INTERACTION OF OXYTETRACYCLINE WITH OVINE SERUM ALBUMIN

Interacción de la oxitetraciclina con la seroalbumina ovina

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SUMMARY

The interaction of oxytetracycline with ovine serum albumin was studied. The apparent association constants, the number of binding sites and the thermodynamic parameters of the interaction were established using the equilibrium dialysis technique at 15, 20 and 25° C. At the three temperatures, one binding site was found for the ovine serum albumin.The apparent association constants ranged from 11845.54 to 13361.10 M^{-1}.The decrease in the constants, due to the decrease in temperature, suggests a loss of affinity between the oxytetracycline and the ovine serum albumin. The spontaneity of the interaction was established, based on the negative value of the standard free energy (ΔG). The decrease of spontaneity when the temperature drops was demonstrated by the variation of ΔG at the different temperatures. This fact arises from the loss of affinity between the oxytetracycline and the ovine serum albumin. Furthermore, the interaction was found to occur endothermically (standard enthalpy ($\Delta H > 0$), with stability (standard entropy ($\Delta S > 0$) and is regulated, fundamentally by electrostatic attraction.

Key words: oxytetracycline, protein binding.

RESUMEN

En el presente trabajo se ha estudiado la interacción de la oxitetraciclina con la seroalbúmina ovina, estableciéndose las constantes de asociación aparente, el número de sitios de unión y los parámetros termodinámicos de la interacción utilizando la técnica de la diálisis de equilibrio a 15, 20 y 25°C. A las tres temperaturas se encontró un sitio de unión para la seroalbúmina ovina, mientras que las constantes de asociación aparentes halladas oscilaron entre 11845.54 y 13361.10 M^{-1}. La disminución del valor de las constantes con la temperatura sugiere una pérdida de afinidad entre la oxitetraciclina y la seroalbúmina ovina. Se estableció la espontaneidad de la reacción, en base al valor negativo de ΔG. La variación del valor de ΔG a las distintas temperaturas del ensayo nos indica que la espontaneidad disminuye con la temperatura, hecho que se deriva del descenso de afinidad de la oxitetraciclina por la proteína. La interacción se produce además de manera endotérmica ($\Delta H > 0$) y estable ($\Delta S > 0$), estando regulada fundamentalmente por fuerzas de atracción electrostática.

Palabras clave: oxitetraciclina, unión a proteínas.

INTRODUCTION

Among the various important functions of plasma proteins is that of binding with many kinds of drugs. The interaction of drugs with plasma proteins is a rapidly reversible process, where the bound fraction of the drug can be considered as a temporary deposit, ready to be released at any time. The protein playing the most important role in this process is albumin, to which a wide variety of drugs binds, especially weak acid and neutral drugs (KRAGH-HANSEN, 1981). The process is similar to an enzyme-substrate reaction,except for the fact that the drug does not splitto produce new molecules(DAVISON, 1981). The interaction can take place through the following kinds of linkage: hydrogen bonds, hydrophobic repulsion, electrostatic attraction or van der Waals'forces (KLOTZ,1973; CURRY,1980; GIBALDI, 1991). When this interaction takes place, the drug circulates around the body in two different forms:free form and bound form, being both in dynamic equilibrium.

To study this process, it is necessary to assess the value of the parameters that determine it: the number of binding sites per molecule of protein and the apparent association constants. The energetic parameters of the interaction; free energy, enthalpy and entropy variation (ΔG, ΔH, ΔS), must also be considered, because they will allow us to establish the dynamic and structural properties of the system (HITZEMANN, 1988).

Terramycin (oxytetracycline hydrochloride) is an amphoteric drug belonging to the tetracyclinegroup, with dissociation constants (pKa) of 3.3, 7.3 and 9.1 (25°C), and therefore, ionized througout the physiological pH range, existing in cationic form at more acidic pH values, in anionic form at more alkaline pH values and in zwitterionic form at relatively neutral pH values.

It is commonly used nowadays in veterinary practice to treat infectious diseases, due to its broad spectrum bacteriostatic action. It is used in most domestic species, particularly in sheep (APPLEYARD AND GILMOUR, 1990; DARGATZ *et al.*, 1990).

The objective of the present study was to evaluate the oxytetracycline-ovine serum albumin (OTC-OSA)interaction at different temperatures,by determining the apparent association constants at each temperature and the number of binding sites per molecule; and by establishing the thermodynamic parameters of the interaction.

