alprazolam. This lack of correlation between in-vitro and in-vivo tests with U-43,465F may be due to: (1) rapid metabolism to a compound with a low affinity to benzodiazepine receptors or (2) its poor penetration into the brain or both. The result of the present investigation indicates that U-43,465F did not achieve adequate concentration in the brain.

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Dependence of drug-protein binding parameters on human serum and albumin concentration

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As early as 1949, Klotz & Urquhart observed a large decrease in the absolute amount of methyl orange bound to bovine serum albumin when the protein concentration was increased five-fold, from 2 g to 10 g litre^{-1}. Thus, the ratios dye bound/total protein are smaller for 10 than 2 g litre^{-1} protein at a given concentration of free dye. Since then, many workers have reported on the artificial (Crooks & Brown 1973) or real dependence of the binding parameters of chemically highly diverse drugs on protein concentration (Brunkhorst & Hess 1965; Westphal 1971; Shen & Gibaldi 1974; Miese et al 1978; Bowmer & Lindup 1978, 1980; Boobis & Chignell 1979; Glasson et al 1980; Tillement et al 1980).

As explanations the Gibbs-Donnan effect, unusual positive cooperativity, inhibition of binding by endogenous ligands and protein-protein interactions have been proposed. Of these, protein-protein interactions seem the likeliest, whilst several authors regard polymerization as an explanation of their findings (Scholtan 1962; Crawford et al 1972; Paubel & Niviere 1973; Müller & Wollert 1976; Zini et al 1976). Pedersen (1962) and Kolb & Weber (1975) have claimed that commercial albumin preparations contain significant amounts of polymers. Recently, Zini et al (1981) have reported on the concentration-dependent polymerization of a widely used human serum albumin (HSA). At a HSA concentration of 1 g litre^{-1}, 99% monomers were detected, but only 60% at physiological concentrations (40 g litre^{-1}). In contrast, it appears that only small amounts of HSA dimers, if any, and practically no other polymers are present in-vivo (Saifer et al 1961; Andersson 1966; Bocci 1967). To evaluate possible albumin polymerization and the presence of endogenous substances in serum on binding, we have compared the binding of metbufen, a new non-steroidal anti-inflammatory drug, to human serum, a commercial HSA, and an HSA sample isolated by us from normal human serum.

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Methods

Human serum (HSA = 600 μM), taken from healthy subjects and frozen, was used. Total proteins and free fatty acids (FFA) were determined (Sampson & Hensley 1975). The mole ratio of 6 FFA/HSA was 0·89. As a commercial source, HSA from Sigma Chemical Co. (Sigma A-1887, fatty acid free), was prepared in 0·067 M phosphate buffer and also stored at −30 °C. All serum or HSA dilutions were similarly made with phosphate buffer. Isolated HSA was prepared from serum via affinity chromatography using a sepharose gel (Zini et al 1981). The mole ratio of 6 FFA/HSA of this albumin was 0·55. [^{14}C]Metbufen (4-*p*-biphenylyl-2-methyl-4-oxo-butyric acid, 1·19 mCi mmol^{-1}, P. Fabre, France) was dissolved in methylpyrrolidone and dilutions made in phosphate buffer. The organic phase never exceeded 5%. Final metbufen concentrations were between 1 and 1500 μM in all cases except when binding to diluted serum (2–2500 μM). All experiments were performed with an equilibrium dialysis technique (Dianorm). Dialysis time was 2 h. The activity in 100 μl of both the dialysate and protein phase were counted in 15 ml of scintillation cocktail (quenching corrected by external standardization). Metbufen recovery after dialysis was always higher than 95%.

Binding was found to be saturable and the number of binding sites, n, and the affinity constant, K_a, were calculated according to their derivation from the law of mass action. The molarity of albumin was substituted for serum since albumin is the only binding protein. Parameters were first estimated via Rosenthal's (1967) linearization procedure and then refined by means of non-linear least squares approximation using a Gauss-Newton algorithm (Zini et al 1979). Binding percentages were calculated according to the formula

$$\%B = \frac{(B) - (A)}{(B)} \times 100 \qquad (1)$$

with (B) the molar concentration of metbufen in the protein compartment and (A) in the protein-free compartment.

