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DETERMINATION OF DIAZEPAM–HUMAN SERUM ALBUMIN BINDING BY POLAROGRAPHY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AT DIFFERENT PROTEIN CONCENTRATIONS

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SUMMARY

The binding of a reducible drug, diazepam, to human serum albumin has been studied by high-performance liquid chromatography and by differential pulse polarography with a static mercury drop, over a wide range of protein concentrations. At low human serum albumin concentration, the influence of drop-time conditions on the differential pulse polarographic results obtained, and their comparison with those of high-performance liquid chromotography, show a decrease in the diffusion current of the free drug in the presence of human serum albumin. This decrease is attributed to a reduction of the surface available for electrochemical reaction due to protein adsorption on the mercury drops. At a given human serum albumin concentration and over a given drop time, the diazepam response is reduced by a constant fraction. Constant reduction of the current above 0.2 g/l protein concentration is the basis for calculating the drug–human serum albumin binding at higher protein concentrations, near the physiological conditions, when high-performance liquid chromotography with UV detection is not sensitive enough. A decrease of the total drug affinity with increasing protein concentration was observed.

INTRODUCTION

Several techniques, such as equilibrium dialysis, ultrafiltration and chromatography, are applied extensively to the determination of the binding characteristics of small molecules to proteins. Polarography has been applied to similar studies, but to a far lesser extent: first, the drug has to be electroactive, and secondly, the interpretations of the results do not seem to be always clear and evident.

Indeed, when a macromolecule is added to a solution of an electroactive substance, a decrease in the diffusion current often takes place, as noticed for the first time by Kolthoff and Lingane[1]. This phenomenon is usually ascribed to complex

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formation between the protein and the small molecule. Well known examples studied are the complexes of albumin with metal ions[2,3] and methyl orange[4]. However, alternative explanations have been offered, *e.g.*, that the depression of the polarographic diffusion current is due to the reduction of the effective surface of the mercury drop by protein adsorption[5,6]. Since albumin is strongly adsorbed on mercury by its disulphide bonds[7,8], both phenomena are probably simultaneously present and should be considered. Thus, Molinier-Jumel *et al.*[9] have recently calculated DNA–anthracyclin binding by differential pulse polarography (DPP) at a very low macromolecule concentration in order to minimize DNA adsorption on the mercury drop.

Moreover, several groups have tried to measure the complexing capacity of natural waters for a metal by DPP, but comparison with results by other methods make it likely that the DPP-estimated constants are not strictly representative of the degree of complexation in the bulk solution, when the water contains some compounds which are adsorbable on the electrode, such as humic acid and fulvic substances[10,11].

The purpose of the work we present here is to collect evidence, at least in the example chosen for study, diazepam–human serum albumin (HSA) binding, concerning factors responsible for the decrease in drug diffusion current. For this purpose, we have studied the binding of diazepam to HSA by reliable high-performance liquid chromatography (HPLC) methods[12,13] and simultaneously by the DPP technique. On the basis of these results the evaluation of binding parameters at high protein concentrations (near the physiological conditions) has been attempted.

EXPERIMENTAL

HPLC apparatus

A Waters Model 6000A pump, a Rheodyne Model 7120 injection valve, and a Waters Model 440 UV detector with 280 nm and 313 nm filters were used. The column (5 cm × 4 mm I.D.) was filled by a slurry-packing technique with 10-μm particle diameter, 100-Å porosity LiChrosorb Diol (E. Merck, Darmstadt, F.R.G.) Unless otherwise stated, the experiments were performed at 37°C. The flow-rate was 0.5 ml/min.

HPLC methods for diazepam–HSA binding

The Hummel and Dreyer method. This method[14] was applied by us to HPLC[12]. The successive eluents used were diazepam solutions in the range $3 \cdot 10^{-7} M$ to $10^{-4} M$. A 50-μl volume of a 2 g/l HSA solution (1.5 nmoles) was injected into the size-exclusion column. For these experiments, the detection wavelength was 280 nm, near the absorption maximum of diazepam.