MATERIAL AND METHODS

OTC-HCl provided by Pfizer,S.A.,ovineserum albumin (FractionV powder),from Sigma,and Wisking dialysistubing, type 8/32, from Serva, were the materials used inthis study.

The equilibrium dyalisis was the method used, in which an aqueous solution containing the protein was placed in cellophane bags which were in turn placed in solutions of the antibiotic at different concentrations, so that the small drug molecules freely passed through the membrane, whereas the protein remained in the bag. The solutions were allowed to equilibrate for 24 hours. Once the equilibrium was reached, the internal and external concentrations of oxytetracycline approached one another, and the concentrations of free and bound drug could be determined.

The cellophane bagswere prepared following the method described by RUDMAN AND KENDALL (1957) and modified by SERRANO *et al.* 1984). Five assays at differents temperatures (15, 20, 25, 30 and 37°C) were carried out. Each assay was composed of three series, from which we obtained the experimental data, one control series, to elaborate the calibration curve, and one more series,to obtain the percentages of recuperation of the antibiotic.

The experimental series were composed of a 50 micromolar ovine serum albumin solution inside the bags, and an oxytetracycline solution at concentrations of 5, 10, 20, 40, 60, 80 and 100 micromoles per litre outside. Both the ovine serum albumin and the oxytetracycline were dissolved in a 0.065 molar pH=7.4 phosphate buffere solutionwith identical physico-chemical characteristics.

The fourth series (control series) just contained the oxytetracycline solutions at the same concentration that the experimental series. The fifth series was identical to the experimental series, but containing the bags buffered solution instead of pro-

tein. The fraction of antibiotic bound to the cellophane was determined from this last series, and then the porcentages of recuperation were deduced.

The quantitative determination of oxytetracycline was carried out using spectrophotometric methods, since this technique does not require sample processing, which prevents any modification of the concentration of protein. The wavelength chosen was 352 nm, at which oxytetracycline presents a maximum peak of absorbance (MOFFAT *et al.*, 1986). In these conditions, the coefficient of molar absorptivity was found to be approximately 20000 L/M/cm.

The free-drug concentrations were directly obtained from the concentration of the buffered solution, and the bound-drug concentrations were calculated by subtracting the free-drug concentration from the concentration inside the bag (total concentration).

To establish the number of binding sites and the association constants, an equation based on the mass action law and expressed as a function of r (number of moles of drug bound to one mole of protein) was used. This hyperbolic equation was transformed into lineal by several procedures, widely recommended: Lineweaver-Burk's (1), Klotz's (2), Scatchard's (3) (KLOTZ AND HUNSTON,1971) and Hill's (4) (CADENAS, 1978).

$$(1)\quad \frac{1}{r} = \frac{1}{n} + \frac{1}{nK}\,\frac{1}{(F)}$$

$$(2)\quad \frac{(F)}{r} = \frac{1}{nK} + \frac{1}{n}\,(F)$$

$$(3)\quad \frac{r}{(F)} = nK - rk$$

$$(4)\quad \ln \frac{r}{n-r} = \ln K - \ln (F)$$

From these equations, the parameters of the interaction, n and K,were obtained. These linearization procedures are shown in table 1.

To calculate these linearized equations,a BASIC programme was used. Data were weighted using the inverse of the square root of the product of the variables of each procedure $C=1/\sqrt{x\text{-}y}$.

The determination of the thermodynamic parameters was carried out using the following equations:

The standard free energy ($\Delta G0$) can be calculated from the equation $\Delta G = -RT \ln K$ (5) (WEPIERRE, 1988), and the standard enthalpy ($\Delta H0$) and the standard entropy ($\Delta S0$) can be calculated from the van't Hoff plot, or another plot such as the following:$\Delta G = \Delta H - T\,\Delta S$ (6) (KLOTZ AND ROSENBERG, 1977).

The statistical analysis was carried out using a t-test in order to find out whether or not the slope of the Hill plot was equal to the unity. The level of significance was $p<0.001$ in the case of the Lineweaver- Burk plot, whereas that found in the case of the Scatchard plot was inferior. These findings are supported by KLOTZ (1973) and WALTER (1974), who reported that the Scatchard plot is the less appropriate for the determination of the parameters of the interaction, although it has other advantages.

RESULTS

The results obtainedat 30 and 37°C were not taken into account due to the high degree of deterioration of the antibiotic,which was greater than 50 percent at 37°C in 22-24 hours.

