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MICROCALORIMETRIC STUDIES ON THE BINDING OF SOME BENZODIAZEPIN DERIVATIVES TO HUMAN SERUM ALBUMIN

P. COASSOLO, M. SARRAZIN, J. C. SARI* and C. BRIAND
Faculté de Pharmacie, 27 Bd Jean Moulin 13385 Marseille, Cédex 4, France

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Abstract—The authors have employed a microcalorimetric method to study the nature of the interactions between several benzodiazepin molecules and human serum albumin. The thermodynamic functions of complexation have been determined at different pH, ionic strength and electrolyte support conditions. Both electrostatic and hydrophobic interactions have been demonstrated to exist in proportions which vary according to the drug's molecular structure.

Human serum albumin (HSA) is known to possess several sets of binding sites for different classes of drugs. In particular, the widely used psychotropic benzodiazepin drugs generally demonstrate extensive binding to HSA whereas other plasma proteins do not appreciably bind these drugs.

The equilibrium constants of complexation and the stoichiometries of the benzodiazepin–HSA complexes have a fundamental role in determining the free drug concentration in the plasma which, in turn, influences the pharmacodynamic activities of these drugs. Similarly, the knowledge of the variations in the enthalpy and entropy values, associated with the complex-forming reactions, enables one to postulate models which approach the exact nature of the chemical interactions, both on the HSA and the benzodiazepin molecules themselves. Moreover, these thermodynamic functions are necessary to correlate the structure of the benzodiazepin molecules and their respective affinity for HSA.

Müller and Wollert [2, 3], Sjöholm and Sjödin [4], and Sjödin *et al.* [5] have calculated, using gel filtration and circular dichroism techniques, the equilibrium constants of various benzodiazepin–HSA complexes. However, the corresponding ΔH and ΔS values of such complexes are still lacking.

In order to facilitate the completion of this series of data, we present below a microcalorimetric method for the direct determination of the variation of thermodynamic functions (ΔH, ΔG and ΔS) and the stoichiometry of all complexes comprising only one class of complexation site and having significant ΔH values. In the case where several sets of binding sites of different affinities are present, it is necessary to use an empiric or a computational treatment [6], using iterative evaluations of the parameters.

We have previously described this microcalorimetric method in the case of complexes involving small molecules [7, 8]. We have also discussed [9] the limitations of such a microcalorimetric method for the determination of K_a and ΔH values. Of late, the progress in the field of microcalorimeter technology has been such as to allow us to apply this technique to the study of the binding with proteins.

The benzodiazepins to be used in the experiments were selected for their respective physical properties. For example, flurazepam and dipotassium chlorazepate are very soluble in water, whereas diazepam, nitrazepam and chlordiazepoxid require an ethanol–water mixture for solubilisation. In solution at pH 7.40, flurazepam is positively charged, dipotassium chlorazepate is negatively charged whereas diazepam, nitrazepam and chlordiazepoxid are electrically neutral.

In order to obtain a more precise characterization of the nature of the sites of complexation on the HSA and the benzodiazepin molecules themselves, the various thermodynamic functions were calculated as functions of ionic strength and support electrolyte concentrations.

MATERIALS AND METHODS

Reagents. Chromatographically pure HSA was purchased from Sochibo (Koch–Light products). It is known that fatty acids compete with some drugs for sites of complexation [11]. To ensure the absence of such species, the HSA was purified by charcoal treatment [12], checked by extensive dialysis against EDTA and gave the same results as the original product. The absence of fatty acids was also checked by Folch's method [13]. The concentration of HSA was determined by measurement of the optical density at 280 nm (A per cent cm = 5.3).

All benzodiazepin derivatives were obtained from Roche not including dipotassium chlorazepate which was obtained from Clin Comar Byla Laboratories. The drugs were used without further purification 5-[^{14}C]diazepam (sp. act. 197 μCi/mg and 5-[^{14}C] nitrazepam (sp. act. 52 μCi/mg) were kindly supplied by Hoffman La Roche, Bâle.

Microcalorimetric technique. The flow microcalorimeter used was a LKB apparatus, type 10700-1. It was placed in a precise thermostated room; all pumps were operated at a rate of 20 cm^3/hr. In these

* To whom all correspondence should be addressed.

conditions, 10^{-3} cal is measured with a precision about 5 per cent.

