of a drug in biological fluids[3-4]. Therefore, the effect of temperature on protein binding of drugs like zidovudine[5], azosemide[6], methohexital[7], phenytoin[8], vancomycin[9], bumatanide[10], haloperidol[11], thiopental sodium[12], methotrexate[13], naproxen[14], pethidine[15], digoxin and its metabolites[16], bile salts[17], p-substituted acetanilides including acetaminophen[18], theophylline[19], quinidine[20], an investigational hepato-protective agent, YH-439[21], furosemide[22], imipramine[23] and valproate[24] have already been worked out. Alteration in temperature tends to induce a change in binding by altering the concentration of unbound (free) drug, binding protein and/or binding sites on a protein in the biological fluids. The magnitude as well as the sign of the thermodynamic parameters derived from the binding data at varied temperatures has been employed to postulate the mechanism of drug-protein complexation[3,4,17,18,25].

Aztreonam (Azthreonam, SQ-26776) is a monobactam potent broad spectrum antibiotic with activity specifically directed against a wide range of gram negative aerobic bacteria[26]. The serum binding[27] and the effects of pH, protein concentration and drug concentration[28] on its albumin binding have already been investigated. In the absence of any report on temperature effects on aztreonam binding, the present study is aimed to study the same and to elucidate the type of drug-albumin interaction involved through thermodynamics of protein binding.

EXPERIMENTAL

Materials : Aztreonam was a generous gift from M/s Squibb Institute for Medical Research, Princeton, NJ, U.S.A. Cellulose dialysis sacs (average width 25 mm, inflated diameter 16 mm and molecular weight cut-off of 12,000) were obtained from M/s Sigma Chemical Co., U.S.A. (Catalog no. 250-9U). Bovine Serum Albumin (BSA) fraction

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V (98.5% pure) was procured from M/s Loba Chemicals, Bombay (Catalog no. 2230). All other reagents were of analytical grade.

Instruments : A Perkin-Elmer UV-visible Lambda 15 spectrophotometer for UV spectral studies, a Spectronic 1201 (Milton Roy, U.S.A.) for routine spectrophotometric determination of unbound ligand, a Control Dynamics APX 175 digital pH-meter equipped with Ingold 455 combination electrode for pH determination, a thermostatically-controlled mechanical shaker (Beutex Instruments, New Delhi) for gentle and uniform shaking of dialysis assembly and a 80486 DX2 PC (Microcare Professionals, Chandigarh) for data treatment were used during the study.

Protein Binding Studies : Binding studies of aztreonam using BSA were carried out by equilibrium dialysis method as described previously[28]. The effect of temperature was studied at five temperatures ranging between 25° and 42°. The studies were carried out at pH 7.4 (phosphate buffer) and at a fixed protein concentration of 4.225 % w/v (~65mM). For construction of Scatchard plots, five varied drug concentrations ranging between 100 and 300 µg/ml were employed.

Data Treatment : The binding parameters, β, n, K as well as the thermodynamic parameters, ΔG° (kJ mol^{-1}), ΔH° (kJ mol^{-1}) and ΔS° (J K^{-1} mol^{-1}) were computed on an in-house PC using a computer program, PROBE[29,30] developed by the authors. For studying temperature effects, the algorithm of PROBE software takes into account a set of equations 1-4. The binding parameters n and K were calculated from Scatchard plots between r and r/D_f using eqn. 1 derived by Scatchard[31] and the thermodynamic parameters using standard thermodynamic eqn. 2-3 and the Van't Hoff equation 4[3,25]. The thermodynamic parameters were computed at the harmonic mean temperature (HMT) of all the temperatures studied and calculated using eqn. 5.

$$r/D_f = nK - Kr \quad \text{.....(1)}$$

$$\Delta G^\circ = - RT \ln K \quad \text{.....(2)}$$

$$\Delta G^\circ = \Delta H^\circ - T.\Delta S^\circ \quad \text{.....(3)}$$

$$\ln K = \frac{\Delta S^\circ}{R} - \frac{\Delta H^\circ}{RT} \quad \text{.....(4)}$$

$$HMT = NT/\Sigma_{c=1}^{NT}(1T_i) \quad \text{.....(5)}$$

Where, r is the ratio of molar concentrations of drug to that protein, D_f is the molar concentration of free drug, T is the absolute temperature, NT is the number of temperatures and R is the Gas constant (taken as 8.3144 J K^{-1} mol^{-1}). The statistical significance of binding results was evaluated using a two-way ANOVA-based factorial analysis on the binding data taking temperature and drug concentration as the factors[32].

RESULTS AND DISCUSSION

The Scatchard plots at all the temperatures were found to be linear ($r^2 > 0.94$, F Ratio > 15, $p < 0.05$) indicating only one class of binding sites on albumin, in agreement with our previous findings[28]. The effect of temperature on BSA binding parameters is summarized in Table 1. Table 2 enlists the thermodynamic parameters at HMT of 33°. Fig. 1 shows a plot of percent unbound drug at different temperatures, while the Van't Hoff plot between log K and 1/T is depicted in Fig.2.