Results

Binding to serum and HSA. Between 0·1 and 100 μg ml^{-1} (0·37–373 μM), binding of metbufen to human serum albumin was linear and 99%. Beyond 100 μg ml^{-1}, metbufen-free fractions increased slowly. Within the linear range, binding percentages of metbufen to HSA were indistinguishable from those of serum. HSA is therefore the only binding protein in serum. Binding parameters were calculated assuming two classes of binding sites. For serum they were: $n_1 = 3 - 5$, dependent on the HSA concentration in serum, $K_1 = 39 \times 10^3$ M^{-1} (for 600 μM HSA) and 40×10^3 M^{-1} (for 50μM HSA) (Table 1).

Binding to dialysed serum. Serum was dialysed against phosphate buffer and HSA readjusted to 40 g $litre^{-1}$. Binding percentages were generally identical with those of non-dialysed serum but the number of binding sites was reduced and the affinity constant increased: $n_1 = 1·77$; $K_1 = 82 \times 10^3$ M^{-1} (Table 1).

Binding to fresh diluted serum. Fresh serum derived from several healthy subjects was diluted (HSA = 20 μM) and binding of metbufen determined. Binding percentages were identical to those obtained with equally diluted frozen serum. Binding parameters were: $n_1 = 1·77$; $K_1 = 306 \times 10^3$ M^{-1}. Neither is significantly different from those obtained with frozen serum (data not shown).

Binding of metbufen to commercial HSA. Results are shown in Table 1: $n_1 = 1·84$ which is identical with the value obtained with dialysed serum. However, the affinity K_1 as well as the binding capacities ($n_1K_1 = 272 \times 10^3$ M^{-1} and 863×10^3 M^{-1}, respectively) were twice (600 μM) and five times (20 μM) higher than those in dialysed serum.

Binding of metbufen to isolated HSA. In terms of binding percentages, binding resembled closely that of commercial albumin. There were two classes of binding sites, but binding parameters were much closer to those of serum than of HSA; $n_1 = 4 - 5$; $K_1 = 40 \times 10^3$ M^{-1}. Again the second class contributed less than 10% of the binding capacity of the first class (Table 1).

Discussion

Binding of metbufen to human serum is higher and linear over three orders of magnitude far beyond the highest drug levels anticipated in antirheumatic therapy. Metbufen being a weak acid, the determination of binding parameters is important for the prediction of its behaviour when other drugs are concurrently administered. Despite HSA being the only binding protein in serum, the number of binding sites and the association constants of metbufen are not identical for albumin solutions and serum of identical albumin content. This discrepancy prompted a more detailed study of the variation of binding parameters as a function of receptor concentration.

First, there was the difference in n_1 and K_1 between serum ([HSA] = 600 μM) and 600 μM commercial albumin. For the former, n_1 is twice as high as for the latter (3·14 versus 1·8). K_1, on the other hand, is three times higher in HSA (148×10^3 M^{-1}) than in serum. Upon dialysis of serum, the number of binding sites was reduced by one half and K_1 doubled. The binding capacity ($n_1K_1 = 145 \times 10^3$ M^{-1}), however, was not affected and was the same as for undialysed serum (123×10^3 M^{-1}). It therefore seems that the normal serum binding of metbufen to albumin is antagonized in a complex, i.e. non-competitive, manner by several dialysable but non-identified endogenous substance(s), resulting in an increase of binding site numbers and a depression of affinity. It is unlikely that the free fatty acids are responsible for this behaviour since simple dialysis reduces the mole ratio of 6 fatty acids/HSA only

Table 1. Binding parameters of metbufen to human serum, commercial serum albumin (HSA) and albumin isolated from human serum. n_1K_1 and n_2K_2 are the products of the number of binding sites (n) of albumin and its corresponding affinity constant (K) for the first and second class of binding sites. For the second class, only the binding capacity, n_2K_2 is given. Since, in human serum, only albumin binds metbufen, the molarity of the receptor protein is 0·6 mM. Mean values ±s.d. All parameters were compared with those of serum according to the Normal Deviation Test (*: $P < 0.05$).