The values of the slope, Y-intercept, correlation coefficient and standard error of the slope for each procedure at 15, 20 and 25°C, are shown in table 1. The number of binding sites can be obtained from the slope of the Klotz plot. Due to its value, close to the unity at the three temperatures, a t-test was carried out to compare the slope with the unity. No differences were found in any case, which is confirmed in the Hill plot when n=1. From this plot, the values of the association constants and the thermodynamic parameters (shown in table 2) were obtained.

The different plots of the oxytetracycline-ovine serum albumin interaction are shown in figures 1-4.

DISCUSSION

The fitness of experimental data depends on the relationship between the dependent and inde-

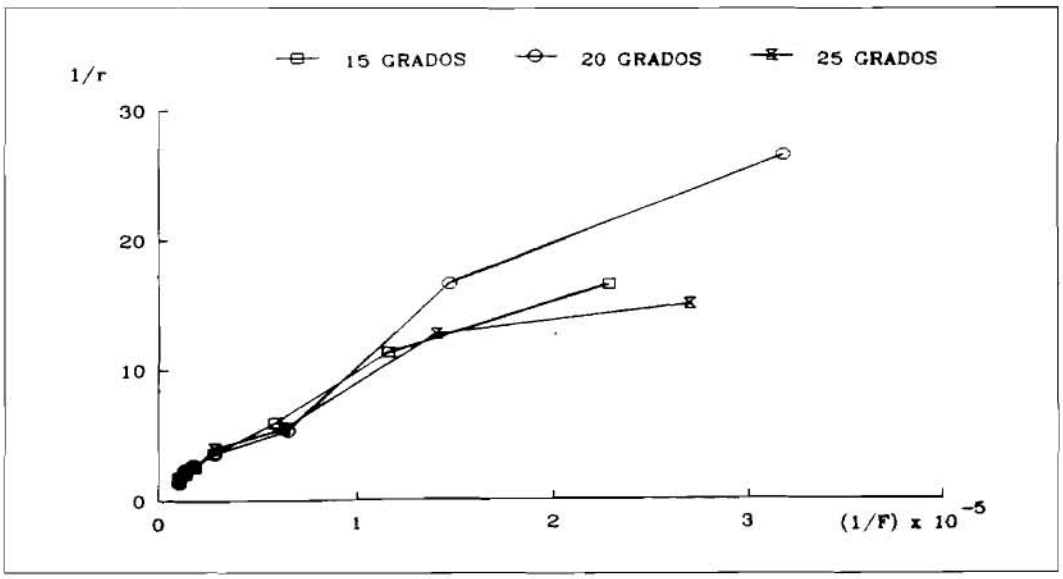

FIGURE 1. Lineweaver-Burk's plot of the interaction of oxytetracycline and ovine serum albumin.

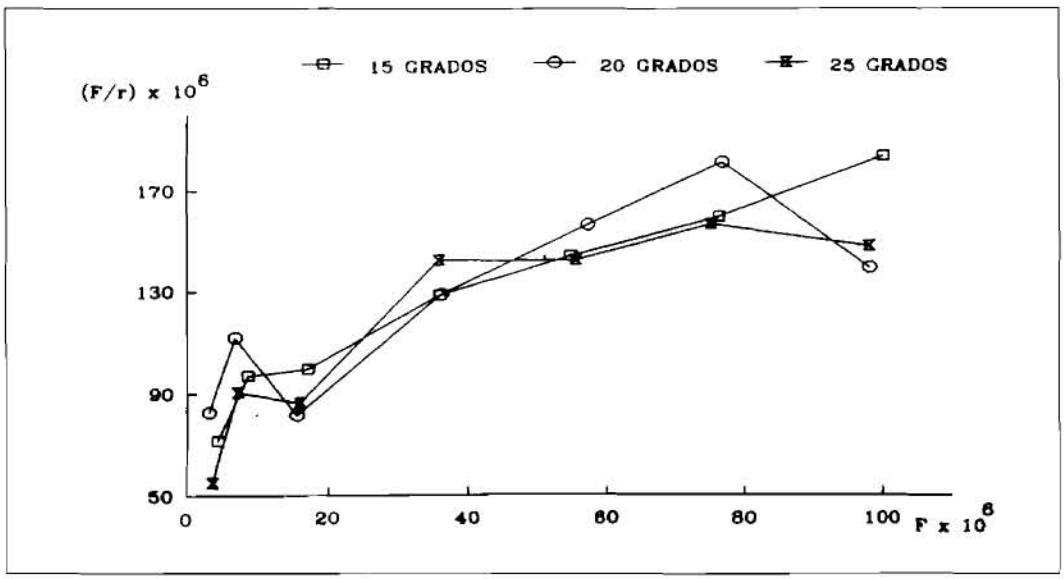

FIGURE 2. Klotz's plot of the interaction of oxytetracycline and ovine serum albumin.

