Age-dependence and free fatty acid modulation of binding kinetics at the benzodiazepine binding site of serum albumin in neonates and adults determined using fast reaction methods

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1 Binding kinetics at the benzodiazepine binding site of albumin have been determined as a function of age and fatty acid concentration in serum from 32 neonates (umbilical cord blood), 28 adults aged 23–65 years and 24 adults aged 66–101 years using fluorescence-time curves obtained in a Durrum-Gibson stopped-flow apparatus and the specific marker ligand dansylsarcosine.

2 The binding reaction can be described by a 2-step mechanism characterised by four constants: the association rate constant k_2 and dissociation rate constant k_{-2} for the rate limiting step-2; the affinity constant K_A' for the formation of an unstable intermediate complex in step-1; the affinity constant K_A for the overall reaction.

3 There were marked differences in k_2 between the three groups of subjects with highest values occurring in neonates (mean: 296 s^{-1}), intermediate values in adults aged 23–65 years (mean: 200 s^{-1} , $P < 0.001$) and lowest values in adults aged 66–101 years (mean: 144 s^{-1} , $P < 0.001$).

4 The dissociation rate constants (k_{-2}) averaged 18.5 s^{-1} overall, showed only minor changes with increasing age and corresponded to dissociation half-lives of 42 ms, 38 ms and 33 ms for neonates, adults 23–65 years and adults 66–101 years respectively.

5 K_A' in neonates and in the group of elderly subjects were lower than in adults aged 23–65 years. K_A for adults aged 23–65 years was similar to that for neonates and double that for elderly subjects: (neonates: $4.90 \cdot 10^5\text{ l mol}^{-1}$; adults 23–65 years: $4.84 \cdot 10^5\text{ l mol}^{-1}$, NS; adults 66–101 years: $2.23 \cdot 10^5\text{ l mol}^{-1}$, $P < 0.001$).

6 The concentration of free fatty acids in serum of the elderly (mean free fatty acid/albumin molar ratio = 1.4) was significantly higher than in the other two groups and, according to measurements made on a system containing purified albumin and oleate, were sufficient to account for the lower k_2 and K_A values in these subjects. Serum free fatty acids were not the cause of the higher k_2 values in neonates.

Keywords protein-binding albumin benzodiazepines neonates ageing kinetics free fatty acids dansylsarcosine

Introduction

Although percentage binding data are available for almost all drugs there is virtually nothing known about the respective association and dissociation rate constants, the actual determinants of the affinity constant. The reason for this is that association and dissociation rates for the binding of a low molecular weight ligand to albumin are exceedingly rapid and thus not measurable using conventional drug protein binding methods. Measurement of binding kinetics requires the use of 'fast reaction' techniques such as stopped-flow or temperature-jump. The first investigations of this type were done by the Nobel prizewinner Manfred Eigen using dyes. Froese & Sehon (1962), coworkers with Eigen, observed that the dissociation half-lives for two azo dyes bound to bovine serum albumin were about 20 and 280 ms and it is likely that dissociation rate data for drugs given in the literature (for example Sjöqvist *et al.*, 1976) are extrapolations of these early studies. Recently fast-reaction binding investigations similar to those of Eigen but using the drugs salicylazosulphapyridine (dissociation $t_{1/2} = 333$ ms; Jansen, 1977) and warfarin (dissociation $t_{1/2} = 70$ ms; Rietbrock & Lassmann, 1980) have been reported.

This paper reports stopped-flow experiments with sera from neonates and adults to investigate whether a relationship exists between age and binding kinetics at the benzodiazepine binding site of human serum albumin. Benzodiazepines bind to albumin with high specificity at binding site II, commonly called the benzodiazepine binding site (Sudlow *et al.*, 1975, 1976; Fehske *et al.*, 1981; Müller & Wollert, 1973). We have used the specific benzodiazepine marker ligand dansylsarcosine which increases its fluorescence intensity on binding to albumin (Sudlow *et al.*, 1975). Isolation of albumin from serum was not necessary since we have shown previously that reliable estimates of association and dissociation rate constants can be obtained using the stopped-flow technique even in the presence of other plasma proteins (Ulrich *et al.*, 1983).