The equilibrium saturation method[13]. Diazepam binding was investigated at two HSA concentrations, 0.4 and 2.0 g/l. The successive eluents used were, respectively, mixtures of 0.4 and 2.0 g/l HSA with (10^{-5} to $10^{-4} M$) diazepam. A 50-μl volume of buffer was injected into the column. The negative peak monitored at 313 nm, at the drug retention volume, corresponded to the free-drug concentration of the mixture studied (used as eluent), $[A]$, according to an internal calibration, as described previously[13]. Two solutions, containing 10 g/l HSA , were tested for diazepam binding at 20–22°C, for comparison with literature results[15].

Frontal analysis[16]. This method has been previously tested by us on another siliceous size-exclusion column[17].

DPP apparatus

The differential pulse polarograms were obtained with a Tacussel polarographic analyser (Type PRG 5) (Villeurbanne, France), a PAR Model 303 static mercury-drop electrode (SMDE) (Princeton Applied Research, Princeton, NJ, U.S.A.), and recorded with a Sefram Model TRP recorder (Paris, France). In the SMDE, the drop is dispensed in a small fraction of the drop time, then the drop area remains constant until the drop is dislodged. A platinum wire counter-electrode and a Ag/AgCl reference electrode were used in a cell, fitted with a glass water jacket. Dissolved oxygen was removed from solutions by bubbling nitrogen through the cell for 20 min and then passing it over the solution during the measurement. About 7–15 ml of solutions were used. Experiments were performed at 20 or 37°C.

DPP measurements

Measurements were made by scanning from -0.8 to -1.3 V (*vs.* Ag/AgCl) at 2 mV sec^{-1} scan-rate with a modulation amplitude of 50 mV and drop times varying from 0.3 to 5 sec. The diazepam solutions used for calibration were in the range from 10^{-7} to $10^{-4}M$; the diazepam reduction peak is observed at -1080 mV. Mixtures of the drug with HSA (from 0.02 to 40 g/l) have been studied. The drug peak is slightly displaced towards negative potentials. The heights of the observed DPP peaks were measured and the background of HSA was subtracted. The free-drug concentration was then calculated by reference to a drug calibration curve under the same drop-time conditions, in the absence of HSA, by considering that the bound drug does not contribute to the peak current, as demonstrated further.

Calculations

The total affinity $\sum_{i-1}^{n} n_i k_i$ was derived from an extrapolated Scatchard plot[18]. Calculations were made on the basis of a molecular weight of 66,500 for HSA.

Reagents

HSA was a Sigma (St Louis, MO, U.S.A.) product (A 1887, essentially fatty acid-free albumin). The HSA solutions were prepared with 0.067 *M* (pH 7.4) phosphate buffer. Diazepam was from Roche (Neuilly, France). Owing to the low solubility of the drug in aqueous buffer, a $10^{-2}M$ solution was prepared in ethanol, and the dilutions used for experiments were made from it, either directly with the phosphate buffer or with HSA solutions.

RESULTS AND DISCUSSION

Measurement of diazepam–HSA binding by HPLC

Diazepam–HSA binding was measured by different HPLC methods. Typical equilibrium saturation method chromatograms are presented in Fig. 1a. Plotting the second peak area *versus* the drug concentration of the injected samples allowed the