Before the outset of each experiment, the base line was drawn when a solution of a given benzodiazepin in a phosphate buffer solution was pumped into the first circuit while the same phosphate buffer was pumped into the second circuit. The time allowed for the system to reach equilibrium was about 20 min, at the end of which a sample of 1 cm^3 of HSA solution was inserted into the phosphate buffer flow. The diffusion effect on the HSA solution was rendered negligible by the presence in the buffer flow of two microbubbles ahead and behind the sample. The volume of the liquid containing the chemical reaction was, therefore, equal to 2 cm^3. The quantity of heat evolved during the complex-forming reaction had to be corrected to take into account the heat of dilution of HSA. This value was determined as previously described but without benzodiazepin in the phosphate buffer solution.

A radiometer 26 pH stat apparatus was always used to confirm that, in a medium without buffer, no variation in the pH value occurs during complex formation. This signifies that no alteration in the pK values of the various ionic species present occurs as a result of the complex-forming reactions. In this manner, the heats of ionization and neutralization were considered as negligible.

Dialysis technique. The equilibrium dialyses were performed in microcells (200 μl) with a Dianorm rotative apparatus, the two compartments of which were separated by a "Visking tube" membrane No. 30/32. At 30°, a condition of equilibrium was reached after 2 hr after which the results were not modified by a longer time of dialysis. Samples of the dialysates were then taken from each compartment for the determination of the concentration of the free and bound drug. They were transferred into 10 cm^3 of Bray's solution and counted over a 10 min period in Beckman scintillation counter. The results of the dialysis equilibria were then plotted according to the method of Scatchard [14].

Experimental conditions. All experiments were performed at 30°. The ionic strength of the solutions were maintained at a constant value of 0.15 by means of a phosphate buffer. The variation of the ionic concentrations were taken into account for the calculations of the thermodynamic function variations. To increase the solubility of the samples of diazepam, nitrazepam and chloridazepoxid, a solution containing 1 or 2% ethanol was used. The pH values of the solutions were maintained at 7.40 except in the case of flurazepam for which the medium was buffered at pH 6.50 (in order to avoid a precipitation of the drug which occurs at pH > 6.50).

RESULTS

Ionic species. The chemical structure and the pK values of the benzodiazepin derivatives are presented in Table 1. It is evident that diazepam, chlordiazepoxid and nitrazepam are neutral at pH 7.40. By contrast, flurazepam has a N_1-side chain which is positively charged at pH 6.50. It is not possible to determine a precise pK_2 value of chlorazepate as a result of chemical structure modifications which manifest themselves under conditions of pH lower than 6. An approximate pK_2 value of 6.0–6.2 has therefore been estimated for this drug which will then be negatively charged under conditions of pH 7.40 [15].

The CNDO/2 method [16] has been employed to estimate the electron densities at all nuclei in order to furnish some insight into the parts of the molecules involved in the electrostatic interactions with HSA. By way of an example, the electron densities at the nuclei of diazepam were calculated using an X-ray cristallographic geometry previously determined [17] and are reported in Table 1.

Enthalpic titration method. This microcalorimetric method allows one to determine directly the association constant (K) and the enthalpy change (ΔH) and, consequently, the free energy (ΔG) and entropy (ΔS) changes for all complex-forming reactions comprising one class of binding sites only. If the stoichiometry of a given complex is known precisely, it is sufficient to record only two heat of complexation determinations, performed under the same experimental conditions but using different substrate concentrations, to determine the values of K and ΔH for the complex.

In this case, let us call:

a—the total HSA concentration,

n—the number of sites, on each HSA molecule which have the same association constant,

b and c—the total drug concentrations corresponding to the first and the second experiments, respectively,

x and y—the bound drug concentrations at equilibrium after the first and second experiments, respectively,

Q_1 and Q_2—the quantities of heat evolved during the first and second experiments, respectively,

ΔH—the change in enthalpy per mole of drug complexed,

V—the chemical reaction volume, expressed in liters.

