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# Polarography: a new tool in the elucidation of drug–albumin interactions

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Many benzodiazepine derivatives are widely used in the treatment of various psychic disorders, and much information is available about their clinical and pharmacological effects [1].

Human serum albumin (HSA) is known to possess several sets of binding sites for drugs. The widely used benzodiazepines generally show extensive binding to HSA, whereas other plasma proteins do not appreciably bind these drugs.

The equilibrium constants and the stoichiometries of the benzodiazepine–HSA complexes have a fundamental role in determining the free drug concentration in the plasma which, in turn, influences the pharmacological activities of these drugs. By using gel filtration, circular dichroism and microcalorimetric techniques, several authors [2–6] have calculated the equilibrium constants of various benzodiazepine–HSA complexes.

Quite recently* we proposed an original a.c. polarographic method for the study of the interaction between chlordiazepoxide and albumin. This method was based on the depression of the polarographic diffusion current of chlordiazepoxide by albumin. A similar phenomenon has been observed for some reducible substances when proteins are added and has been ascribed to complex formation between the reducible molecules and proteins [7–13]. We herein report the determination of the formation constant for the complex clorazepate–albumin using an a.c. polarographic method.

HSA (concentration 5%, mol. wt 69,000) was a product of the Sigma Chemical Co. Drugs were obtained from Lab. Chile, S.A. Santiago-Chile. The clorazepate concentration used was 0.25 mM. Sorensen buffer (pH 6–9) was used throughout these studies. The ionic strength was kept constant at 0.3 M with KCl. Polarographic measurements were performed in the TACUSSEL assembly previously described [14]. Potentials (Ep) were measured against a saturated calomel electrode.

The polarographic behaviour of clorazepate (CZP) has been well studied [15]. By a.c. polarography, we obtained an irreversible, diffusion-controlled and pH-dependent peak with a peak potential of −1.08 V at pH 7.4. The height of this peak showed a linear correlation with the CZP concentration. When increasing quantitites of HSA were added to a solution containing CZP, we observed a decrease in the height until a limiting value was reached. This effect was due to the interactions between HSA and CZP and could be used to calculate the most important binding parameters.

$Ip$ and $(Ip)_o$ are the diffusion currents of the drug in the presence and the absence of HSA, respectively, and $C$, $C_b$ and $C_f$ are the concentrations of total, bound and free drugs, where

$$C = C_f + C_b \tag{1}$$

$$Ip/(Ip)_o = C_f + kC_b/C \tag{2}$$

the fractional coefficient, $k$, is the value of $Ip/(Ip)_o$ when enough excess HSA has been added to result in all the CZP being bound. This $k$ value is obtained by an extrapolation method using the following condition:

$$\lim_{C_f \to 0} Ip/(Ip)_o = k \tag{3}$$

The experimental data were obtained by plotting $Ip/(Ip)_o$ versus HSA concentration at a fixed CZP concentration. Furthermore, decreasing CZP concentrations resulted in a decrease in the $Ip/(Ip)_o$ ratio at a constant HSA concentration. If protein adsorption to the drop, or another non-specific factor, were responsible for the diffusion current decrease, $Ip/(Ip)_o$ should, at a given HSA concentration, be independent of the concentration of CZP. Probably with a short drop time the HSA adsorption was non-competitive, with a highly favored interaction between HSA and CZP.

* J. A. Squella, E. Papic and L. J. Nunez-Vergara, presented at the Eighth International Symposium on Bioelectrochemistry and Bioenergetics, Bologna, June 1985.

Defining the average number of clorazepate moles bound per mole of HSA as:

$$r = C_b/[\text{HSA}] \quad (4)$$

it is possible to employ the well-known Lineweaver–Burk equation [16] to plot $1/r$ against $1/C_f$ in order to obtain the stability constant, $K$, and the coordination number, $n$, from the slope and the intercept. Figure 1 shows the straight line obtained for this plot at physiological conditions of pH and temperature. Several values of $n$ and $K$ at different pH and temperature are shown in Table 1. The reproducibility of the $K$ values obtained by this polarographic method was calculated for four independent sets of experimental points at physiological conditions. The obtained $K$ value was $1.61 \cdot 10^4 \pm 0.25 \cdot 10^4$ M, showing adequate reproducibility.

From the thermodynamic equation:

$$\Delta G = -RT \ln K \quad (5)$$

it is possible to obtain the free energy change for the HSA–CZP interaction. We obtained negative values for this parameter under all the experimental conditions, showing that the interaction process was spontaneous. Furthermore, we observed a linear correlation (Arrhenius type) between ln $K$ and the reciprocal value of the absolute temperature, obtaining the $\Delta H$ value from the slope. This method of obtaining $\Delta H$ assumes that $\Delta H$ is constant in the temperature range studied (20–40°). Thermodynamic parameters for the interaction are reported in Table 2. From the negative values of the enthalpy change, it can be concluded that the interaction process was exothermic.