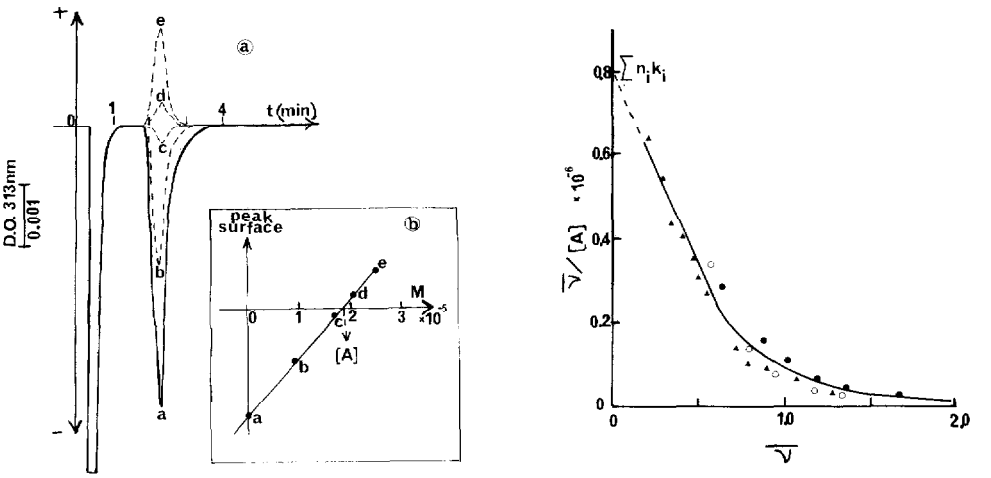


Fig. 1(a). Equilibrium-saturation chromatographic method. Eluent: 2 g HSA per liter of $5 \cdot 10^{-5}$ *M* diazepam-buffered (pH 7.4) solution. Injection: a, 50 µl of buffer; b–e 50 µl of, respectively, 9, 17, 20 and 25 µ*M* diazepam solutions in buffer. (b) Internal calibration: peak surface *versus* diazepam concentration of injected samples. D.O. = optical density.

Fig. 2. Scatchard plot for the binding of diazepam to HSA, as measured by HPLC: ▲, Hummel and Dreyer method; ● (○) Equilibrium-saturation method at, respectively, 0.4 and 2.0 g/l HSA concentration. ν = Mean number of moles of drug bound per mole of protein.

estimation of [*A*], the free drug concentration of the mixture, as reported in Fig. 1b. The data obtained for two low HSA concentrations (0.4 and 2 g/l) are in good agreement with those of the Hummel and Dreyer method as presented in Fig. 2. With the Hummel and Dreyer method, lower molar binding ratios can be determined than with the equilibrium saturation method, and it gives a more precise total affinity, $\sum_{i=1}^{n} n_i k_i$, equal to $0.8 \cdot 10^{-6}\ M^{-1}$. But only the saturation method supplies the determination of the [*A*] value at known total albumin and drug concentrations, which permits a comparison with further DPP experiments.

Two diazepam–HSA mixtures were studied, at 20°C with a higher protein

TABLE I

COMPARISON BETWEEN MEASURED FREE-DRUG CONCENTRATIONS FROM DPP, HPLC AND GEL FILTRATION EXPERIMENTS

The HSA concentration was 10 g/l and the temperature was 20–22°C. Gel filtration values were obtained from a literature Scatchard plot[15].

Total drug concentration (µM)	*Calculated free-drug concentration (µM)*			*Ratio*	
	Gel filtration	*HPLC*	*DPP*	*DPP by gel filtration*	*DPP by HPLC*
80	2.1		0.90	0.43	
101	3.5		1.45	0.41	
130	8.2	7.8	3.4	0.41	0.43
180	30.0	32.0	15.0	0.50	0.47

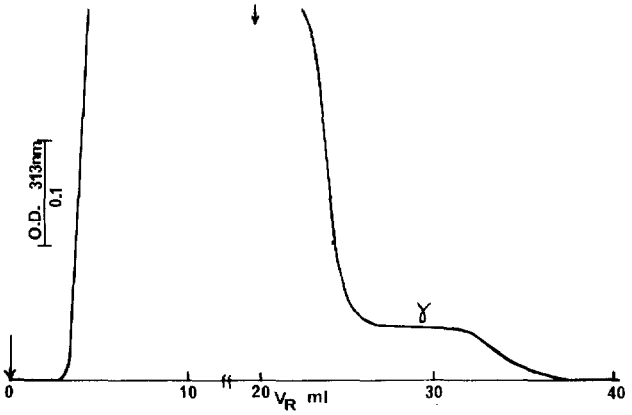
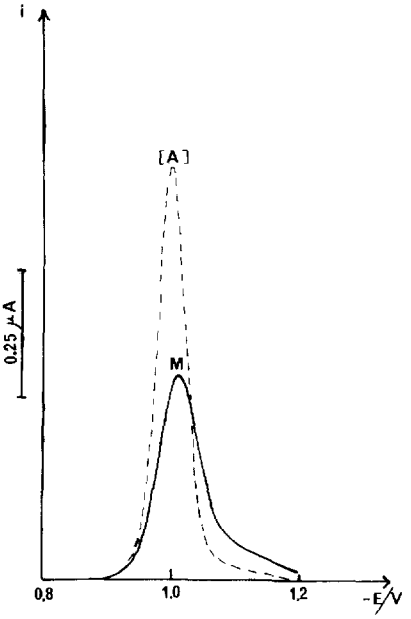