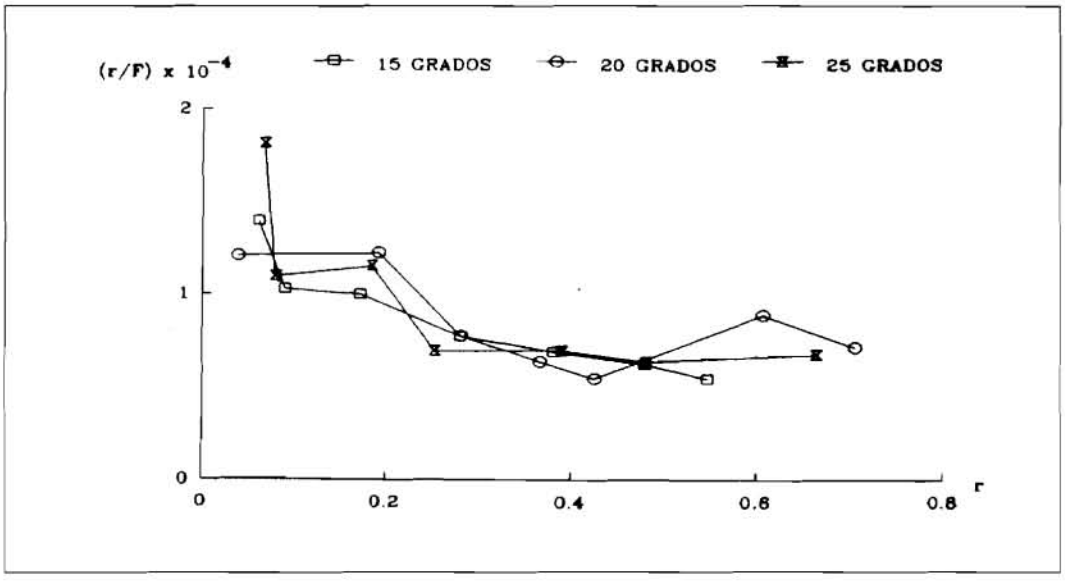

FIGURE 3. Scatchard's plot of the interaction of oxytetracycline and ovine serum albumin.

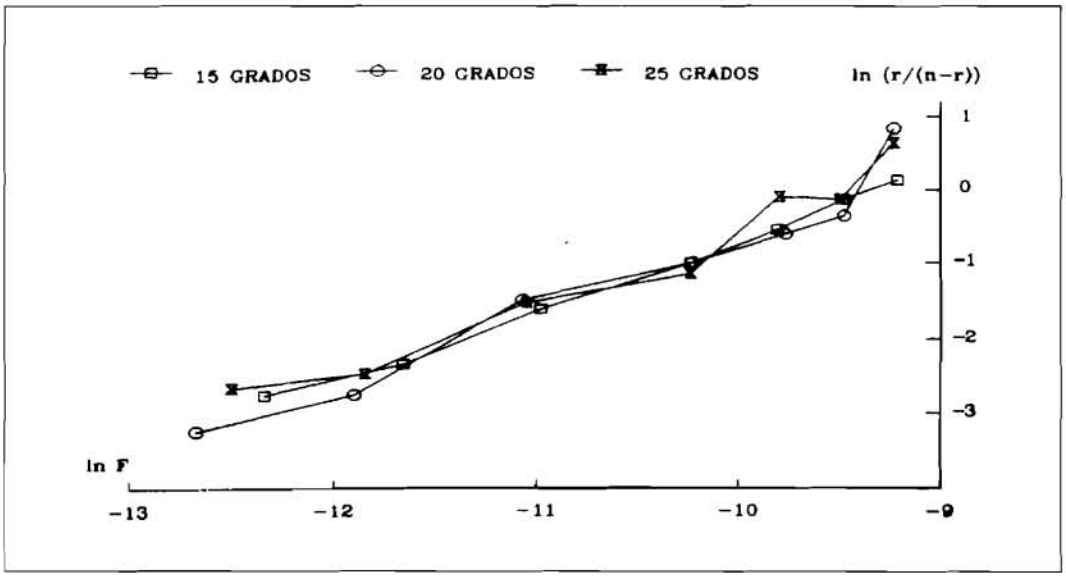

FIGURE 4. Hill's plot of the interaction of oxytetracycline and ovine serum albumin.