Coassolo *et al.* [6] have studied the CZP–HSA interaction using a microcalorimetric method. They found a value of $1.95 \cdot 10^4 \pm 0.15 \cdot 10^4$ M for $K$ and a value of $-9.9 \pm 0.1$ kcal/mol for $\Delta H$ at pH 7.4, 30° and ionic strength of 0.25. These values are in accordance with the ones obtained in this work: $1.61 \cdot 10^4 \pm 0.25 \cdot 10^4$ M for $K$ and $-9.50 \pm 1.00$ kcal/mol for $\Delta H$ at pH 7.4 37°, and ionic strength of 0.30. Despite the high deviation obtained in the $\Delta H$ polarographic value, the above results provide good support for the validity of our polarographic method.

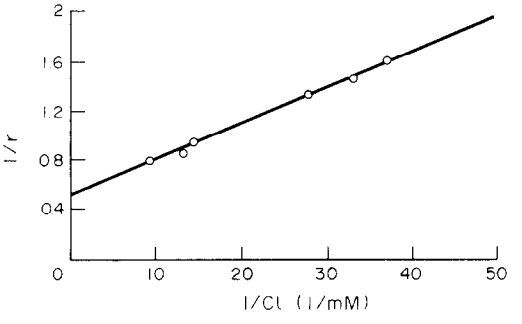


Fig. 1. Lineweaver–Burk plot for clorazepate–human serum albumin interaction at pH 7.4 and 37°.

Table 1. $n$ and $K$ values obtained from Lineweaver–Burk plots at different pH and temperature

| | $K \cdot 10^{-4}$ (M) | $n$ |
|---|---|---|
| pH 6.0 | | |
| 25° | 3.43 | 2.09 |
| 37° | 2.54 | 2.43 |
| pH 7.4 | | |
| 25° | 2.98 | 1.50 |
| 37° | 1.61 | 1.87 |
| pH 8.0 | | |
| 25° | 1.15 | 4.99 |
| 37° | 2.33 | 4.01 |

$K$ = the stability constant, and $n$ = the coordination number.

Table 2. Thermodynamic data for the HSA–clorazepate interaction at different pH values, 37°

| pH | $\Delta G$ (kcal/mol) | $\Delta H$ (kcal/mol) | $\Delta S$ (cal/mol $K$) |
|---|---|---|---|
| 6.0 | −6.2 | −4.75 | −4.77 |
| 7.4 | −5.95 | −9.5 | 11.45 |
| 9.0 | −6.17 | −11.04 | 15.47 |

Experiments comparing interactions between HSA or plasma solutions and CZP produce similar results indicating that albumin is the only plasma constituent that interacts with CZP.

The proposed polarographic method is a useful alternative method in the study of HSA–drug interactions. The polarographic electroactivity of the drug is the only necessary requirement of the method.

In summary, applicability of a.c. polarography to the study of drug–albumin binding interactions has been demonstrated. A study of the binding of CZP to HSA by this technique gave results similar to those obtained by a microcalorimetric technique in a previous paper [6]. The method makes it possible to determine the association constant, the binding sites, and all the thermodynamic parameters describing the interaction.

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# Anti-aggregatory and contractile activity of analogues of prostaglandins $E_1$, $D_1$ and $H_1$

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The prostaglandins (PGs) are a family of potent biological compounds derived from arachidonic acid (AA; all *cis*-5,8,11,14-eicosatetraenoic acid). The naturally occurring PGs, the two-series metabolites, include $PGE_2$, $PGF_{2\alpha}$, $PGD_2$, $PGI_2$ (prostacyclin) and $TXA_2$ (thromboxane), and are derived from the endoperoxide intermediate $PGH_2$. The production of unique PGs from novel AA analogues has permitted the study of how subtle differences in agonist structure affect their interactions with PG receptors located in different tissues. It has been reported previously that the TX analogues derived from four fatty acids differing in carbon chain length and position of the fatty acids' double bonds could be used to distinguish between vascular and platelet TX receptors [1]. Of interest to this study was that PGDs, PGEs and PGHs have been shown to have an effect on both platelets and smooth muscle [2, 3]. The possibility exists that PG analogues that would be potent anti-aggregatory agents could be synthesized, but they would be less active in stimulating smooth muscle. Such analogues could be potentially useful as anti-thrombotic agents. The present study describes the relative potencies, and the possible selectivity, of one series PGH and PGE analogues for inhibition of platelet aggregation and stimulation of smooth muscle contraction. The effects of the corresponding $PGD_1$ analogues on platelet aggregation are also presented.