Under conditions whereby the temperature, the pH and ionic strength values remain constant during the two experiments, then the association constant for a particular complex, at a particular ionic strength, is expressed as:

$$K = \frac{x}{(na-x)\ (b-x)} = \frac{y}{(na-y)\ (c-y)}. \quad (1)$$

The quantities of heat, experimentally determined are

$$Q_1 = x.\Delta H.V \text{ and } Q_2 = y.\Delta H.V. \quad (2)$$

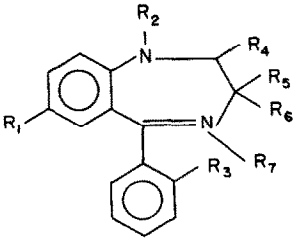
Combining the expressions (1) and (2) one obtains:

$$K = \frac{\left(\frac{Q_1}{\Delta H.V}\right)}{\left(na - \frac{Q_1}{\Delta H\ V}\right)\left(b - \frac{Q_1}{\Delta H.V}\right)} = \frac{\left(\frac{Q_2}{\Delta H.V}\right)}{\left(na - \frac{Q_2}{\Delta H.V}\right)\left(c - \frac{Q_2}{\Delta H.V}\right)} \quad (3)$$

$$\Delta H = \frac{(c-b)\ Q_1Q_2 \pm \sqrt{[Q_1^2Q_2^2(b-c)^2 - 4na(Q_1c - Q_2b)\ (Q_1Q_2^2 - Q_1^2Q_2)]}}{2na\ (Q_1c - Q_2b)\ V}. \quad (4)$$

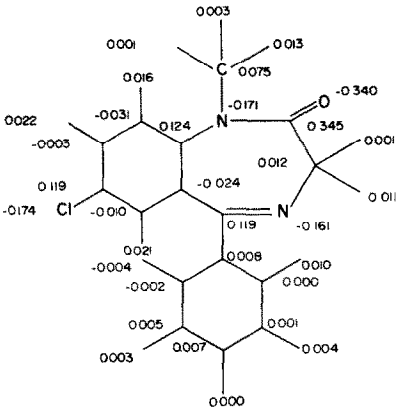
If the stoichiometry of the complex is not known, we assume a stoichiometry and the values for K and ΔH are determined using several sets of two different substrate concentrations, with the same experimental

Table 1. Chemical structure, pK values of studied benzodiazepins and electron densities of diazepam



	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Flurazepam	Cl	$(CH_2)_2$-*N*-$(C_2H_5)_2$	F	O	H	H	
Chlordiazepoxid	Cl	H	H	$-NHCH_3$	H	H	O
Diazepam	Cl	CH_3	H	O	H	H	
Dipotassium chlorazepate	Cl	H	H	O	H	$-COOK\cdot KOH$	
Nitrazepam	NO_2	H	H	O	H	H	

	pK_1	pK_2	pK_3	
Flurazepam	1.60	8.40		From manufacturers
Chlordiazepoxid	4.30			From manufacturers
	4.60			Barrett *et al.*, 1972
Diazepam	3.17			From manufacturers
	3.30			Barett *et al.*, 1972
Dipotassium chlorazepate		6.0–6.2	11.1	
Nitrazepam	2.88	10.88		From manufacturers
	3.20	10.80		Barett *et al.*, 1972



conditions by applying equations (3) and (4). If the resulting values of K and ΔH remain constant whatever the pair of drug concentrations chosen, then the assumed value for the stoichiometry of the complex is correct.

To confirm the validity of the derived values for the stoichiometry and the K and ΔH values, it is necessary to compare the heat saturation curves plotted from the calculated n, K and ΔH values, with those found experimentally. The theoretical and the experimental heat saturation curves represent the corresponding quantities of heat (Q) evolved from the experiments under conditions of constant HSA concentration (a) and variable drug concentrations (b_i). The mathematical expression for the heat saturation curve $Q = f(b_i)$ is obtained from equations (1) and (2):

$$Q = \left[\frac{1}{K} + na + b_i - \sqrt{\left(\frac{1}{K} + na + b_i\right)^2 - 4nab_i}\right]\frac{\Delta H.V}{2} \quad (5)$$

Dialysis results. When readioactive compounds are available, the stoichiometries of the complexes are determined by means of the dialysis equilibrium technique. For example, the stoichiometry observed for the nitrazepam–HSA complex is equal to one. The Scatchard plot [14] of diazepam–HSA complex indicates the presence of two complexing sites on the HSA molecule, the equilibrium constant of the second site is about forty times lower than equilibrium constant of the high affinity binding site (Fig. 1). Consequently, it was considered that only the HSA complexation site of high diazepam affinity comes into play under conditions whereby the concentration of the diazepam ranges from once to twice that of the HSA (approx. 10^{-4} M). In spite of an addition of 2% ethanol the solubility of the diazepam was insufficient to determine the variations of the thermodynamic functions of the second site of complexation on the HSA.