It is apparent from Fig. 1, that an increase in temperature is associated with a decrease in protein bound fraction of aztreonam. This may be due to the decreased affinity of the drug to the albumin molecule as reflected by a constant fall in the values of the association constants, while the number of binding sites remain practically constant over the temperature range studied (Table 1). The decrease in albumin binding of YH-439 with an augmentation in incubation temperature has similarly been proposed to be due to decreased binding affinity[21]. As shown in Fig. 1, the binding initially decreases at a slower rate ($d\beta/dT = 0.0061$) and only 25% reduction in bound fraction is observed between 25° to 37°. However, a relatively faster rate of increase in albumin-bound fraction ($d\beta/dT = 0.0352$) is observed between the higher temperature range of 37° to 41° with almost three-fold diminution in binding discernible in the entire range.

Most literature reports[5-8,10,13,15,18-23] also indicate significant effect of temperature on *in vitro* protein binding, while some others[9,11,16] construe temperature-independent binding. Invariably in all these studies, the protein binding has been found to diminish with increasing temperature, except with furosemide[22] where binding tended to increase with temperature. Our results are in consonance with these findings. ANOVA-based two-way factorial analysis of binding data in the current study indicates statistically

Table 1
In vitro Protein Binding Parameters of Aztreonam at Various Temperatures

Temperature (°C)	Percent Bound (Mean ± SD)	Association Constant (mol^{-1})	Number of Binding Sites
25	29.5 ± 8.8	5680	0.97
29	28.0 ± 7.6	3629	1.09
37	22.2 ± 5.2	1698	1.12
39	15.6 ± 2.6	1292	1.06
41	7.6 ± 1.6	844	1.02

significant effect of temperature ($p < 0.001$) as well as drug concentration ($p < 0.05$) on binding of aztreonam. That the drug concentration has significant effect on the fraction of aztreonam bound to serum albumin has already been reported [26]. Since aztreonam is administered to patients with Gram negative infection(s) with associated pyrexia, the temperature range beyond 37° is clinically more relevant to interpret. It can be anticipated that higher blood levels of unbound aztreonam (as shown in Fig. 1) would be present in patients with high fever. However, the magnitude of total albumin binding of aztreonam being small (i.e.,only 22%), about 8-9% augmentation in the concentration of the pharmacodynamically active unbound (free) drug is observed in the temperature range between 37° and 39° and also between 39° and 41°. This may result in only inconsequential alteration in the clinical outcome of the aztreonam therapy in hyperthermic patients.

The Van't Hoff plot (Fig. 2) in the present study was also found to be linear ($r^2 = 0.984$, F ratio = 61.5, $p < 0.01$). The linearity of the Van't Hoff plots has long been used to indicate that no conformational change in binding sites on protein molecules takes place within the temperature range studied[25,31]. However, some authors[3,33] are skeptical about drawing any conclusion from this characteristic. Besides the linearity of Van't Hoff plot, the almost constant values (table 1) of number of binding sites, ranging between 0.97 and 1.12 (Mean 1.07), clearly demonstrate that BSA not show any conformational change on binding in the temperature range studied.

Of the thermodynamic parameters, the entropy change ($\Delta S°$) on binding has been found to be most useful in predicting the nature of interaction between the drug and the binding site on protein molecule[34]. Since the total entropy change may involve several contributions, like hydrophobic bonds, van der Waal's forces, electrostatic, hydrogen bonds, conformational change in protein or drug molecule and changes in the ionization of drug or protein, it is difficult to hypothesize a single mechanism of drug-protein interaction. But it is well known that the strong hydrophobic affinity between protein molecule and the ligand is characterized by high and positive entropy

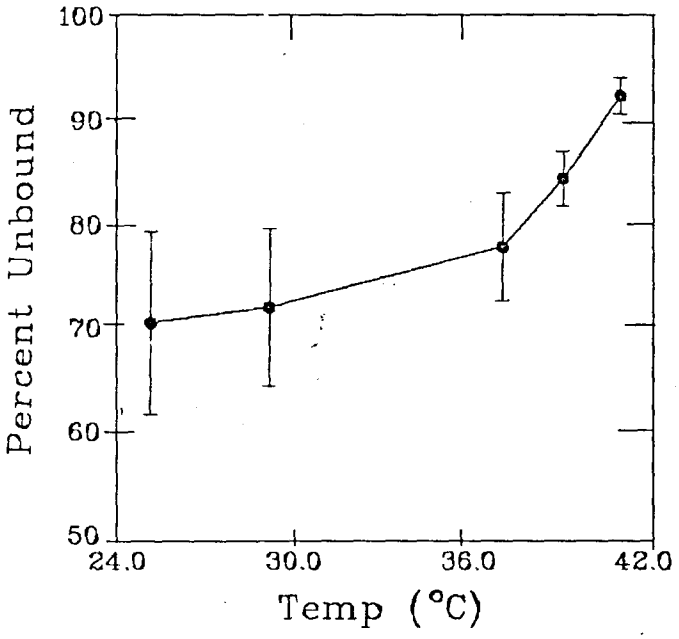
Table 2
Thermodynamic Parameters for Protein Binding of Aztreonam with BSA at Varied Temperatures