Fig. 3. Frontal analysis elution profile. Eluent: 0.067 *M* (pH 7.4) phosphate buffer. Injection: 20 ml of (10 g HSA per liter of 180 μM diazepam) solution in buffer. Temperature: 20°C. V_R = Retention volume.

concentration (10 g/l), both in the equilibrium saturation method and by frontal analysis. Fig. 3 presents the chromatographic profile obtained by the latter technique. Both the low and reversible adsorption of HSA and the strong retention of diazepam on LiChrosorb Diol are responsible for the appearance of a well defined, free ligand plateau, γ. The results so obtained are in good agreement with previous ones, ob-



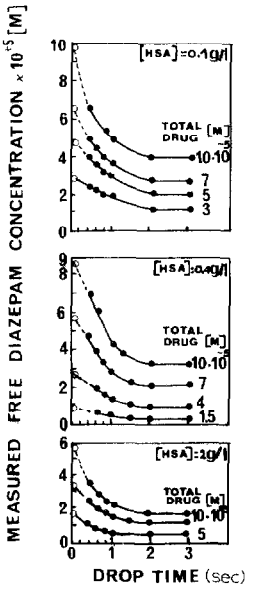
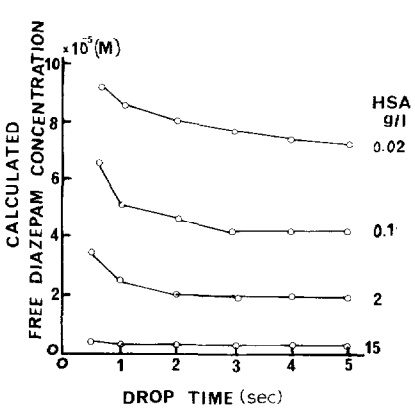


Fig. 4. Effect of HSA on diazepam DPP peak. —, 2 g HSA per l of $5 \cdot 10^{-5}$ *M* diazepam mixture (Solution M); ---, $1.6 \cdot 10^{-6}$ *M* diazepam solution (measured as free-drug concentration of mixture M by HPLC). The drop time was 1 sec, and the temperature 37°C.

Fig. 5. Influence of drop time on calculated free diazepam concentration (●) and comparison with [*A*] values from HPLC (○). DPP calculations were made by reference to drug calibration curves at similar drop time without HSA.



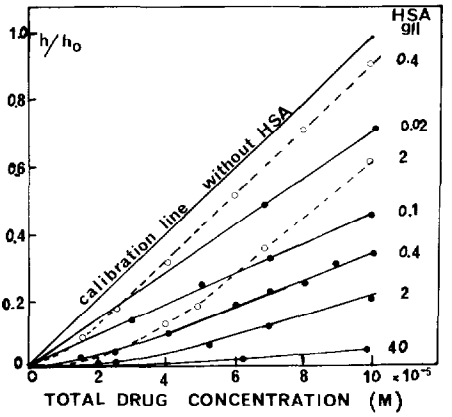


Fig. 6. Influence of drop time and concentration of albumin on the value of free diazepam concentration. Total diazepam concentration is 10^{-4} *M*.

Fig. 7. Change of relative peak height (h/h_0) obtained during DPP titrations of HSA with diazepam at 5-sec drop time. The full lines refer to DPP experiments: h and h_0 are the DPP peak heights of diazepam solutions in the presence and absence of HSA, respectively. The dashed lines refer to HPLC experiments: h is the DPP peak height of diazepam solutions of concentration [*A*], determined as the free drug concentration of the mixture by HPLC.

tained under the same temperature and concentration conditions by gel-filtration experiments[15], as reported in Table I.

It was not possible to study lower drug–HSA ratios and more concentrated HSA solutions, because in both cases, the optical density of the mixture, close to detector saturation, reduces the accuracy of the free ligand determination.

Polarographic behaviour of diazepam in the presence of HSA

All solutions investigated for free drug concentration determination by the equilibrium saturation chromatographic method were also tested by the DPP technique.