Microcalorimetric results. In the case of bimolecular complexes we have demonstrated [9] that the

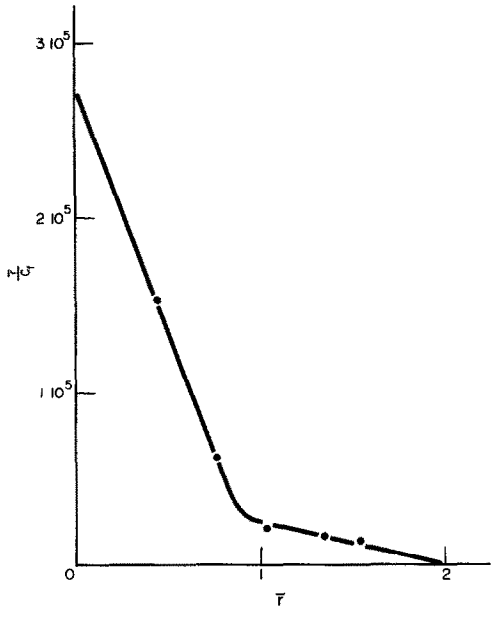


Fig. 1. Scatchard plot of the binding of 5 [^{14}C]diazepam (from 5.10^{-5} to 4.10^{-4} M) with HSA (10^{-4} M) at pH 7.40, 30° and ionic strength 0.15. Each point represents the S.E.M. of ten experiments.

most accurate thermodynamic results are obtained when the Q_1 measurements correspond to $b/a = 1$ and those of Q_2 correspond to $c/a \geq 2$. However, the conditions must be chosen so that $b/a \leq 1$ while $c/a \leq 2$ as a result of the poor solubility of the drugs in the present study. In order to facilitate the statistical analysis of the results of the thermodynamic functions variations, calculated under such concentration conditions, two series of ten experiments were performed.

The enthalpy titration method was employed to determine the stoichiometry of the complexes formed between the drugs chlorazepate, chlordiazepoxid, flurazepam and HSA. For each of these drug–HSA complexes a value of 1 was obtained for the stoichiometry. The experimental results and the derived thermodynamic functions variations for the HSA–chlorazepate complex are listed in Tables 2 and 3, respectively. The experimentally found heat saturation curve (solid line in Fig. 2) for this complex is exactly superimposed by the corresponding theoretical curve (dotted line in Fig. 2) plotted with the aid of equation (5) using the following input parameters: $K = 13\,000\ M^{-1}$, $\Delta H = -6.6$ kcal/mole, $n = 1$. The resulting thermodynamic functions for all the complexes investigated are listed in Table 4.

To evaluate the influence of the medium on the thermodynamic functions variations of the HSA–chlorazepate complex the ionic strength of the reaction solutions were varied by altering the concentration of the phosphate buffer, and the measurements then recorded. The resulting thermodynamic functions are presented in Table 5. Assuming that the activity coefficients remain constant in the range of ionic strength between 0.10 and 0.25, then it would appear that there is only a negligible effect on the affinity between chlorazepate and HSA, by the presence of the sodium and phosphate ions.

That chloride ions are strongly complexed with

Table 2. Thermodynamic quantities of dipotassium chlorazepate–HSA interaction*

Series of experiments	Number of experiments	Total chlorazepate concentration in reactional volume	Experimental calorimetric determination (10^{-3} cal)
1	5	$0.707.10^{-4}$ M	-0.741 ± 0.032
2	7	$1.296.10^{-4}$ M	-1.164 ± 0.032
3	4	$3.534.10^{-4}$ M	-1.920 ± 0.040
4	4	$7.068.10^{-4}$ M	-2.195 ± 0.082
5	7	$1.072.10^{-3}$ M	-2.225 ± 0.078
6	4	$1.414.10^{-3}$ M	-2.290 ± 0.090
7	4	$3.652.10^{-3}$ M	-2.350 ± 0.100

* In experimental conditions described in Fig. 2.

