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EFFECT OF DEXTRAN 70 BLOOD SUBSTITUTE ON DIAZEPAM BINDING BY HUMAN SERUM ALBUMIN

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ABSTRACT

Diazepam binding studies with, dextran 70 and human serum albumin (HAS) were carried out using centrifugation and a membrane ultrafiltration technique to separate the drug protein complex and the free microsolute. The molecular filtration method was found to be precise and reproducible with very little variations. The binding of diazepam to dextarn was significant but considerable smaller when compared with the extensive binding of diazepam to HAS. The results of these studies demonstrate that diazepam binding of HSA is altered in the presence of dextran. Diazepam hinds to dextran, resulting in displacement of diazepam from albumin binding sites. To contributing effect of dextran to overall diazepam binding increased as the dextran concentration increased and the HAS concentration decreased. However, the net result of HSA dilution with dextran 70 was still an increase in the percent free diazepam. Various concentrations of dextran 70 significantly displaced HSA hound diazepam in all the mixtures studied.

INTRODUCTION

The presence of blood substitutes may alter the pattern of protein binding and the pharmacokinetic parameters of drugs. The overall changes may be Mc result of certain variations in the binding of Mugs to the components in blood substitutes as well as to plasma proteins. Consequently, the extent of plasma protein binding of drugs can affect their distribution elimination, and thus the intensity and duration of their pharmacological action. A number of studies with blood substitutes demonstrating variations in pharmacokinetics of drugs have been reported *in vivo* (1-3). Further, an *in vitro* study was conducted to assess the effect of a perfluorochemical (PFC) emulsion blood substitute on the binding of diazepam to albumin (Graben and Parson 1988). The result of that study showed an increase in the percent free diazepam as the concentration of emulsion decreased. The PFC emulsion significantly displaced albumin bound diazepam in all mixtures examined the displacing effect of some endogenous substances such as fatty acids, uric acid and bilirubin on diazepam binding to human semi albumin was repotted in other *in vitro* studies (Wong & Sellers, 1979 and Tsutsumi et al. 1974).

Many synthetic blood substitutes and plasma volume expanders are infused into the vascular system for various medical reasons. Isotonic dextran solutions such as 10% Dextran 40 (MW 40,000) and 6% Dextran 70 (MW 70.000) am commercially available both in saline and 5%dextrose solution. Artificial colloids such as dextran, gelatin and

hydroxyethyl starch are free of transmittable diseases and arc readily available at a low cost. Dextran 40, 70 and 110 solutions are administered in hypovolemic and hypotensive shock, hemorrhage and in burns. In addition to their volume effect, dextran solutions provide antithrombotic properties (Messmer, 1988). Human patients were infused with dextran 60 and 70 pre- and postoperatively to investigate the effect on plasma proteins, blood coagulation and fibrinolysis due to dextrans. No patients exhibited any clinical signs of thrombosis or embolism (Bergman el al. 1990).

Dextran 70 and hypertonic saline were infused into bled dogs to assess the systemic and regional oxygen delivery and uptake. Dextran in hypertonic saline is efficacious only when used as a short term resuscitative measure (Curtis & Cain, 1992).

PFC blood substitute emulsions are reported to change the pharmacokinetics and disposition of drugs due to the variation in plasma protein binding. Reports examining the use of dextran solutions as blood substitutes in humans and animals tend to suggest some pharmacokinetic and hemodynamic changes, especially in the level of plasma proteins. Dings normally transported by plasma proteins may be affected by interaction with the component of a blood substitute administered simultaneously (Parson. 1986). The plasma protein binding of drugs in the presence of blood substitutes may be altered by several mechanisms. This may be due to plasma protein dilution or displacement of the bound drug by the components or additives of the blood substitutes (Graben & Parson, 1988). The aim of this project was to study *in vitro* binding of HSA to diazepam in the presence of dextran employing an ultrafiltration dialysis technique, with the intent of pursuing further *in vitro* and *in* vivo studies.