## *Materials and methods*

*Synthesis of fatty acids and PG analogues.* Fatty acids (19 : 3ω5, 20 : 3ω7, 21 : 3ω6, and 21 : 3ω7) were prepared by total organic synthesis [4], e.g. 20 : 4ω6, is AA, a 20 carbon fatty acid with 4 unsaturations starting 6 carbons removed from the terminal methyl group. The identical compounds labeled in position 1 with [$^{14}$C] were also synthesized (except 20 : 3ω6). Unlabeled 20 : 3ω6 and 20 : 4ω6 were purchased from NuChek Prep., Elysian, MN. The [1-$^{14}$C] compounds of 20 : 3ω6 (57 Ci/mole) and [1-$^{14}$C]20 : 4ω6 (56 Ci/mole) were obtained from new England Nuclear, Boston, MA. PG standards were a gift from Dr. John Pike, Upjohn Co., Kalamazoo, MI. 2′,5′-Dideoxyadenosine was purchased from P.L. Biochemicals Inc., Milwaukee, WI.

Endoperoxide analogues (PGHs) were formed from the corresponding fatty acids using sheep seminal vesicle (SSV) microsomes [homogenized in fatty acid free bovine serum albumen (BSA)] as a source of cyclooxygenase [5]. Approximately 1 μCi of the respective [1-$^{14}$C]fatty acid was added to monitor recovery and assist in the identification of products formed during incubation. Purification of the PGHs was performed by the method of Gorman *et al.* [6]. The PGH analogs were identified by monitoring their respective $R_f$ values during TLC chromatography and their spontaneous breakdown to the corresponding PGEs and PGDs after incubation of the product in 0.1 M $KPO_4$ buffer, pH 7.4 [7]. Additionally, the PGEs were identified by HPLC using retention times and radioactivity as markers [8]. For the $PGE_1$ analogues, a single u.v. band at 192 nm was observed corresponding to between 84 and 94% of the injected radioactivity.

PGE analogues were prepared by incubating the corresponding fatty acids with BSA treated SSV microsomes in the presence of 1 mM epinephrine and 1 mM reduced glutathione at 37° for 30 min. After isolation of the PGEs from unreacted fatty acid using an open bed silicic acid column [7], products were isolated by TLC in solvent systems containing $CHCl_3$–$CH_3OH$–acetic acid–$H_2O$ (90 : 8 : 1 : 0 : 8, v/v) [9] and the aqueous phase of ethyl acetate–isooctane–acetic acid–$H_2O$ (110 : 50 : 20 : 100, v/v) [10].

PGD analogues were synthesized by incubating the appropriate fatty acid with BSA treated SSV microsomes and a rat basophilic leukemia (RBL-1) cell supernatant (a rich source of PGH → PGD isomerase) [11]. Conversion of the SSV-generated PGHs to PGEs was minimized by inhibition of the PGH → PGE isomerase present in the SSV with 0.3 μM parahydroxymercuribenzoate [5]. PGDs were separated by TLC as described above and were eluted from the silica gel with chloroform–methanol (2 : 1). HPLC confirmed the presence of one major peak representing between 57 and 78% of the total injected radioactivity.

*Assay of biological activity.* Anti-aggregatory activity of the generated PG and endoperoxide analogues was tested against AA-induced aggregation of 0.5 ml human platelet-rich plasma (PRP) as previously described [12]. The contractile activity of the $PGH_1$ analogues was tested on superfused rabbit aorta, whereas $PGE_1$ analogues were studied on rat stomach strip using the method described by Leduc *et al.* [1]. Spiral strips of the rabbit aorta or strips of rat stomach fundus were superfused at 37° with oxygenated Krebs–Henseleit buffer at a flow rate of 10 ml/min. To assess whether the $PGE_1$ and $PGD_1$ analogues inhibited aggregation by elevating platelet cyclic AMP levels, the PRP was preincubated with 100 μM 2′,5′-dideoxyadenosine, and inhibitor of adenylate cyclase, for 1 min before addition of the AA.

## *Results*

The biological activities of several analogues of $PGE_1$, $PGH_1$ and $PGD_1$ were tested as inhibitors of AA-induced platelet aggregation (Table 1). The most potent anti-aggregatory compounds tested were the $PGE_1$s derived from 19 : 3ω5, 20 : 3ω6 and 21 : 3ω7. The common feature of these compounds is the presence of double bonds at positions, 8, 11 and 14 in the fatty acid substrate. Other PGEs tested were less active.

In contrast to the PGEs, the anti-aggregatory activity of the PGDs was not dependent on the presence of the bonds at positions 8, 11 and 14. The most potent $PGD_1$ tested was generated from 20 : 3ω5. Unfortunately, no other ω5-PGDs were available for testing. The $PGH_1$ compounds were considerably less potent than their corresponding $PGE_1$ metabolites though the rank order of potency was generally maintained (Table 1). In all cases, the analogues were found to produce their anti-aggregatory effects via a cyclic AMP mediated mechanism since pretreatment with 2′,5′-dideoxyadenosine (100 μM), 2 min prior to the addition of