Fig. 4 shows the typical polarographic curves obtained, respectively, with a diazepam–HSA mixture and a diazepam solution of concentration [*A*] equal to the free-drug concentration of the mixture, as determined by HPLC. The results demonstrate the depressing effect of HSA on the diazepam electrode response. A similar inhibitory effect of albumin on the metal-ion polarographic current has been observed and attributed to a reduction of electrode-accessible surface by albumin adsorption[19]. Thus, we have studied the influence of two parameters able to change HSA adsorption on the electrode, the mercury drop time and the HSA concentration.

Influence of drop time on polarographic current of diazepam. Fig. 5 shows a higher response of the electrode when the drop time decreases. The observed free drug concentration tends toward the [*A*] value determined previously by HPLC when the drop time goes to zero. Moreover, with increasing drop times, the polarographic response reaches a constant value. Fig. 6 reports the influence of the protein concentration on the minimum drop time at which a constant response is obtained. It is observed that the higher the HSA concentration, the smaller is the minimum drop time needed. For example, with a 15 g/l HSA solution, a constant polarographic

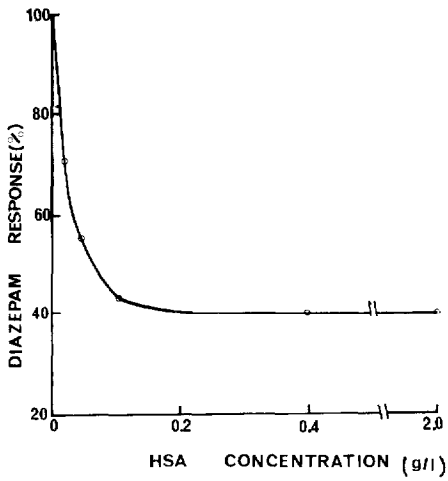


Fig. 8. Electrode response to diazepam in the presence of HSA at 5-sec drop time. The response is expressed in percentage, and represents the ratio between the free-drug concentration, calculated from DPP experiments according to the drug calibration curve without HSA and the free-drug concentration from HPLC experiments.

response is obtained with a 1-sec drop time. Conversely, with very dilute protein solutions a 5-sec drop time is necessary. We interprete the constancy of the response for drop times long enough that are as indicating a maximum coverage of the mercury drop with protein molecules. With concentrated protein solutions, this coverage is reached very quickly, therefore, and a short drop time is sufficient.

Influence of HSA concentration. Fig. 7 presents the titrations of HSA with the drug, obtained at a 5-sec drop time. This drop time was chosen to reveal a maximum effect of the presence of HSA in the whole range of concentrations studied. HPLC results are also shown in Fig. 7, for comparison. All plots begin at a curvilinear portion related to the binding of the drug to HSA. When protein saturation is reached, both the DPP peak height and the free concentration measured by HPLC increase linearly with the total amount of drug added. For the lines corresponding to HPLC measurements, the slope is 1, as expected when the protein is saturated, while the slopes of the lines by DPP are lower and decrease as the HSA concentration increases. This discrepancy between the results by different techniques can be explained by a reduction of the drug electrochemical signal by protein adsorption on the mercury electrode.

Owing to the proportionality of the response of polarographic and HPLC techniques with the drug concentration above protein saturation, we propose that this ratio gives the adsorption correction factor to be applied in polarographic measurements for obtaining the correct free drug concentration. We have verified that this correction factor is independent of drug concentration for a given HSA concentration, even in the domain below protein saturation. Thus, this observation is consistent with the lack of electro-activity of the bound drug and confirms the hypothesis made at the beginning of this work.