The concentration of free fatty acids was determined in the serum in each subject since it is known that these substances can compete with drugs binding to serum albumin (Spector *et al.*, 1973) and that their concentration may vary, e.g. in pregnancy (Ogbum *et al.*, 1980; Keele *et al.*, 1966).

Methods

Collection of serum

Fresh blood was obtained from the umbilical

cord of healthy neonates ($n = 32$) at delivery and from an antecubital vein of adults aged between 23–101 years ($n = 52$) and who had fasted for 12 h overnight. Blood chemistry parameters showed no evidence of any underlying hepatic or renal pathology. Six of 24 subjects aged 65–101 years were receiving drugs (nitroglycerin, digitalis, theophylline, glibenclamide and doxycycline) but no anionic drug likely to bind to the benzodiazepine binding site on human serum albumin (Kragh-Hansen, 1981).

Protein content of serum was measured by the biuret-method. Albumin concentrations were determined by electrophoresis or using the bromocresol green method of Rodkey (1965). Free fatty acid concentrations were determined with the method of Trout *et al.* (1960) or using the equivalent but more convenient method of Shimizu *et al.* (1980).

1. Measurement of binding rate

The binding rate of dansylsarcosine to albumin in sera was determined using the stopped-flow method with fluorescence detection. A detailed description of the Durrum-Gibson stopped-flow apparatus has already been published so only a brief outline will be given here (Lassmann *et al.*, 1983; Lassmann & Rietbrock, 1982). The reactants, dansylsarcosine ($2.5 \mu\text{mol l}^{-1}$) and serum diluted with Sörensen phosphate buffer pH = 7.4 to produce albumin concentrations in the range $2.8\text{--}54 \mu\text{mol l}^{-1}$, are contained in syringes which are emptied by compressed air into a measuring cell via a mixing jet within a 'deadtime' of 2 ms. When the flow has stopped the fluorescence (Figure 1) using excitation at 351 ± 5 nm (xenon high pressure lamp, Osram, Munich with interference filter, Schott and Sons, Mainz) was recorded at 475 nm with the aid of a digital storage oscilloscope (Nicolet Explorer, Offenbach). The best fitting biexponential function for each signal was determined by on-line computation using a rotating iterative procedure giving the hybrid constants k_1 (observed) and k_2 (observed), (Equation 1). Since the single term k_1 (observed) accounted for more than 90% of the fluorescence developed, calculation of association and dissociation rate constants were based on k_1 (observed) alone (Lassmann *et al.*, 1983).

Fluorescence
intensity

$$= Ae^{-k_1(\text{observed})t} + Be^{-k_2(\text{observed})t} \quad (1)$$

Figure 1 Binding of dansylsarcosine to human serum albumin in native serum. Time course of fluorescence (475 nm, excitation at 351 nm) during a stopped-flow experiment. Concentrations: $2.5 \times 10^{-6} \text{ mol l}^{-1}$ dansylsarcosine and $5.5 \times 10^{-6} \text{ mol l}^{-1}$ human serum albumin (Serum obtained from 35 year old male adult). Arrow shows when reactants were injected into the measuring cell.