	LINEWEAVER BURK	KLOTZ	SCATCHARD	HILL[$]
15ºC				
Slope	7.2893-10^{-5}	1.1759	-12993.60	0.9752
Intercept	1.2923	7.6447	12283.15	9.1467
Standard Error	5.2906-10^{-6}	0.1703	3000.26	0.0552
Correlation coefficient	0.9534***	0.8456***	-0.7048***	0.9709***
20ºC				
Slope	8.4771-10^{-5}	0.9073	0-7433.06	1.0322
Intercept	0.9947	8.6762-10^{-5}	10558.23	9.7232
Standard Error	6.5247-10^{-6}	0.2187	3977,72	
Correlation coefficient	0.9474***	0.6894***	-0.4833*	0.0922
				0.9319***
25ºC				
Slope	6.0577-10^{-5}	1.1663	-14231.82	0.9839
Intercept	1.3571	6.5926-10^{-5}	13892.51	9.3474
Standard Error	5,1924-10^{-6}	0.1872	3883,57	0.0696
Correlation coefficient	0.9387***	0.8194***	-0.6425**	0.9556***

* Significant at 5% level ($p<0.05$)
** Significant at 1% level ($p<0.01$)
*** Significant at 0.1% level ($p>0.001$)
$ Considering one binding site per ovine serum albumin molecule

TABLE 1. Regression analysis of Lineweaver-Burk's, Klotz's, Scatchard's and Hill's equations at the temperatures of 15, 20 and 25° C (number of pairs = 21).

pendent variables in each method of linearization. What is more, data are evaluated in different ways if they are obtained from the first or the last segment of the plot. For these reasons, the three methods frequently give differently values of the parameters. So, weighting of the data is necessary. The inverse of the square root of the product of the variables was the coefficient used in this study, for there is variation in the values of both r and free oxytetracycline concentration.

As for the parameters of the interaction, the ovine serum albumin was found to have one binding site, since the Y-axis intercept in Lineweaver-Burk's, the slope of Klotz's and the X-axis intercept of Scatchard's plot were close to the unity.To check this fact, a statistical test was carried out, in which the null hypothesis was that the slope of Klotz's plot was equal to the unity. t-values of 1.033, 0.424 and 0.888 were found at 15, 20 and 25°C respectively, and the null hypothesis was accepted. This was confirmed by establishing, as a new null hypothesis, that the slope of Hill's plot was equal to the unity. This hypothesis was accepted for n=1 and rejected for n=2 and n=3.

This fact had been reported previously by SERRANO AND CARCELES (1986), who found the number of binding sites to be one per bovine serum albumin molecule.

The apparent association constants,that were obtained from Hill's plot ranged from 11845.54 to 13361.10 M^{-1}. The values found were smaller than those reported by SERRANO AND CARCELES (1986).

The decrease in the value of the constants was due to the drop in temperature, and suggests a

	15° C	20°C	25°C
Ka	11845.54	1.232.58	13361.10
n	1	1	1
AG			
(Kacl Mol-1)	-5.3709	-5.4876	-5.6287
AH			
(Kcal Mol-1)	2.0617	2.0617	2.0617
AS			
(Cal °K Mol-1)	25.78	25.78	25.78

TABLA 2. Parameters of the oxitetracycline-ovine serum albumin interaction at 15, 20 and 25° C.

loss of affinity between the oxytetracycline and the ovine serum albumin.

From the interpretation of thermodynamic parameters, we can conclude that the interaction between oxytetracycline and ovine serum albuminis spontaneous (ΔGnegative), endothermic (ΔH positive) and stable (ΔS positive). The spontaneity of the interaction decreased when the temperature did, due to the loss of affinity between OTC and OSA.

The enthalpy stabilization can be usually associated with the formation of new bonds (hydrogen bonds and van der Waal´s attraction) whereas the entropy stabilization is usually caused by the displacement of previously arranged water molecules and the formation of new hidrophobic interactions (HITZEMANN, 1988).

KLOTZ (1973) and KLOTZ AND FIESS (1951) reported that the following equations:

$$-T\,\Delta S = 1.35\,\Delta G$$

$$-\Delta H = 0.35\,\Delta G$$

are satisfied when electrostatic interaction takes placeat a temperature of 298°K (25°C).

By applying this fact to our data, AS and AH were found to be the same experimentally and theoretically. Therefore, we can affirm that electrostatic interaction occured in the OTC-OSA interaction, possibly between the nitrogenous group of OTC and one of the carboxilic free groups of OSA situated on the surface of the molecule.

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