Temperature (°C)	$\Delta G°$ ($kJ\ mol^{-1}$)	$\Delta S°$ # ($J\ mol^{-1}\ K^{-1}$)
25	-22.44	-216.60
29	-20.73	-219.39
37	-19.18	-218.73
39	-18.60	-219.19
41	-17.65	-220.81
Mean*	-19.75	-219.75

* At Harmonic Mean Temperature (HMT) of 33°C.

Computed using average value of $\Delta H°$ as -87.019 $kJ\ mol^{-1}$ by Eqn.4

Fig.1 : Degree of protein binding of aztreonam at various temperatures



Each point represent the mean of experimental values and the crossbars indicate ± S.E.M

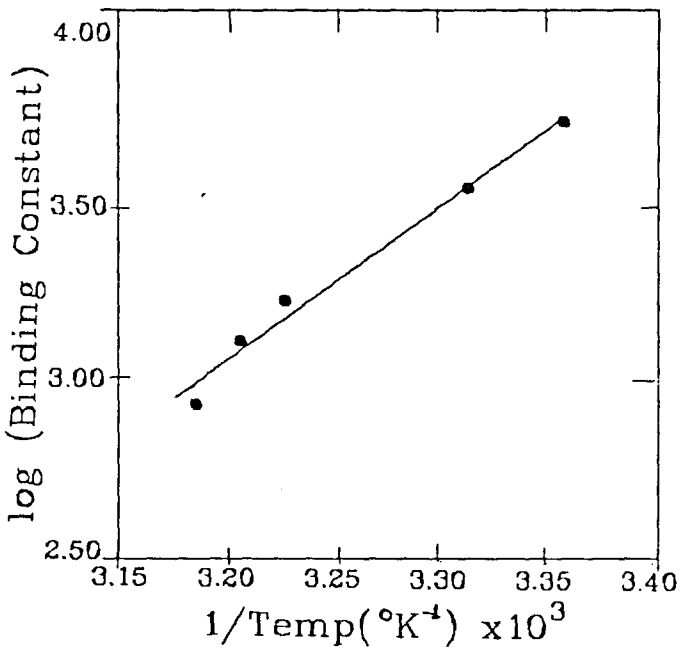


Fig.2 : Van't Hoff plot between log (association constant, mole^{-1}) of aztreonam and inverse absolute temperature (°K^{-1}) at pH 7.4.

values[34]. Positive entropies coupled with small negative enthalpies observed in the thermodynamic studies conducted on phenothiazines, have been construed to involve hydrophobic drug-protein interactions[35]. As usually highly protein bound drugs tend to be lipophilic, their degree of protein binding has been reported to correlate well with the lipophilicity[36,37]. However, in the present investigation, the large negative values of entropy rule out any major involvement of hydrophobic bonding. The negative value of enthalpy obtained from the slope of Van't Hoff plot, characterize exothermic interactions leading to the formation of stable drug-protein complexes.

The negative value of enthalpy together with the negative values of entropy observed with drug-protein binding have usually been hypothesized to involve hydrogen bonding[38]. In the current study, however, the high affinity of aztreonam to albumin molecule characterized by large magnitudes of binding constants (Table 1) can not be attributed only to relatively weak hydrogen bonds. On the other hand, Nemthy and Schrega[39] indicated that when the negative values of entropies and enthalpies are associated with high association constants (usually >10^3), the kind of interaction between the ligand and the protein should be concluded as predominantly ionic[39]. Aztreonam being a weak triprotic acid with pk_1=0.7, pk_2=2.75 and pk_3=3.91, remains essentially dissociated at biological pH. In our earlier study, the extensively higher magnitude of the fraction of aztreonam bound to albumin (0.22) than the fraction unionized (6.23×10^{-25}) at physiological pH of 7.4 and normal body temperature of 37° has also indicated the significant binding of aztreonam in the cationic form[28]. Thus the present thermodynamic study corroborates that the aztreonam-albumin binding is primarily ionic in nature. Such binding of ionic forms of drugs to plasma proteins have already been reported with imidazole[25], β-blockers[40] and fentanyl[41]. The moderately high and negative value of standard free energy indicates that ionic interactions between aztreonam and albumin are spontaneous in nature. Since the binding of drugs governed by such ionic interactions with plasma proteins is known to be altered in the presence of inorganic ions[41-43] like Ca^{++} and Cl, the likely influence of high concentration of such endogenous

ions on serum binding of aztreonam needs to be investigated.

ACKNOWLEDGMENTS

The authors thank the Squibb Institute for Medical Research, Princeton, NJ, U.S.A. for the generous gift of aztreonam and the valuable literature on the drug.

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