2. Calculation of binding constants

We have shown previously (Rietbrock & Lassmann, 1980) that warfarin binds to the warfarin binding site (site I) on human serum albumin according to a two-step binding model of the form:

- L : Ligand
 k_1, k_{-1} : binding rate constants for step-1
 k_2, k_{-2} : binding rate constants for step-2

where drug and human serum albumin associate to form an unstable intermediate complex (L-HSA*), which rearranges to a more stable state (L-HSA) involving specific binding forces. Step-1 is controlled by diffusion of the ligand and binding at the surface of the albumin. The kinetics of this nonspecific binding are so fast that the preequilibrium occurs within the 'dead-time' of the stopped-flow apparatus and velocity constants (k_1, k_{-1}) cannot be determined by this method. The time course of the observed fluorescence change thus reflects step-2 with rate constants k_2 and k_{-2} for conversion into the stable state. This 'rearrangement' is therefore the rate limiting step. If conformational changes on the albumin molecule are involved then the rate constants will be sensitive to allosteric perturbations or modulation and the extent to which

this occurs would be of particular clinical and pharmacological interest.

The affinity constant K_A obtained by applying this model to stopped-flow measurements on warfarin ($1.6 \cdot 10^5 \text{ l mol}^{-1}$ at 37°C) agreed well with the affinity constant ($2.03 \cdot 10^5 \text{ l mol}^{-1}$ at 37°C) obtained by a gel-filtration method. The model also appears valid for calculating binding rate constants of dansylsarcosine since we were able to demonstrate a linear relationship between k_1 (observed) and $k_2, k_{-2}, k_2 K_A'$ and K_A where:

$$\frac{1/[k_1 (\text{observed}) - k_2]}{1/[k_2 \cdot K_A' \cdot (C_{\text{DS}} + C_{\text{HSA}})]} = 1/k_2 + \quad (3)$$

- K_A' : affinity constant for step I
 K_A : affinity constant for overall reaction
 C_{DS} : molar concentration of free dansylsarcosine at equilibrium
 C_{HSA} : molar concentration of free human serum albumin at equilibrium

The constants k_2, k_{-2} and K_A' are estimated by curve fitting (least square method) using k_1 (observed), C_{DS} and C_{HSA} as variables. The affinity constant K_A , which is equivalent to the apparent association constant obtained with commonly used protein binding methods, is calculated using equation 4.

$$K_A = K_A' \cdot \frac{k_2}{k_{-2}} \quad (4)$$

Statistics

The Kruskal-Wallis non-parametric test for differences between three unequal size groups was used to compare subjects according to age (new-borns, adults 23–65 years, adults 66–101 years). When the null hypothesis was rejected, multiple comparison analysis (Dunn, 1964) with alpha-correction (Holm, 1979) was carried out. Correlation coefficients between paired observations were calculated using Spearman's Rho method.

Results

The association rate constant (k_2) measured in sera from neonates and adults decreased with increasing age ($r^2 = 0.59$, $P < 0.01$, Figure 2). The mean value of 296 s^{-1} for neonates was significantly ($P < 0.001$) higher than in adults aged 23–65 years (200 s^{-1}). In the group of adults aged 66–101 years velocity of the associative process was further reduced to 144 s^{-1} ($P < 0.001$) (Table 1). Of the 20 subjects older than 75 years, k_2 in 13 cases was lower ($< 120 \text{ s}^{-1}$)

Figure 2 Association and dissociation rate constants k_2 (●), k_{-2} (○) (s^{-1}) obtained with serum for dansyl-sarcosine-albumin binding as a function of age in 32 neonates and 52 adults aged 23–101 years.

than in all other subjects investigated. No sex-dependency of k_2 in any age group was apparent.

The dissociation rate constant (k_{-2}) was generally low, averaged $18.5 s^{-1}$ overall, and differed little between the groups (Figure 2) reaching statistical significance only in the comparison between infants and the elderly subjects (Table 1). The rate constants correspond to dissociation half-lives of 42 ms, 38 ms and 33 ms for neonates, adults 23–65 years and adults 66–101 years respectively.

The affinity constant K_A (Figure 3) in adults decreased with increasing age, mainly because of its dependence on k_2 , but there was no difference between neonates and the group of adults aged 23–65 years because of the lower K_A' in step-1 for neonates (Table 1). K_A' values (Figure 4) in neonates and subjects aged 66–101 years were similar and both significantly lower than in adults aged 23–65 years.