Fig. 8 describes the ratio of the DPP response at a 5-sec drop time to HPLC free-drug measurements as a function of the protein concentration in the bulk so-

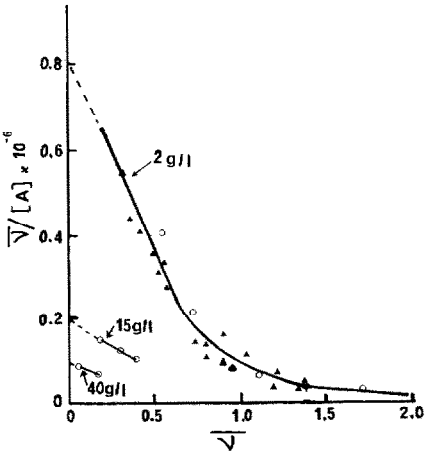


Fig. 9. Scatchard plots for diazepam–HSA binding, at various HSA concentrations. The DPP (○) experiments have been corrected for HSA adsorption inhibition. △, 2 g/l HSA, HPLC experiments.

lution. This percentage decreases with increasing protein concentration towards a limit value of 40% at about 0.4 g/l HSA concentration and remains constant over this concentration.

We have performed additional DPP experiments at a 10-g/l protein concentration and 22°C and compared them with HPLC and previous gel-filtration results[15]. Table I reports the values of free-ligand concentrations found by these techniques and shows that the response of polarography for free-drug concentration is equal to 0.44 times (mean value) that by chromatography. This value is not far from the value 0.40, determined above, and agrees with the independence of the response reduction above a 0.4-g/l HSA concentration.

Previous studies[2–4] have shown that both free and bound drug could contribute to the polarographic signal with a bound-ligand response about 20% of that of the free drug. This lower response was attributed to a smaller diffusion coefficient of the drug bound to the protein. This interpretation of polarographic data is not ap-

TABLE II

INFLUENCE OF HSA CONCENTRATION ON DIAZEPAM AFFINITY

HSA concentration (g/l)	$\sum_{i=1}^{n} n_i k_i \cdot 10^{-6} (M^{-1})$	*Experiments*	*Temperature (°C)*
2.5	1.24	ref. 20	
10	0.53	ref. 15	22 25
40	0.22	ref. 21	
2.0	0.8	Our DPP experiments with correction for adsorption	
15	0.2		37
40	0.1		

plicable to the example we report, since calculated signals would be greater than those we have observed, especially for mixtures with high binding percentage. Moreover, in the papers cited, the authors have neglected albumin adsorption on the electrode.

Thus, on the basis of our experiments, it seems allowable to neglect the contribution of bound drug to the polarographic signal because, as mentioned above, we have observed a constant ratio between polarographic and chromatographic results on the free-drug concentration regardless of the proportion of bound drug. Thus, we attribute, in the example studied, the origin of the correction factor to be applied for interconversion of HPLC and DPP results to the inhibition of the free drug response by the decrease of mercury surface available due to the HSA adsorption on the electrode.

Determination of diazepam-binding affinity in concentrated HSA solutions

In order to measure the binding of diazepam to HSA in concentrated protein solutions, close to physiological conditions, we have used the polarographic technique since, as mentioned above, HPLC cannot be used conveniently.

As the background of the protein electrochemical signal is low enough at the drug potential, we have been able to make accurate measurements of the diazepam peak height in the presence of 15 and 40 g/l HSA, at 37°C. On the basis of the results reported above, we have applied a correction factor of 0.40 to obtain the value of free drug concentration, $[A]$, according to the equation.

$$[A] = (1/0.40) \cdot [\text{Drug}]_{\text{DPP}}$$

$[\text{Drug}]_{\text{DPP}}$ is the calculated value from the calibration curve obtained in the absence of protein.

In Fig. 9 we have drawn the portions of Scatchard plots so obtained. The corresponding total affinity $\sum_{i=1}^{n} n_i k_i$ reported in Table II, together with previous results[15,20,21] clearly show a decrease in the affinity of diazepam to HSA when the concentration of the protein increases. Such a phenomenon has been observed for several other drugs[22,23]. A tentative interpretation of this phenomenon is based on the hypothesis of protein aggregation in concentrated solutions.

Some of us have recently adduced evidence for a concentration-dependent polymerization of the commercial HSA (Sigma A-1887) used in these experiments[24]. Moreover, recent work of Nakano *et al.*[25] has revealed that the binding capacity of the HSA dimer for some drugs is less than that of the monomer. The decrease of diazepam binding in concentrated HSA solutions is probably relevant in light of the same considerations. For this reason, it is important that the binding characteristics of a drug be determined experimentally over a wide range of albumin concentrations, in order to obtain sufficient information for forecasting *in vivo* binding properties.

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