Table 3. Thermodynamic quantities of dipotassium chlorazepate–HSA interaction* calculated from two series of experiments using the stoichiometry value equal to one

Series of experiment chosen	$K(M^{-1})$	ΔH (kcal/mole)	ΔG (kcal/mole)	ΔS (e.u.)
2 and 5	$13\,000 \pm 800$	-9.90 ± 0.20	-5.65 ± 0.05	-14 ± 1
1 and 3	$13\,900 \pm 1100$	-10.10 ± 0.12	$-5{,}69 \pm 0.05$	-15 ± 1
1 and 4	$11\,900 \pm 800$	-10.16 ± 0.14	-5.59 ± 0.04	-15 ± 1
1 and 6	$11\,500 \pm 700$	-10.40 ± 0.11	-5.57 ± 0.04	-16 ± 1
2 and 4	$13\,800 \pm 1000$	-9.82 ± 0.14	-5.68 ± 0.05	-14 ± 1
2 and 6	$13\,700 \pm 1000$	-9.70 ± 0.15	-5.68 ± 0.05	-14 ± 1
Means	$13\,000 \pm 1000$	-10.00 ± 0.20	-5.60 ± 0.10	-15 ± 1

* In experimental conditions described in Fig. 2.

Note: to determine the values of K, ΔH, ΔG and ΔS, each heat measurement of one serie is associated successively with all the others of another series. In this way we define the statistical, K, ΔH, ΔG and ΔS values; all the results are given with estimations of the errors from the mean value $P = 0.05$.

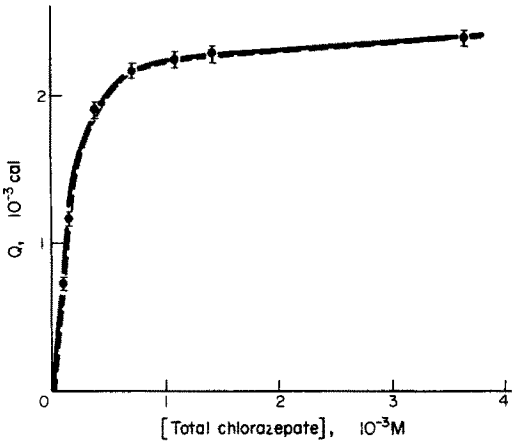


Fig. 2. Dipotassium chlorazepate–HSA interactions; total HSA concentration $1.178.10^{-4}$ M; reaction volume $1.95.10^{-3}$ L; pH 7.40, ionic strength 0.15, 30°, phosphate buffer.

albumin, has been fully demonstrated [18, 19]. For example, Lovrien and Strutevant [19] have shown that each Bovine Serum Albumin (BSA) molecule complexes a total of nine chloride ions. The results, obtained from microcalorimetric measurements, indicated that the primary site of complexation occurred with thermodynamic values of -8.3 ± 3.0 kcal/Cl^- for ΔH and -4.62 kcal/Cl^- for ΔG whereas the eight remaining secondary sites exhibit values of -3.1 ± 1.0 kcal/Cl^- for ΔH and -2.73 kcal/Cl^- for ΔG. Interestingly, Tauja-Chareyre *et al.* [20] have shown that HSA also complexes chloride ions. In order to ascertain whether chloride ions would act in competition with the chlorazepate molecules for the sites of complexation on the HSA, a series of heat of complexation experiments were performed using reaction media containing NaCl. The ionic strengths of the complexation medias were maintained at 0.15 by a compensatory decrease in the concentration of the sodium phosphate. The resulting values of the thermodynamic functions are reported in Table 6. If it is assumed that the same values of enthalpy of complexation are exhibited by the HSA-Cl^- and BSA-Cl^- complexes, then one can state that chloride ions compete with chlorazepate for the secondary binding sites on HSA.

DISCUSSION

Let us compare the thermodynamic functions variations of the HSA complexes with the three types of benzodiazepin derivatives. The large negative enthalpic and entropic values observed for the chlorazepate–HSA complex suggest that the interactions between the two molecules, under conditions of pH 7.40, are dominated by electrostatic rather than hydrophobic forces. The negative ΔS value, which indicates an increase in the orderliness of the system on complexation, is thermodynamically unfavourable. As a result, the ΔG value indicates only a small favourability for the process of complexation despite the relatively large ΔH value.