Receptor proteins	HSA concentration in μM	n_1	$K_1 \times 10^{-3}$ (M^{-1})	$n_1K_1 \times 10^{-3}$ (M^{-1})	$n_2K_2 \times 10^{-3}$ (M^{-1})
Serum	600	3·14 ± 0·2	39 ± 4·7	123 ± 24	4
Serum	50	5·37 ± 2·2	40 ± 26	125 ± 227	11 ± 14
Dialysed serum	600	*1·77 ± 0·5	82 ± 25	144 ± 85	15 ± 17
Commercial HSA	600	*1·84 ± 0·3	*148 ± 32	272 ± 103	21 ± 32
Commercial HSA	20	*1·84 ± 0·2	*468 ± 166	863 ± 421	47 ± 8
HSA isolated from serum	300	3·78 ± 0·4	40 ± 8·4	153 ± 49	9 ± 6
HSA isolated from serum	20	*4·97 ± 0·6	44 ± 14	220 ± 98	2 ± 2
HSA isolated from serum	10	2·95 ± 0·1	*91 ± 12	*270 ± 45	—[a]

[a] at 10 μM, only the binding sites of the first class are occupied.

to 0·75 versus 0·89 in non-dialysed serum. Similar increases in binding capacity of dialysed serum have been noted before in regular (Andreasen 1974) and especially in uraemic serum. Likewise, Sjöholm et al (1979) have found that warfarin and salicylic acid binding is less in normal and particularly so in uraemic serum than in albumin solutions of equal albumin content.

Fresh diluted serum, on the other hand, shows two binding sites (first class) and a rather high affinity constant subject to large error. Both n_1 and K_1 are not statistically different from frozen serum and, generally, closer to HSA of equal dilution than to undiluted serum. A different range of concentrations of metbufen and a different effect of putative endogenous substances at high dilutions of serum may account for that. Finally, binding of metbufen to isolated HSA closely matches binding to undiluted serum. The first class comprises 3–5 binding sites and, most importantly, the affinity constant is identical with that of serum, around 40×10^3 M^{-1}. The respective binding capacities do not differ from those of serum, either. It therefore seems that binding parameters obtained with serum more truely reflect the binding behaviour of acidic compounds in-vivo than an isolated HSA solution, even if the latter is equimolar with serum. It may, however, still be advantageous to characterize a drug's interaction with its binding proteins in isolated form to facilitate comparisons of results obtained in different laboratories or with different techniques. These results pose the question of the mechanism by which albumin polymerization might affect n and K. Due to a large albumin polymer content of HSA at physiological concentrations, the number of binding sites is reduced, in this case by one half to $n = 2$. This might be a direct consequence of the loss of binding sites due to spatial hindrance. Upon dilution of HSA, however, the affinity is affected (augmented) preferentially and n remains unchanged (Table 1). Increases of n with diminished albumin concentration have, however, also been noted (Bowmer & Lindup 1978).

In conclusion, the results may be summarized as follows: normal human serum (non-dialysed) and isolated albumin bind metbufen at three sites of high affinity and exhibit affinity constants of 40×10^3 M^{-1} (first class), whereas commercial albumin has two sites of high affinity, but a threefold higher affinity K_1. The latter fact may be due to the preparation techniques of HSA leading to albumin polymerization which has been shown recently to occur in the albumin sample used (Zini et al 1981).

Finally, binding parameters obtained with commercial albumin samples differ from those obtained with serum and should not be used to evaluate possible displacement reactions due to concomitantly administered drugs in-vivo. The presence of various endogenous substances and the almost complete lack of polymerized albumin in serum seem to account for that.

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