Mean albumin concentration in sera from neonates and adults aged 66–101 years was 10–20% lower (5.56 and $5.85 \cdot 10^{-4} mol l^{-1}$) than in adults aged 23–65 years ($6.76 \cdot 10^{-4} mol l^{-1}$,

Table 1 Rate constants and affinity constants for dansylsarcosine-albumin binding and free fatty acid/albumin molar concentration ratio in neonates and adults (mean $\pm$ s.d.).

	Neonates n = 32	Age group 23–65 years n = 28	66–101 years n = 24
k_2 (s^{-1})	296 ± 63	200 ± 32	144 ± 45
	————— $P < 0.001$ —————	————— $P < 0.001$ —————	————— $P < 0.01$ —————
k_{-2} (s^{-1})	16.3 ± 3.0	18.4 ± 3.9	21.0 ± 4.7
	————— $P < 0.001$ —————	————— $P < 0.001$ —————	
K_A' ($10^4 l mol^{-1}$)	2.69 ± 0.83	4.24 ± 1.05	3.19 ± 1.28
	————— $P < 0.001$ —————	————— $P < 0.001$ —————	
K_A ($10^5 l mol^{-1}$)	4.90 ± 1.63	4.84 ± 1.72	2.23 ± 0.95
	————— $P < 0.001$ —————	————— $P < 0.001$ —————	
C_{HSA} ($10^{-4} mol l^{-1}$)	5.56 ± 0.52	6.76 ± 0.57	5.85 ± 0.79
	————— $P < 0.001$ —————	————— $P < 0.001$ —————	
C_{FFA}/C_{HSA}	0.64 ± 0.30	0.61 ± 0.27	1.40 ± 0.56
	————— $P < 0.001$ —————	————— $P < 0.001$ —————

Figure 3 Relationship between the affinity constant K_A for dansylsarcosine-albumin binding and age in 32 neonates and 52 adults aged 23–101 years.

Figure 4 Relationship between the affinity constant K_A' for dansylsarcosine-albumin binding and age in 32 neonates and 52 adults aged 23–101 years.

Table 1). However, C_{FFA}/C_{HSA} molar ratios in subjects aged 66–101 years were more than two-fold greater than in the other two groups. Highest values (> 2 mol free fatty acid per mol albumin) occurred in three subjects aged 82, 89, and 95 years.

The low values for k_2 in elderly subjects could be attributed to the higher C_{FFA}/C_{HSA} molar ratio (Figure 5) since the addition of oleate in the molar ratio 3:1 to purified albumin reduced k_2 to 25% and K_A to 10% (Figure 6). An approximately four fold increase in the C_{FFA}/C_{HSA} molar ratio (0.5 to 2.0) produces a two fold decrease in k_2 in adults. Free fatty acids were without effect on the dissociation constant k_{-2} and affinity constant K_A' (Figure 6).

Figure 5 Relationship between the association rate constant k_2 , dissociation rate constant k_{-2} for dansylsarcosine-albumin binding and the free fatty acid/albumin molar concentration ratio, C_{FFA}/C_{HSA} , in 32 neonates ($\bullet$) and 52 adults (Δ) 28 subjects aged 23–65 years; $\blacksquare$ 24 subjects aged 66–101 years). $\diamond$ k_{-2} .

Figure 6 Relationship between the association rate constant k_2 , ($\bullet$) dissociation rate constant k_{-2} ($\circ$) (upper figure), affinity constants K_A ($\blacklozenge$) and K_A' ($\blacksquare$) (lower figure) for dansylsarcosine-albumin binding and the oleate/albumin molar concentration ratio, C_{OL}/C_{HSA} in a synthetic system consisting of purified human serum albumin and varying concentrations of oleate.