MATERIALS AND METHODS

All studies were conducted at room temperature (24.5°C) and centrifuge (Beckman Centrifuge, Model J-21) temperature never exceeded 30°C. All the diazepam reference standards and all final solutions employed 0.1 N H_2SO_4 as a solvent. The samples were measured at 284 nm with a spectrophotometer (Beckman DU-6, UV visible). A phosphate buffer system was used consisting of KH_2PO_4 and Na2HPO_4 with a pH of 7.4 (± 0,1). The phosphate buffer was used in the dilution of dextran 70 and HSA. Human serum albumin (HSA), essentially fatty acid free (Sigma, St. Louis, MO) and Dextran 70 (Kendall McGaw Laboratories. Irvine, CA) were used as binding agents. Diazepam (Sigma, St. Louis, MO) dissolved in 0.5 ml methanol was evaporated over a water bath. The film of the drug in a 10 ml beaker was dissolved in phosphate buffer, followed by the addition of HSA and/or dextran 70 which were thoroughly stirred to obtain a clear solution.

To standardize the technique of filtration, a 1 ml sample was introduced into the sample reservior of a Centrifree device (Amicon Micropartition system, 30.000 DA) with a 1 ml Hamilton Gastight syringe. The lower part of the device was firmly inserted into

the filtrate cup before the introduction of the sample and the reservior cup was replaced over the end of the tube after the introduction of the sample. One to three successive ultrafiltrates ranging between 200-750 µl were obtained upon centrifugation at 1960 x g and analyzed for contents of free diazepam. The concentration of diazepam increased progressively with the size of the sample till it was constant. The retentate in the tube was replaced with the second 1 ml portion of the same sample. The concentration of the ultrafiltrate from the second sample at the longest time interval was found to be similar to the one obtained earlier during the first run at the same time interval. Four such runs were made and the concentration of diazepam recovered was found to be constant. For subsequent experimental studies, the same procedure was adopted using a 1 ml portion of the sample and collecting about 500 id of filtrate, and then replacing the remaining sample in the tube with the fresh 1 ml portion and collecting 500 µl of filtrate in a fresh cup for diazepam quantitation. Quadruplicate measurements were made for each sample and average percent free diazepam concentrations are reported.

RESULTS AND DISCUSSION

One to three ultrafiltrates ranging between 200 and 350 el were obtained from each 1 µl sample which contained only diazepam. The diazepam concentration obtained from these ultrafiltrates and from ultrafiltrates from two additional 1 µl samples ranged between 84.45 and 87.90% of the initial diazepam concentration. In one to three rinse filtrates from the same sample, the amount of diazepam increased progressively from the first to the third ultrafiltrate. When subsequent ultrafiltrates exceeded 400 µl in volume, the diazepam concentration in the ultrafiltrate approached that in the initial sample. This may be due to the membrane being loaded and saturated with the microsolute or free ligand after the first ultrafrltration. Similar observations were made in other studies while filtering diazepam (DeMuynck et al., 1988) and other drugs (Whitlam & Brown. 1981) through membranes during ultraftltration determinations in serum protein binding. The concentration of the membrane permeable microsolute was the same in the filtrate after an initial sample had been filtered.

Diazepam was used in these studies because it is extensively bound by HSA and is an excellent marker for the second major high affinity drug binding site on HSA (Graben, 1988). The diazepam concentrations of 32, 64, 128 and 192 µg/ml used in these binding studies correspond to a ratio of 166, 33.3. 50 0, 66.6 and 100% v/v respectively for a 4% HSA solution of 1.12 mole diazepam/mole HSA. The results for diazepam binding by cloth-an 70 are presented in Table 1. It is evident that diazepam was greatly bound to dextran. The binding was dependent on polymer concentration. The percent of free diazepam decreased as the dextran concentration increased. Different drug concentrations from 32 to 192 µg/ml indicate that there exists an equilibrium between the free and the bound diazepam if the ascending order of drug concentration is taken into consideration. The extent of binding controls the equilibrium as a small amount of drug binding was noted with smaller diazepam concentrations. Table 2 presents the results of diazepam

binding to various concentrations of HSA. Diazepam was extensively bound to HSA and that the extent of binding by 4%HAS solution to 192 μg/ml diazepam approached 100%. The amount of free diazepam increased as the concentration of USA decreased. These findings are in agreement with the results of other studies reporting diazepam binding to HSA (Fellskc et al., 1981 and Klotz et al., 1975).