Flurazepam, which is positively charged at

Table 4. Thermodynamic functions of some benzodiazepin–HSA complexes at pH 7.40, ionic strength 0.15 and in phosphate buffer

	ΔH (kcal/mole)	ΔG (kcal/mole)	ΔS (e.u.)
Flurazepam	$+10 \pm 1$	-2.0 ± 0.2	$+40 \pm 4$
Chlordiazepoxid	-5.7 ± 0.1	-6.6 ± 0.1	$+3 \pm 1$
Diazepam	-3.9 ± 0.2	-7.6 ± 0.2	$+12 \pm 1$
Dipotassium chlorazepate	-10.0 ± 0.2	-5.6 ± 0.1	-15 ± 1
Nitrazepam	-5 ± 1	$+5.8 \pm 0.3$	$+3 \pm 1$

Table 5. Influence of ionic strength on chlorazepate–HSA complex formation at pH 7.40, in phosphate buffer

Ionic strength	$K(M^{-1})$	ΔH (kcal/mole)	ΔG (kcal/mole)	ΔS (e.u.)
0.050	9800 ± 400	-12.3 ± 0.2	-5.5 ± 0.1	-18 ± 1
0.100	$17\,000 \pm 1000$	-10.1 ± 0.1	-5.8 ± 0.1	-14 ± 1
0.150	$13\,000 \pm 1000$	-10.0 ± 0.2	-5.6 ± 0.1	-15 ± 1
0.200	$12\,000 \pm 1000$	-9.9 ± 0.1	-5.6 ± 0.1	-14 ± 1
0.250	$19\,500 \pm 1500$	-9.9 ± 0.1	-5.8 ± 0.1	-14 ± 1

Table 6. Influence of chloride ions on chlorazepate–HSA complex formation at pH 7.40, 30°, ionic strength 0.15 and in phosphate buffer

Ionic strength brought by chloride ions	$K(M^{-1})$	ΔH (kcal/mole)	ΔG (kcal/mole)	ΔS (e.u.)
0	$13\,000 \pm 1000$	-10.0 ± 92	-5.6 ± 0.1	-15 ± 1
0.022	7800 ± 600	-5.4 ± 0.1	-5.4 ± 0.1	0
0.077	7700 ± 600	-5.3 ± 0.1	-5.3 ± 0.1	0
0.132	7200 ± 800	-5.3 ± 0.2	-5.3 ± 0.1	0

experimental pH, presents a hydrophobic chain probably surrounded like phenyl rings by the so-named "icebergs" [21, 22]. The attractive coulombic interactions between the sites of complexation on the HSA and the negatively charged nuclei of drug molecule (which are situated near *N*4; see Table 1) are considerably hindered by the positively charged side chain.

Therefore, we assume that the exothermic effects of complexation, which arise from the attractive coulombic interactions, are smaller than the endothermically promoted disrupture of "icebergs" which would occur on complexation. The summation of these quantities results in a positive enthalpy variation of complexation. Consequently, at pH 6.50, the driving force of the complexation process is the increase in entropy of the system. It is interesting to note that Müller and Wollert [3] have observed that the affinity of HSA for flurazepam increases when the pH value increases from 6.60 to 8.20; a result which would be expected by the neutralization of the nitrogen on the side chain which favorizes the electrostatic interactions.

The process of complexation between HSA and diazepam, which is electrically neutral at pH 7.40, is enthalpy rather than entropy driven. However, both of these thermodynamic functions act in the same sense, such as to increase the favourability of complex formation. Similarly, the enthalpy and entropy values are favourable towards the complexation processes for all the neutral benzodiazepins. These are consequences of complementary hydrophobic and electrostatic interactions.

In conclusion, we can state that the nature of the attractive forces in the complexes between HSA and both neutral and negatively charged benzodiazepin derivatives is essentially of electrostatic character. That is, the negatively charged portions of the drug molecules are bound by attractive electrostatic interactions to the positively charged sites on HSA.

The hydrophobic interactions are not always evident in terms of the observed thermodynamic values but they would play a role in determining the stability of the complexes formed in solution. However, hydrophobic interactions are quite evident in the case of the complexation process between positively charged flurazepam and HSA.

The ΔH and ΔS values for the complexation processes between HSA and the three types of benzodiazepin drugs lend support to these conclusions. Further support comes from the fact that chloride ions compete with chlorazepate for sites of complexation whereas sodium ions exert no effect on the complexation processes.

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