Discussion

The commonly used protein-binding methods such as equilibrium dialysis, gel-filtration or ultracentrifugation enable the affinity constant, K_A , to be measured but give no information on the mechanisms involved. For example, a decrease in K_A can arise through a decrease in the association rate without change in the dissociation rate, or conversely through an increase in the

dissociation rate without change in the association rate. This study has shown that the decrease with increasing age of the affinity constant for the binding of dansylsarcosine to albumin is attributable to a decrease in the association rate constant k_2 without change in the dissociation rate constant.

The low k_2 values in the elderly can be attributed to binding modulation by the elevated free fatty acid concentrations found in this group of subjects. Although long chain free fatty acids, (K_A ca. 10^8 l mol⁻¹), bind to albumin with an affinity constant approximately 1000 times greater than that for benzodiazepines (Kragh-Hansen, 1981), modulation is more likely to result from a conformational change than competitive displacement because fatty acid binding sites are distant from the benzodiazepine site and there is little chance of interference (Goodman, 1958; Spector *et al.*, 1973; Berde *et al.*, 1979). Thus only the rate constant of step-2, and not the affinity constant, K_A' , of step-1, is decreased when the oleate concentration is increased in solutions of purified albumin. Fatty acid induced conformational changes have been implicated in the marked increase in warfarin binding at binding site 1 with isolated albumin when the two specific fatty acids binding sites are occupied (Rietbrock *et al.*, 1985).

The rate of formation of the stable complex (k_2) is highest in neonates indicating that the binding properties of neonatal albumin differ from those of adult albumin. K_A for neonatal albumin is the same as that for albumin in adults aged 23–65 years because formation of the unstable intermediate complex in neonates, reflected in the value of K_A' , is slower. We have looked into the possibility that this might be a measurement artifact but repeat measurements on a further group of neonates confirmed that the results are reproducible. Thus, it appears that neonates do not form the intermediate complex as rapidly as expected but that which is formed is converted with the highest velocity into the stable complex. We consider that this phenomenon is caused by differences in the outer sphere of the protein rather than by alterations in the benzodiazepine binding site *per se* and the report of Miyoshi *et al.* (1966) showing that the albumin in cord and adult blood differ in their

physical properties, supports this interpretation. Alternative explanations include competitive inhibition by an unknown ligand or alterations in the tertiary structure of albumin due to associated ligands or other ions in the milieu (Kragh-Hansen, 1981; Wilting *et al.*, 1980).

The lower K_A values in the elderly reported here represent an increase of approximately 100% in the free fraction of dansylsarcosine. For a drug having binding properties identical to dansylsarcosine and a concentration of 100 ng ml⁻¹ in blood the unbound fraction would have a concentration of 0.35 ng ml⁻¹ in neonates and adults aged 23–65 years and 0.77 ng ml⁻¹ in the elderly. An increase of 100% in the free fraction is considerably greater than that reported for benzodiazepines hitherto. For example, Greenblatt *et al.* (1981) and Divoll *et al.* (1981) reported increases in the free fraction of only 25% and this was partly due to an age related 10% reduction in serum albumin concentration in the elderly subjects. It is conceivable that previous investigators have overlooked large changes in the free fraction of benzodiazepines because of the release of free fatty acids by lipoprotein-lipases during equilibrium dialysis experiments where the duration of dialysis can be up to at least 18 h (Greenblatt *et al.*, 1985). According to Giacomini *et al.* (1980) fatty acids released by lipoprotein-lipases during equilibrium dialysis can lead to false estimates of the percentage binding. This criticism does not apply to K_A determined using fast reaction techniques because of the rapidity with which the measurement is carried out. If lipoprotein-lipase action *in vitro* should be shown to reduce differences in protein binding between subjects of different age groups, then pharmacokinetic parameters based on free drug, e.g. $CL_{(unbound)}$, would not be valid and age related modifications in drug-albumin binding under these circumstances may be overlooked. Likewise, the significance of a lower albumin concentration and the resulting rise in the free drug concentration in sera of the elderly may also be greater than previously expected (Woodford-Williams *et al.*, 1964/65).

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