The percent free diazepam concentrations resulting from binding of 192 μg/ml in mixtures of dextran 70 and 4% HSA solution are prescribed in Table 3. Table 3 shows that an increase in dextran concentration at a constant HSA concentration results in an increase in the percent of free diazepam and a decrease in HSA concentration at a constant diazepam concentration also generally results in an increase in the percent of fret diazepam. Therefore increasing dextran while decreasing HSA results in an additive increase in free diazepam. The situation is encountered *in* vivo when the infusion of increasing amounts of dextran 70 in clinical situations results in a fall in the plasma protein level (Olin Ed., 1994). In a cross-over study in rabbits and pigs, equal volumes of dextran 40 and saline were infused (Adam, 1989). The infusions resulted in a reduction of plasma protein in both species. With dextran 40 infusions the reduction of plasma protein was greater than simple hemodilution in both species because of the stearic exclusion of protein. This effect is supported by our study which shows that the higher amounts of free diazepam resulting in our studies may be due to the displacement caused by dextran 70. Any decrease in the plasma protein levels is likely to affect the pharmacokinetics of concomitantly administered drugs and hence may elicit an altered pharmacological response. While maintaining the v/v ratio of 4% HSA concentration constant, an increase in the concentration of dextran 70 in a mixture undergoing binding with 192 μg/ml demonstrated a substantial increase in the percent free diazepam displacement until dextran also reached a v/v concentration of 100% (Table 3). Situations like this can arise in cases of hvpovolemia warranting blood volume expansion with blood substitutes that could further result in hemodilution, thus changing the sequence of protein binding and the pharmacodynamics of drugs. Hemodilution with blood substitutes and normal saline have been reported to change the pharmacokinetics of some drugs in the rat because of reduced plasma protein binding (Shrewsbun, 1986). This is supported by our studies which demonstrate that a decrease in HSA concentrations results in increases in the percent free diazepam because of reduction in the capacity of HAS Table 4 shows that 192 μg/ml of diazepam is completely bound by 100% v/v of 4% HSA solution and gradual increases in dextrin results in successive increases in percent free diazepam. With 75% v/v of 4% HSA and 25% v/v dextran in a mixture. the percent free diazepam was only 2.65 whereas the gradual increase of dextran resulted in higher amounts of free diazepam. A similar trend of diazepam displacement was noted with 25 and 50% v/v of 4% HSA in the mixture. The results of a study assessing the effect of dextran 75 on the coadministration of cholesterol diet suggest that dextran 75 is able to displace and reduce serum cholesterol (Lefrance and LeBlanc, 1967). This study supports the experimental results of *in vitro* displacement of diazepam observed during our studies. The data presented in Table 5 show percent free diazepam resulting from various concentrations of dextran 70 and 4%HSA alone and in mixtures. Comparison of the results demonstrate

highly significant diazepam binding by dextran in the presence of HSA. The contributing effect of dextran 70 to overall diazepam binding increased as the dextran concentration increases and the albumin concentration decreases as shown by the increase in percent free diazepam concentration. The binding of diazepam to dextran was significant but considerably smaller when compared with the binding of diazepam to HSA. Nonetheless, the net result of HSA dilution with dextran 70 was still an increase in the percent free diazepam and the difference became more significant as the amount of dextran in the mixture increased.

The use of blood substitutes including dextran is critical to compensate and resuscitate trauma victims for blood or plasma loss. Patients who receive blood substitutes usually will also receive ding therapy depending on the clinical symptoms. When the drugs are co-administered with a blood substitute, the pharmacokinetics of these drugs may be altered to such an extent that the drugs are toxic or ineffective. A studs is reported in the literature where an *in vivo* interaction in rats occurred between dextran 75 and tolbutamide and resulted in an increase in the toxicity of tolbutamide (Doug Interactions, 1972). The increase in toxicity may be due to some alternations in the pattern of Iolbutamide binding.

The data obtained from the binding studies of diazepam to HSA in the presence of dextran 70 may have important implications regarding blood substitute use in conjunction with concomitantly administered drugs.

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Table 1

The effect of dextran 70 concentration on the binding of diazepam

% v/v of Dextran	Diazepam concentration. µg/ml	Mean percent free Diazepam (± s.d.)
25	192	31.07 (1.16)
50	192	28.21 (1.14)
75	192	23.01 (1.35)
100	32	59.31 (3.30)
100	64	49.57 (1.91)
100	96	35.16 (1.84)
100	128	32.74 (1.01)
100	192	26.25 (0.78)

Table 2
The effect of human serum albumin concentration of the binding of diazepam

% v/v of 4% HSA solution	Diazepam concentration, ug/ml	Mean percent free Diazepam (± s.d.)
100	192	0.001 (0.001)
75	192	2.65 (0.32)
50	192	5.06 (1.10)
25	192	15.56 (1.22)

Table 3
The effect of mixtures of dextran solution and human serum albumin on the binding of diazepam (192 μg/ml)

% v/v of Dextran	% v/v of 4% HSA solution	Mean percent free Diazepam (± s.d.)
25	100	2.47 (0.40)
50	100	6.80 (0.45)
75	100	10.68 (0.63)
100	100	12.81 (0.99)
25	75	3.44 (0.20)
50	75	5.78 (0.90)
75	75	8.53 (0.65)
100	75	11.76 (0.94)
25	50	5.41 (0.82)
50	50	7.45 (0.39)
75	50	12.31 (0.69)
100	50	18.32 (0.72)
25	25	17.03 (0.76)
50	25	26.65 (0.90)
75	25	32.05 (1.45)
100	25	27.52 (1.23)

Table 4
The effect of human serum albumin and mixtures of human serum albumin and dextran solution on the binding of diazepam (192 μg/ml)

% v/v of 4% HSA solution	Mean percent free diazepam (± s.d.)	% v/v of mixtures of 4% HSA: dextran solution	Mean percent Free diazepam (± s.d.)
100	0.001 (0.001)	100:25	2.47 (0.40)
		100:50	6.80 (0.45)
		100:75	10.68 (0.63)
		100:100	12.81 (0.99)
75	2.65 (0.32)	75:25	3.44 (0.20)
		75:50	5.78 (0.90)
		75:75	8.53 (0.65)
		75:100	11.76 (0.94)
50	5.06 (1.10)	50:25	5.41 (0.82)
		50:50	7.45 (0.39)
		50:75	12.31 (0.69)
		50:100	18.32 (0.72)
25	15.56 (1.22)	25:25	17.03 (0.76)
		25:50	26.65 (0.90)
		25:75	32.05 (1.45)
		25:100	27.52 (1.23)

Table 5
The effect of dextran solution, human serum albumin and the mixtures on the binding of diazepam (192 μg/ml)

% v/v when present		Mean percent free diazepam			
Dextran solution	4% HSA solution	Dextran Solution alone (± s.d.)	4% HSA Alone	Mixtures of HSA and dextran Solution (± s.d.)	t-test
25	75	31.07 (1.16)	2.65 (0.32)	3.44 (0.20)	$p < 0.1$
50	50	28.21 (1.14)	5.06 (1.10)	7.45 (0.39)	$p < 0.05$
75	25	23.01 (1.35)	15.56 (1.22)	32.05 (1.45)	$p < 0.001$

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